Contamination with BSE -
Inactivation Mechanism and Risk Assessment for Fat Derivatives

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Chapter I

Introduction

I.1 The prion hypothesis

Prions are *pro*teinaceous *in*fectious particles (**Prusiner, 1982**). Although not yet characterised in structural detail, the only macromolecule being specifically associated with prions is a host-coded, hydrophobic glycoprotein, the so-called *pr*ion *p*rotein (PrP). Nucleic acids as an essential part of infectivity were ruled out by quantitative analyses (**Kellings *et al.*, 1992; Safar *et al.*, 2005**) and excluded finally because of the infectivity of fibrils from pure recombinant PrP (**Legname *et al.*, 2004**). The observation that PrP is present in infected but also in non-infected mammals indicated that PrP can adapt at least two different isoforms: a harmless conformation, designated PrP^{C} (for cellular) and a harmful and infectious conformation, designated PrP^{Sc} (for scrapie). The protein-only hypothesis postulates that solely PrP^{Sc} is the infectious agent of transmissible spongiform encephalopathies (TSEs). The key event of TSEs is that as consequence of infection PrP^{Sc} acts as template for the posttranslational conversion of PrP^{C} into PrP^{Sc}, thereby amplifying the infectious isoform (**Prusiner, 1984;** *for review:* **Cohen, 1999; Riesner, 2004**). This concept is extended by the appearance of different pathology-associated isoforms referred to as prion strains.

I.1.1 Structure, biosynthesis, and function of PrP^{C}

The cellular prion protein (PrP^{C}) is an evolutionary highly conserved membrane protein which is encoded on chromosome 20 by a single copy gene (PRNP) (**Kretzschmar *et al.*, 1986**). The primary translation product of PRNP is composed of 253 amino acids. The 22 N-terminal amino acids represent a signal peptide for direction into the endoplasmatic reticulum (ER). In the ER lumen, a second signal sequence comprising 23 amino acids at the C-terminus is replaced by a glycosyl-phosphatidyl-inositol-anchor (GPI-anchor) (**Stahl *et al.*, 1987**). PrP^{C} contains two highly branched glycosyl groups with sialic acid substitutions which are attached to N 181 and N 197, respectively, as well as a disulfide bridge between C 179 and C 214. The primary sequence of the Syrian gold hamster prion protein (SHaPrP) and all posttranslational modifications are presented in **Fig. 1.1**. Circular dichroism and infrared spectroscopy revealed that PrP^{C} is dominated by roughly 43 % α-helices and has only little β–sheet content. The NMR structure of recombinant PrP in solution consists of three α-helices, a small anti-parallel β-sheet, and a more or less flexible N-terminus (**Pan *et al.*,**

1993; Riek *et al.* , 1996; Liu *et al.*, 1999; Zahn *et al.*, 2000). The tertiary structure of the human prion protein (hPrP 121-230) is presented in **Fig. 1.2** according to **Zahn *et al.*, 1999**. For the NMR study, recombinant PrP was utilised which had been expressed in *Escherichia coli* cells. Due to this prokaryotic expression system, recombinant PrP does not contain posttranslational modifications. Nevertheless, the tertiary structure obtained does not differ markedly from the three-dimensional structure of natural PrP^C in solution. Posttranslational modifications do not influence the tertiary structure of PrP^C (**Hornemann *et al.*, 2004**).

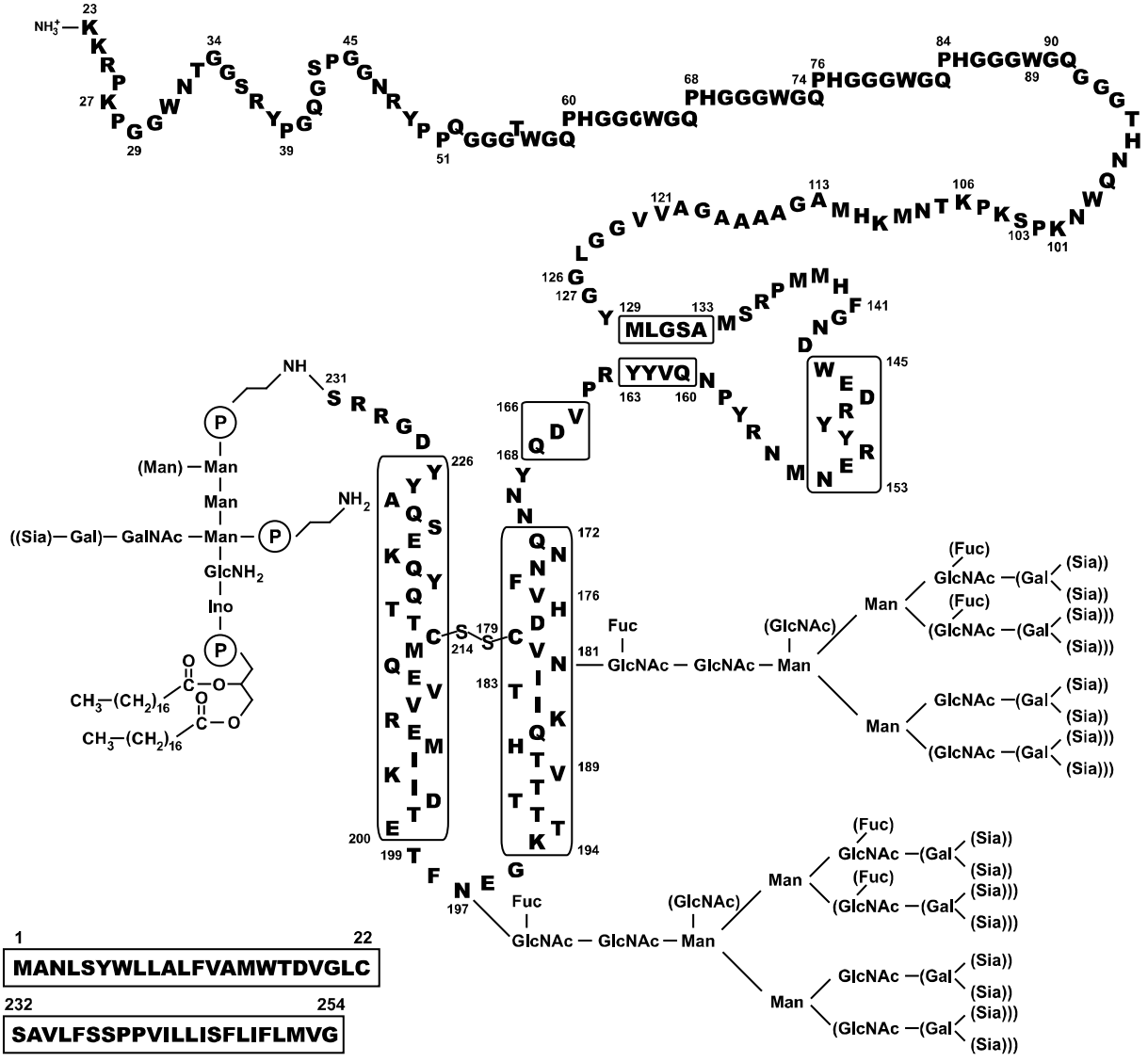

Fig. 1.1: Schematic representation of amino acid sequence and posttranslational modifications of Syrian gold hamster PrP (SHaPrP). Signal peptides: aa 1-22 and aa 232-254; α-helical structures: aa 145-153, aa 166-168, aa 172-194, and aa 200-226; β-sheets: aa 129-133 and aa 160-163; disulfide bridge Cys 179-Cys 214; N-glycosylations: Asn 181 and Asn 197; GPI-anchor: Ser 231. Modified after **Dumpitak & Riesner, 2005.**

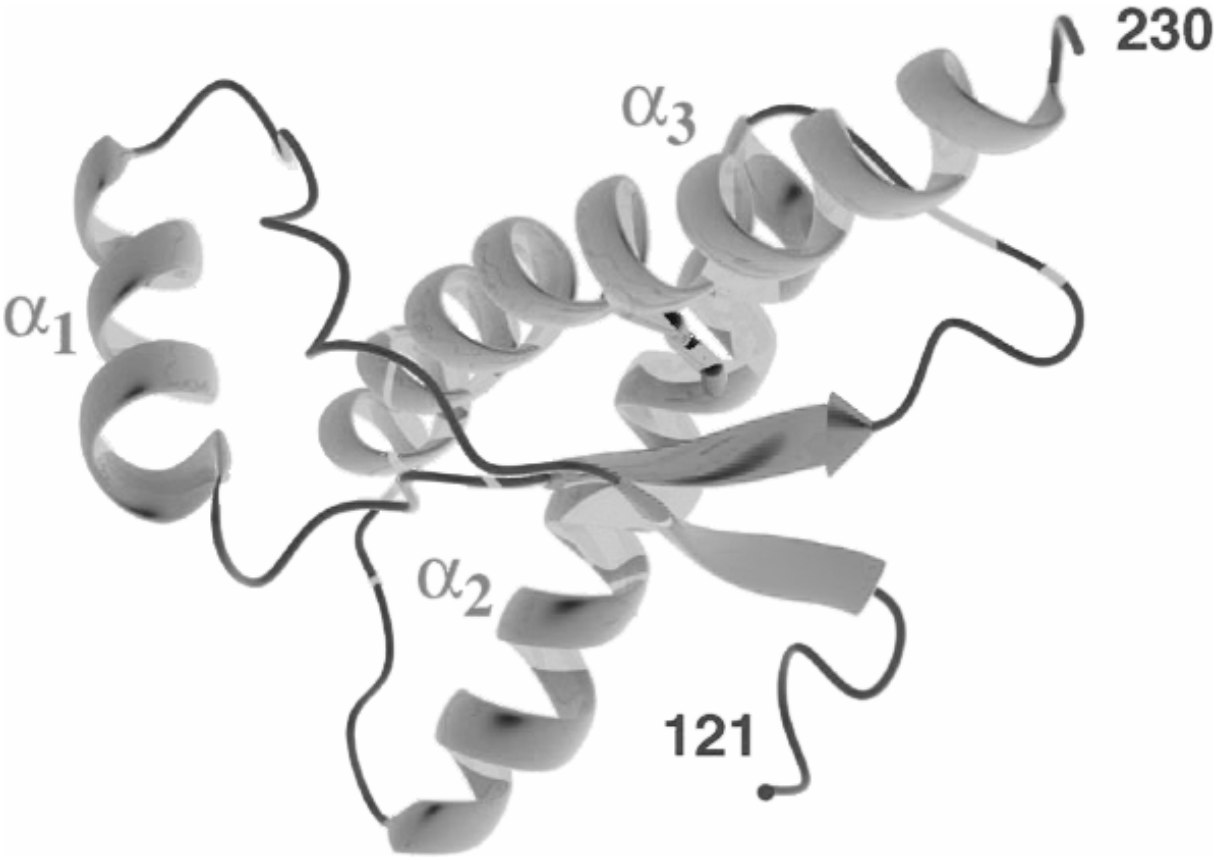

Fig. 1.2: Tertiary structure of the human prion protein (hPrP 120-230) as revealed by NMR. Modified after **Zahn *et al.*, 2000**.

After complete posttranslational modification, the remaining 208 amino acid PrP^C with a molecular weight between 33 and 35 kDa is transported to the cell surface where it is embedded in the outer membrane via its GPI-anchor. Most probably, the anchoring within lipid bilayers prevents an aggregation of PrP^C. Like other GPI-anchored proteins, PrP^C is mainly incorporated into caveolae- or raft-like microdomains of the plasma membrane which are rich in cholesterol and sphingolipids (**Vey *et al.*, 1996; Naslavsky *et al.*, 1997)**. Whereas the glycosyl-groups of PrP^C do probably not alter the three dimensional structure significantly, the attachment to the membrane by the GPI-anchor might have more influence. Recent advances in solid-state NMR spectroscopy are promising to elucidate the structural influence of the membrane attachment.

PrP^C is mainly expressed in the central nervous system but also in most other organs of the body with exception of liver and pancreas. It is also found in T- and B-lymphocytes, monocytes, dendritic cells, macrophages, and platelets (**Kretzschmar et al., 1986; Weismann et al., 1993; Li *et al.*, 2001**). Although

the amount of PrP^C in brain is high compared to other tissues, it represents less than 0.1 % of the total nervous system proteins. PrP^C passes through a subcellular cycle between plasma membrane and endosomal compartments and seems to recycle to the interior of the cell with a transit time of approximately 60 min. (**Shyng *et al.*, 1993**).

It is unclear, however, which role PrP^C plays in the cellular metabolism once it has reached the cell surface. The high grade of amino acid sequence homology among mammalian prion proteins of 85 % - 97 % (**Gabriel *et al.*, 1992**) suggests a role of functional relevance. Surprisingly, $PRNP^{0/0}$-mice phenotypes were not altered neither in their development and reproduction nor in their behaviour. This demonstrates that PrP^C is not essential for those features (**Büler *et al.*, 1992; Büler *et al.*, 1993; Prusiner *et al.*, 1993; Weissmann *et al.*, 1993; Manson *et al.*, 1994**). The comparison of five different $PRNP^{0/0}$-mice lines, however, indicated slight differences between $PRNP^{0/0}$-mice and $PRNP^{+/+}$-mice, namely an lowered long-term potentiation of neurons (**Collinge *et al.*, 1994**), progressive ataxia and a loss of Purkinje cells (**Sakaguchi *et al.*, 1996**), and minor disturbances in the circadian rhythm (**Tobler *et al.*, 1996**). Experimental evidence suggested a role in the copper metabolism in the synaptic cleft (**Brown & Besinger, 1998**) or a role similar to superoxide dismutase, i.e. in degradation of radicals (**Brown *et al.*, 1999**). Besides that, it may play a role in signal transduction or in apoptosis (**Martins *et al.*, 2002**). In the last years an increasing number of different putative PrP receptors have been described such as the laminin receptor (**Gauczynski *et al.*, 2001**) or Src kinase (**Mouillet-Richard *et al.*, 2000**) but so far this has not led to a convincing explanation of the biological function of PrP^C.

I.1.2 PrP^C vs. PrP^{Sc}: a comparison of biochemical properties

The pathology-associated prion protein (PrP^{Sc}) is derived from PrP^C by a posttranslational process involving non-convalent modifications. Since both PrP^C and PrP^{Sc} protein forms are encoded by the same cellular gene, the primary sequence of both isoforms is chemically identically and there are no covalent modifications in PrP^{Sc} that cannot be found in PrP^C (Stahl *et al.*, 1993). Because a quantitative comparison of glycosyl groups is still impossible, the proposed chemical identity of PrP^C and PrP^{Sc} is restricted to the peptide moiety.

Nevertheless, the conformational transition of PrP^{C} to PrP^{Sc} drastically changes the physicochemical properties of the protein (*for review:* **Cohen & Prusiner, 1998**). PrP^{C} is soluble, exhibits a mainly α-helical secondary structure, and is sensitive to degradation by proteolytic digestion, e.g. with proteinase K (PK). In contrast, PrP^{Sc} forms β-sheet-rich aggregates which are insoluble in mild detergents and partially PK resistant (res PrP). Since one infectious unit correlates with 10^{5} to 10^{6} PrP^{Sc} molecules, it is improbable that monomolecular, soluble infectivity exists (**Bolton & Bendheim, 1991**).

The exact PrP^{Sc} structure is still unknown because of the failure of biophysical structure analysis such as NMR and X-ray on insoluble proteins. Spectroscopic data, however, suggest that at least one α–helix is converted to a β-sheet structure and that the N-terminus is also structured probably due to intermolecular interactions. An α-helical content of roughly 20 % was obtained for PrP^{Sc}, whereas the β-sheet content was increased to about 34 % (**Pan *et al.*, 1993; Cohen & Prusiner, 1998**). A new approach was followed by H. Wille who studied by electron microscopy two-dimensional crystalline-like arrays of PrP^{Sc}. Electron density maps could be fitted best if instead of β-sheets a β-helix was assumed. As depicted in **Fig. 1.3**, the β-helical N-terminus is located in the inner part of trimeric subunits and two remaining α-helices are at the outer side. The glycosyl groups point into the space between the trimers (**Govaerts *et al.*, 2004**).

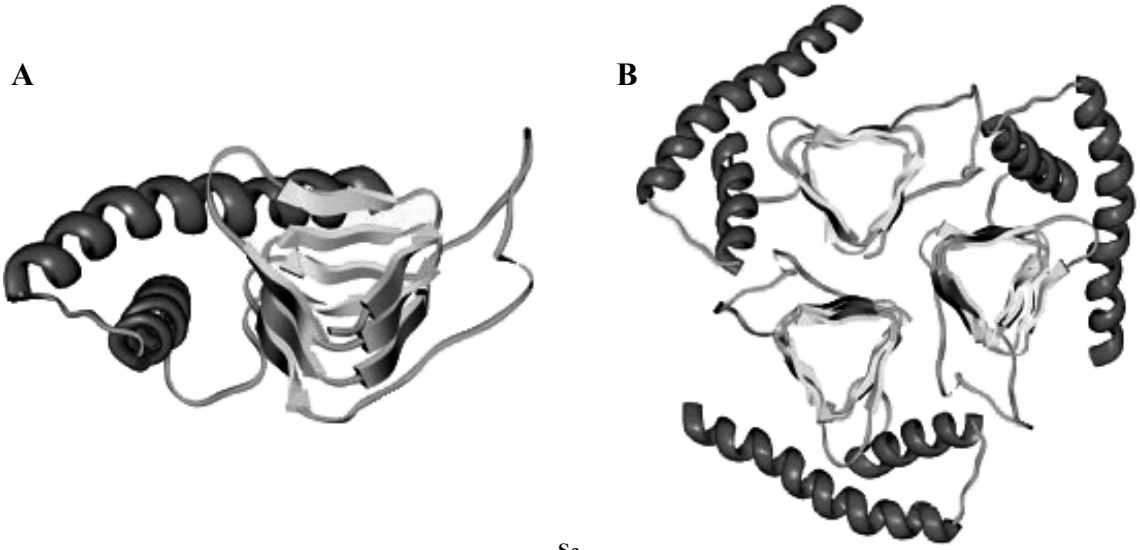

Fig. 1.3: Model for the structure of PrP^{Sc} oligomers based on electron microscopic analysis of two-dimensional crystals. (**A**) Model of the monomer of PrP 27-30. (**B**) Trimeric model of PrP 27-30 built by superimposing three monomeric models. For reasons of clarity, the sugars at the outer side are omitted. Modified after **Govaerts *et al.*, 2004**.

The multimeric PrP^{Sc} aggregates accumulate *in vivo* to various forms of structures ranging from diffuse deposits to amyloidic fibres, condensed plaques, or florid plaques which are most evident within the central nervous system. In contrast to PrP^{C}, PrP^{Sc} is deposited in cytoplasmatic vesicels and extracellular plaques (**McKinley *et al.*, 1991**). The subcellular sites where PrP^{Sc} is formed and the trafficking pathways leading to these sites are still under investigation.

To the current knowledge, PrP^{Sc} is the only component correlating unequivocally but not always quantitatively with prion infectivity (**Cohen & Prusiner, 1998; Lasmezas *et al.*, 1997; Riesner, 2004**). Upon purification using detergents and limited digestion by proteinase K, PrP^{Sc} is transformed into a N-terminally truncated but still infectious form consisting of residues 90-231. This 27-30 kDa fragment is denoted PrP 27-30 and is highly resistant to further PK digestion. It forms rod-shaped fibrils, so called prion rods, with the ultrastructural and tinctorial properties of amyloid (**McKinley *et al.*, 1991**).

Since these aggregates result from hydrophobic interactions, they were assumed to interact with other lipophilic compounds. Indeed, small amounts (~1 %) of sphingomyelin, galactosylceramide, and cholesterol which are known as components of the cell membrane in caveolae-like sites are attached to the PrP molecules in prion rods (**Klein *et al.*, 1998**). Additionally, substantial amounts (~10 %) of a polymeric sugar consisting of α-1,4-linked and 1,4,6-branched polyglucose have been found associated with prion rods (**Dumpitak *et al.*, 2005**). It could not be shown, however, that the lipids or the polyglucose scaffold are essential for prion infectivity. Nevertheless, the specific lipids might indicate the origin of prions, namely the site of PrP^{C} accumulation in the outer cell membrane, whereas the structural scaffold might contribute profoundly to the enormous physical and chemical stability of prions.

I.1.3 Models for prion replication

According to the protein only – hypothesis, solely PrP^{Sc} is the replicating agent responsible for prion diseases. The conformational transition of PrP^{C} to PrP^{Sc} is an autocatalytic process that occurs as long as new PrP^{C} is present. It leads to an exponential amplification of the infectious agent (**Prusiner, 1984; Riesner, 2004; Cohen, 1999**). As mechanistic idea for the replication of prions, four models are currently discussed. The general problem in creating a mechanistic

model is the necessity to explain transmissible cases but also sporadic and genetic etiologies.

Prusiner and his group proposed the "heterodimer model" comprising a transformation of a PrP^{C}-PrP^{Sc} heterodimer into a PrP^{Sc} homodimer and a following formation of amyloid fibrils (**Cohen *et al.*, 1994**). Since genetic evidence indicated the existence of an auxiliary factor in the transformation process, the model was later extended to the "template assistance" model (**Telling *et al.*, 1995; Cohen & Prusiner, 1998**).

Eigen (1996) generalised the Prusiner model to a co-operative model resembling an allosteric binding of a ligand. The catalytically active state are intermediates of PrP^{Sc} oligomeres.

Lansbury and his colleagues proposed that monomeric PrP^{C} is in a fast equilibrium with a PrP^{Sc}-like conformation. In contrast to the "heterodimer model", in the "seeded nucleation" model it is assumed that only if a rate-limiting nucleus of n PrP^{Sc}-like molecules is formed, the growth of infectious PrP^{Sc} aggregates is faster than dissociation (**Come *et al.*, 1993**).

The models mentioned above assume a conformational transition of PrP in solution. The two-phase model take into account that PrP^{C} is attached due to its glycolipid anchor to the outer cell membrane. Only if it is released from the surface into solution, a conformational transition due to the presence of a prion particle can occur (**Riesner, 2001**).

I.1.4 Prion strains

The prion model might be well explained on the basis of conformational alterations of PrP^{C} as consequence of a direct or indirect induction by PrP^{Sc}. The prion concept, however, has to be extended due to the appearance of so-called prion strains. When different scrapie isolates are transmitted to experimental animals, a diversity of phenotypic properties is observed, despite of the genetic identity of PrP in the recipient animal. The distribution and the pattern of severity of spongiform change, the clinical symptoms, as well as the incubations periods vary according to the strain of agent. In further passages, the characteristic strain features are retained despite transmission through a variety of hosts that differ in PrP genotype (**Bruce, 1991**). It was also demonstrated that

PrP of different prion strains has distinct stabilities against denaturation by chaotropes and degradation by proteinase K (**Kimberlin *et al.*, 1983; Safar *et al.*, 1998; Peretz *et al.*, 2002; Legname *et al.*, 2005**). Furthermore, individual TSE strains exhibit thermostabilities deviating from each other which also do not depend on the PrP amino acid sequence (**Dickinson & Taylor, 1978; Somerville *et al.*, 2002; Taylor *et al.*, 2002**). The molecular size and the glycosylation profile of PrP^{Sc} have been used to describe different prion strains by a technique referred to as glycotyping (**Parchi, *et al.*, 1997; Wadsworth *et al.*, 1999**). In this method, the extent of glycosylation of the di-, mono-, and un-glycosylated res PrP is examined by immunoblotting. However, a detailed study demonstrated that glycotyping of PrP isoforms is difficult to perform and to interpret (**Vorberg & Priola, 2002**).

Different TSE strains are characterised by exactly the same PrP amino acid sequence. Therefore, it is assumed that the information for strains is enciphered in the quaternary structure of prions, i.e. in different interactions between PrP molecules, being based on different conformations of the prion protein (**Bessen & Marsh, 1994; Telling *et al.*, 1996; Safar *et al.*, 1998; Peretz *et al.*, 2002; Legname *et al.*, 2005**). In accordance with the protein only – hypothesis, a strain-specific conformation of PrP^{Sc} is proposed to transfer this conformation to a host PrP with different primary sequence. No mechanism, however, has been proposed so far to explain how so much biological information could be encoded by conformational alteration, can be replicated within and between species, and can interact in a strain specific manner with host metabolism to control phenotypic properties. Also, the mystery of the phenotypic stability of the BSE agent in mice regardless of whether transmission is directly from cattle to mice or via intermediate species is unsolved.

I.2 Transmissible spongiform encephalopathies (TSEs)

Fatal neurodegenerative diseases have been known for centuries. As early as 1759, scrapie was mentioned as a lethal and transmissible disease of sheep (**Leopoldt, 1759**). It has been known as epidemic for at least 250 years. The first human prion disease was described between 1920 and 1921 by the neurologists Alfons Jakob and Hans Creutzfeldt as progressive neurodegeneration and was later named Creutzfeldt-Jakob-disease (CJD). Due to a low incidence of CJD nearly no attention was paid, until the Kuru disease of the Fore people on Papua

Neuguinea was described (**Gajdusek & Zigas, 1957**). At this time, first clinical and histopathological similarities between all of these neurological disorders became obvious. The bovine spongiform encephalopathy (BSE) as the most prominent representative of prion diseases was recognised in 1986 in the United Kingdom (**Wilesmith *et al*, 1988**). The formerly unknown cattle disease led to an epidemic peak in 1992. Up to now, more than 182,000 British cattle were affected and it is assumed that prion-contaminated products of about one million cattle have entered the human food chain. In 1996, an association between BSE and a new variant of CJD became evident (**Collinge *et al.*, 1996; Bruce *et al.*, 1997; Hill *et al.*, 1997**). Besides BSE and CJD, a variety of fatal, late-onset, neurodegenerative diseases of mammals belong to the group of transmissible spongiform encephalopathies (TSEs) (**Tab. 1.1**).

Tab. 1.1: Transmissible spongiform encephalopathies (TSEs) of man and animal.

disease	host	pathogenesis
Kuru	man	transmission of PrP^{Sc} due to ritual cannibalism
Creutzfeldt-Jakob disease (CJD)	man	
iatrogenic (iCJD)	man	transmission of PrP^{Sc} due to contaminated surgical equipment
familial (fCJD)	man	germline mutation
sporadic (sCJD)	man	somatic mutation or spontaneous conversion of PrP^{C}
variant (vCJD)	man	transmission of PrP^{Sc} due to BSE-contaminated products
Gerstmann-Sträussler-Schenker syndrom (GSS)	man	germline mutation or spontaneous conversion of PrP^{C}
fatal familial insomnia (FFI)	man	germline mutation
scrapie	sheep goat	transmission of PrP^{Sc} or spontaneous conversion of PrP^{C}
bovine spongiform encephalopathy (BSE)	cattle	transmission of PrP^{Sc} due to contaminated feed
transmissible mink encephalopathy (TME)	mink	transmission of PrP^{Sc} due to contaminated feed
chronic wasting disease (CWD)	deer elk	unknown
feline spongiform encephalopathy (FSE)	cats	transmission of PrP^{Sc} due to contaminated feed
exotic ungulate encephalopathy (EUE)	nyala kudu	transmission of PrP^{Sc} due to contaminated feed

Whereas other neurodegenerative diseases like Alzheimer's disease, Parkinson's disease, or Huntington's disease are exclusively of sporadic or genetic etiology,

the name TSE indicates that these diseases may also be acquired by transmission of an infectious agent. All neurodegenerative diseases are characterised by clinical symptoms that are caused by a loss of certain brain functions. Typical symptoms of TSEs are dementia and ataxia which appear after a long time without symptoms (**Wilesmith *et al*, 1988**). Incubation periods of Kuru seem to last up to fifty years (**Collinge *et al*., 1996**). Histopathologically, TSEs are characterised by the presence of spongiform modifications of the grey matter, i.e. a neuronal vacuolisation based on cell death of neurons, astrocytic gliosis, and microglioses. As characteristic for all neurodegenerative diseases, proteinaceous plaques of amyloid morphology can be detected (**McKinley *et al.*, 1991; Prusiner, 1998; Caughey & Lansbury, 2003**). Therefore, these disorders are sometimes called protein misfolding diseases to which also disorders with other misfolded proteins belong. In contrast to other amyloidic diseases, the amyloid deposits in neurodegenerative diseases are restricted to the central nervous system. In the case of TSEs, these protein deposits are mainly composed of the aggregated prion protein PrP^{Sc}.

I.2.1 The occurrence of BSE and vCJD

Since no scrapie-like disease of cattle had ever been recorded previously, a natural, earlier undetected disease of cattle with the potential to establish an epidemic is unlikely. There is no scientific doubt that spongiform encephalopathies in cattle, mink, felidae, and exotic zoo ruminants are the result of the simultaneous presence of five factors (**Wilesmith *et al.*, 1991; Kimberlin & Wilesmith, 1994; Taylor *et al.*, 1998**):

- an increased sheep population in the UK, relative to that of cattle
- a sufficient prevalence of endemic scrapie in sheep
- possibly, a mutation in the PRNP gene of one cattle individual as origin for an epidemic
- the use of meat and bone meal (MBM) in cattle feeds
- changes in rendering conditions that allowed the recycling, i.e. the survival, interspecies transfer, and intraspecies expansion, of a more thermostable strain of scrapie at a sufficiently high infectivity titre to represent an effective dietary challenge for cattle.

Rendering procedures are boiling procedures that melt the fat in animal tissues and allow it to be drawn off as tallow. Subsequently, the lipid fraction is separated from tissues by pressing, centrifugation, or filtration. The remaining solids (greaves) are pulverised to produce meat and bone meal for incorporation into animal feedstock as a protein supplement. Until the late 1970s it was a common practice in Great Britain to subject these solids to hot solvents like benzene, hexane, petroleum etc. followed by dry heat and/or steam at a temperature of 100 °C because this procedure resulted in low fat meat and bone meal. The application of this solvent extraction process might have provided sufficient additional inactivation of scrapie and BSE infectivity to prevent meat and bone meal of being an effective oral dose. However, changes in the British manufacturing practice led to a widespread abandonment of these boiling procedures. In contrast, since 1939 the German "Tierkörperbeseitigungsgesetz", i.e. an animal disposal law, stated that all dead animals and slaughterhouse by-products, deemed unfit for human consumption, are to be subjected to a temperature of 132 °C and saturated steam before being used as animal feed (**von Ostertag *et al.*, 1958**). Surprisingly, BSE and scrapie infectivity was inactivated by the solvent extraction process only with minor efficiency. The slight inactivation achieved was mainly due to exposure to heat (**Taylor *et al.*, 1997; Taylor *et al.*, 1998**). This suggested that the abandonment of the solvent extraction process by British renderers was not the key factor that permitted the emergence of BSE. More likely, all five factors listed above must have conspired.

The association between BSE and meat and bone meal led to the introduction in 1988 of a ban in Great Britain on feeding ruminant-derived proteins to ruminants. In 1994 the ban was extended to all mammalian proteins and applied throughout the EU. BSE-affected animals as well as high risk tissues comprising skull and spinal cord material were excluded from the food and feed chains in 1994 and 1997, respectively. Since 1996, the over-30-month-scheme guarantees that only cattle younger than 30 months enter the human food chain. Finally, since 2000, cattle older than 24 months and sheep and goats older than 18 months have to be subjected to a rapid test for BSE.

Although a substantial declining in BSE incidence was achieved by these countermeasures, concern has been heightened in 1996 when an association between BSE and a new variant of CJD (vCJD) in humans became evident. In

1990, ten cases of vCJD were identified in the UK, but not in other countries. In contrast to other prion diseases, the PK-digested PrP^{Sc} (res PrP) of BSE and vCJD were recognised to display identical glycosylation patterns (**Collinge *et al.*, 1996**). Histopathological changes typical for vCJD were observed in BSE-infected mice (**Bruce *et al.*, 1997; Hill *et al.*, 1997**). Since BSE and vCJD have identical phenotypic properties dissimilar to those of other TSE agents, the emergence of vCJD is most likely be attributed to transmission via dietary exposure to BSE from cattle to man (**Will *et al.*, 1996**). **Almond** and **Pattison (1997)** concluded "in effect, vCJD is human BSE".

The countermeasures implemented in response to BSE prevent further spread of the disease to man. Additional challenges for public health result from the risk of human-to-human transmission (**Brown *et al.*, 2001; Beekes *et al.*, 2004**). Transmission of vCJD via insufficiently inactivated surgical equipment, tissue implantation, and contaminated medical products resulted in more than 300 iatrogenic CJD cases (*for review:* **Brown *et al.*, 2000; Beekes *et al.*, 2004; Sutton *et al.*, 2006**). CJD can apparently be transmitted by blood transfusion (**Llewelyn *et al.*, 2004**). Up to now, prion infectivity was detected in a variety of tissues from the 161 confirmed cases of vCJD patients (**DH, 2005**) such as brain, spinal cord, lymphatic system, peripheral nervous system, and skeletal muscles (**Bruce *et al.*, 2001; Glatzel *et al.*, 2003; Ramasamy *et al.*, 2003**). All vCJD patients have been homozygous for methionine at the PRNP human codon 129. Although a susceptibility to disease of all three genotypes cannot be excluded, it seems unlikely that they all develop clinical symptoms in the course of the normal life span (**Peden *et al.*, 2004; Bishop et al., 2006; Ironside JW., 2006**). Nevertheless, subclinical carrier of CJD may pose a risk to individuals with more susceptible PRNP genotypes. The corollary is that not only several health-care related questions and problems remain to be solved but that a statistical appraisal of future cases of BSE-derived human CJD disease and of the probability of human-to-human transmission remains difficult.

I.2.2 Amyloidic deposits

As characteristic for all TSEs, proteinaceous deposits consisting of PrP^{Sc} aggregates of amyloid morphology can be detected (**McKinley *et al.*, 1991; Prusiner, 1998; Caughey & Lansbury, 2003**). The term "amyloid" was introduced by **Virchow (1854)** and originally defined "cellulose-like" properties

of deposits **(Sipe & Cohen, 2000)**. Nowadays, the term "amyloid" is used to describe neurodegenerative diseases associated with protein deposits defined by:

- a specific labelling using the dyes congo-red or thioflavin T resulting in specific gold-green birefringence under polarised light,
- linear, rigid, and unbranched fibrils with a diameter of 10 nm and varying length,
- a β-structure with β-strands at intervals of 0.47 nm perpendicular to the long axis of the fibril and backbone hydrogen bonds parallel to the long axis of the fibril. At present, the arrangement of β-strands in a helical conformation of 20 amino acids per turn is favoured (**Peretz *et al.*, 2002; Wille *et al.*, 2002**).

Important to note, the protein aggregation is believed to be rather the cause than the effect of neurodegeneration. Recent data indicate that the fibril final product may not be the proximate mediators of cyto- and neurotoxicity. Rather than fibrils, oligomeric intermediates or protofibrils may actually be the neurotoxic agent (**Caughey & Lansbury, 2003**). These prefibrillar aggregates are temporally unstable and rapidly change into mature and eventually fibrillar forms. Mechanisms proposed to be responsible for the cellular damage caused by these soluble, oligomeric aggregates comprise (*for review:* **Meredith, 2005**):

- disruption of cell membranes, e.g. by inserting into membranes and disrupting ion gradients
- inactivation of native proteins, e.g. by a failure of sequestering transcription factors to the correct cell compartment
- inactivation of components of the quality-control system of cells like chaperones, chaperonines, or the proteasome

It is noteworthy, that protein aggregates appear to represent the lowest free energy state. They are therefore regarded as intrinsically more stable than the native, monomeric states (**Thirmualai *et al.*, 2003; Dobson, 2005**). Actually, it has been shown that proteins and peptides not associated with disease can also be converted intro fibrillar structures with the same properties as amyloid fibrils. This suggests that the ability to form amyloid fibrils is inherent in all polypeptide sequences (**Dobson, 2005**). These findings, however, raise the question of what is the native state of proteins at all?

I.3 Decontamination of prions

I.3.1 Thermodynamic stability

In order to inactivate prion-contaminated specimen, i.e. to reduce the infectivity of PrP^{Sc} as the main component of prions, the thermodynamic stability of PrP^{Sc} is to be overcome. Thermodynamic stability of a protein is the difference in the free energies between the folded state and the unfolded states. Proteins fold up solely as a result of thermodynamic drive leading to a native state that is more stable than the unfolded states, i.e. is characterised by a minimum of free energy. The process of folding starts from a large number of conformations which condense rapidly and reduce the number of pathways available. The resulting compact intermediate state, in which substantial proportions of the secondary structure and the hydrophobic core are assembled, is called 'molten globule'. The molten globule still lacks the tertiary interactions characteristic of the native state. It finally folds to the functional state through something like a single path that constitutes the rate-limiting step of folding. The folded protein is held together by a delicate balance of non-covalent forces accompanied by an only slight energetic preference of the native state.

In case of prions, i.e. the obvious presence of both PrP^{C} and PrP^{Sc} as two isoforms of the same polypeptide chain, this concept has to be extended. PrP^{C} might be the lowest free energy state leading to the assumption that a single PrP^{Sc} molecule is only metastable but stabilised by its tendency to form aggregates. Otherwise, PrP^{C} might only be kinetically stable indicating that PrP^{Sc} is the most stable isoform. The latter hypothesis would imply that all PrP^{C} molecules are inclined to eventually fold into the infectious isoform what is definitely not the case. Obviously, not only the thermodynamic stability of both isoforms has to be evaluated but also the energy barrier to unfolding, i.e. the activation energy to obtain the other isoform.

Whatever the most stable form is, prions are well known for their strong resistance to physical and chemical procedures which inactivate conventional pathogenic agents (**Taylor, 1994; Taylor, 2000;**). Indeed, it was the extraordinary high resistance to inactivation methods such as radiation that led to the suggestion of a new type of infectious agent (**Alper *et al.*, 1966**). The inactivation of prion-contaminated specimen can only be achieved by a combination of heat treatment with as harsh substances as strong alkali,

chaotropic salts, or oxidising agents (**Taylor *et al.*, 1997; Taylor, 2004**). The resistance of pathogens facing external influences is always associated with the chemical properties and the structure of the agents. Consequently, what might account on a molecular level for the outstanding stability of prions?

In the literature, only one other kind of proteins are generally described to be very stable, namely proteins from hyperthermophilic organisms. Hyperthermophiles grow at temperatures above 80 C. The most thermophil organism known so far is the archaeon *Pyrolobus fumarii* which grows at temperatures of about 113 °C. A significant proportion of *P. fumarii* cells taken from the exponential growth phase survived even autoclaving for 1 h at 121 °C (**Blöchl *et al.*, 1997**). Enzymes of organisms like that developed the unique property of high thermostability which is additionally associated with a higher resistance to chemical denaturants. The most thermostable protein characterised to date is rubredoxin from *Pyroccocus furiosus* with an extrapolated melting temperature of about 185 °C. Interestingly, the N-terminus of this monomeric 53 amino acid protein is involved in β-sheet formation resembling the dominating secondary structure of PrP^{Sc}. Nevertheless, hyperthermophilic and mesophilic proteins are highly similar because the sequences of homologous hyperthermophilic and mesophilic enzymes are 40 % to 85 % alike, the three-dimensional structures are superposable, and the catalytic mechanisms are the same (**Sterner & Liebl, 2001**). The difference in the free energies between the folded and unfolded states of mesophilic proteins are in the range of 30 – 60 kJ/mol. This corresponds to a few hydrogen bonds or hydrophobic interactions. Surprisingly, values for thermophilic proteins at 25 °C are not higher (**Pfeil, 1998**).

The major driving force of protein folding is considered to be the hydrophobic effect (**Dill, 1990**). The hydrophobicity of amino acid chains lead to the collapsed structure of the molten globule, from which the native structure is achieved by the contribution of all types of interactions, i.e. hydrogen bonds, salt bridges, and Van der Waals interactions. It was observed that mesophilic and hyperthermophilic proteins have the same basic stability in their highly conserved and most efficiently packed protein cores. This finding explains why stabilising interactions in hyperthermophilic proteins are mainly found in the less conserved areas, namely near the protein surface. Experimental evidence indicates that no single mechanism is responsible for remarkable protein

stability. Increased thermostability is based, instead, on a small number of highly specific interactions. Interactions that nature uses to render proteins extremely thermostable are listed below (*for review:* **Sterner & Liebl, 2001**; **Vieille & Zeikus, 2001**).

Factors of minor importance for the thermostability of proteins are:

- additional aromatic interactions
- reduction of the content of the thermally labile amino acid asparagine, glutamine, cysteine, and methionine
- increase in the numbers of disulfide bridges
- optimised hydrophobic interactions within the protein core
- increase in the number of hydrogen bonds
- increased compactness or packing density (Van der Waals interactions)
- posttranslational modifications e.g. glycosylation

Factors of moderate importance for the thermostability of proteins are:

- increased polar compared with non-polar surface areas
- increases in α-helical content and α-helix stability
- improved fixation of the polypeptide chain termini to the protein core
- truncation of solvent-exposed loops
- (improved) binding of metal ions
- a higher number of prolines and a lower number of β-branched amino acids in loops (valine, isoleucine, threonine)

Factors of major importance for the thermostability of proteins are:

- additional or improved electrostatic interactions caused by salt bridges or particularly networks thereof
- association to oligomers and contact of subunits due to hydrophobic and electrostatic interactions

An increased number of electrostatic interactions on the surface of PrP^{Sc} aggregates are not known. The tendency of the tertiary structure of PrP^{Sc} to form hydrophobic aggregates, however, may be the key property to elucidate the mystery of the extraordinary thermodynamic stability of prions. The polyglucose scaffold found associated with prion rods (**Dumpitak *et al.*, 2005**) or the binding of copper ions to the N-terminal octarepeat region (**Zanusso *et al.*, 2001**) might additionally contribute to the stability of prions. Of course, high thermostability

might also be based on environmental factors such as anorganic salts, high protein concentration, or unknown ligands.

I.3.2 Thermal inactivation

a) Theoretical aspects

The majority of inactivation reactions of complex systems such as prions in liquid solutions can be described by a first order kinetics with the concentration of the original infectious agents decreasing exponentially over time (**Appel *et al.*, 2001; Oberthür, 2001**). According to the Arrhenius equation, the velocity of the inactivation reaction depends on temperature. All molecular systems including proteins are characterised by thermal energy-dependent equilibrium fluctuations of their covalent and non-covalent bonds, induced by Brownian motions of the liquid. Heat increases the kinetic energy of this atomic motion. At higher temperatures, energy barriers between different folding states are more easily overcome as the average atom motion is raised. At further increased temperatures and dependent on the amino acid sequence of a given polypeptide chain, the energetic preference of the native structure is lowered. The kinetic energy is high enough to disrupt non-convalent bonds like hydrogen bonds and hydrophobic interactions. Thus, secondary and tertiary structure elements get lost and the protein denatures. The loss of secondary and tertiary structure is concomitant with an inactivation of biologic activity. At a prolonged inactivation time or at further increased temperature, sufficient kinetic energy is available to even disrupt covalent bonds and the protein is degraded. Although chemical reactions proceed slowly when the reaction centres are not accessible to the solvent as in polymeric prion rods, the destruction rates are expected to increase proportional with temperature.

The transition of a monomeric native protein into its unfolded state is a reversible equilibrium reaction. Only aggregation of the already unfolded protein renders the inactivation of reversibly denatured proteins irreversible. This leads to inactive but stable structures. Actually, most proteins tend to exhibit a tendency toward association reflecting the high proportion of their unfolded surface that is non-polar. In the case of folding intermediates, which are characterised by a somewhat higher proportion of non-polar residues exposed to the solvent, aggregation is a frequently experienced hazard. Thus, unfolded proteins often form aggregates. These aggregates are intrinsically even

more stable than the folded, monomeric state because they are stabilised profoundly by hydrophobic interactions with other unfolding protein molecules. The aggregated state of a polypeptide chain is characterised by the absolute minimum of free energy. That implies that aggregates are energetically more favourable than the native state (**Dobson, 2001; Thirmualai *et al.*, 2003**). In addition to aggregation, chemical modifications take place once the protein is unfolded. These modifications also contribute to make denaturation irreversible. Chemical modifications comprise the deamidation of Asn and Gln residues, the β–elimination of disulfide bridges, the thiol oxidation of Met and Cys residues, the formation of the racemised D-form of Asp and Ser residues, the Maillard reaction between Lys residues and reducing sugars, and the hydrolysis of peptide bonds.

b) Practical considerations

The rate constant of chemical reactions is not only dependent on temperature but also on other conditions such as pH value, ionic strength, and the presence of stabilising or destabilising substances. All of these environmental parameters may have significant influence on the decontamination of prions. For instance, for the Syrian hamster-adapted 263K strain of scrapie, a range of titre losses of 3.2 $\log_{10}$ units (**Brown *et al.*, 1990**) to 7.5 $\log_{10}$ units (**Taylor *et al.*, 1994**) have been reported for a 30 min. incubation at 134 °C under comparable conditions. In practice, prions reside in a heterogeneous physico-chemical environment. This environment consists of aqueous phases, fatty phases, and different solid phases including the surface of decontamination vessels. The presence of prions within a certain phase determines the inactivation rate experienced.

Surfaces effects, particularly drying or smearing onto steel surfaces, can additionally have a protecting effect (**Taylor, 1999**) as well as interfaces between hydrophobic and hydrophilic environments (**Prusiner *et al.*, 1993**). The lower efficiency of inactivating smeared and dried specimen is related to the rapid heating that occurs in the film of dried material and the consequently rapid fixation of PrP^{Sc}. The survival of prion infectivity is also affected by the mode of sample preparation, i.e. the presence of prions in undiluted tissue, in tissue homogenate, or in preparations thereof (**Taylor *et al.*, 1998**), and by the fixation and dehydration state of the agent (**Taylor, 1996**). For example, a previous fixation with formaldehyde or ethanol reduces the sterilising effect of subsequent autoclaving due to an enhanced tissue thermostability (**Taylor &**

McConnell, 1988; Taylor, 1996). Even the size of the tissue specimen subjected to autoclaving seems to influence the inactivation achieved (**Taylor, 1999**). It is known that the heat inactivation of conventional infectious agents like bacteria is impaired by the presence of fat (**Sendhaji, 1977; Sendhaji & Loncin, 1977**). In a similar way, preliminary results indicated that the structural integrity of the PrP 27-30 backbone against hydrolysis is also protected by the presence of lipids (**Appel *et al.*, 2001**).

Additionally, it was recognised that prions strains are also characterised by differences in their heat inactivation resistance and kinetics. All TSE are sensitive to exposure to heat but to varying extents (**Somerville *et al.*, 2002**). Moreover, when a heating procedure is only partially-inactivating, a tailing type of inactivation curve is obtained. In these cases, after an initial large decline in infectivity titre, the residual infectivity resists further inactivation for a prolonged period of time. This biphasic inactivation curve may reflect the protective effect of aggregation or a population heterogeneity. After autoclaving, it was even demonstrated that the surviving amount of scrapie or BSE infectivity can be constant regardless of the starting titre (**Rohwer, 1984; Taylor *et al.*, 1994; Taylor, 1999; Somerville *et al.*, 2002**).

The results of decontamination studies undertaken so far revealed important properties of the heat inactivation of prions. Most importantly, the thermal inactivation of prions is less efficient under dry conditions than under moist conditions. As consequence, the current recommendations from the World Health Organisation (WHO) for the sterilisation approaches for preventing iatrogenic transmission of CJD include a previous treatment with chemical disinfectants followed by a heat exposure in a saturated steam environment, either in a gravity-displacement autoclave at 121 °C or in a porous-load autoclave at 134 °C (**WHO, 1996; Taylor, 2004; Sutton *et al.*, 2006**). A sufficient margin of consumer safety appears to be achieved. Nevertheless, for the understanding of the mechanism of thermal prion decontamination, it is indispensable to gain systematic insight into heat inactivation on a structural and molecular level. The currently available knowledge solely describes the environmental parameters to achieve an efficient inactivation. An underlying systematic approach to understand the molecular characteristics of the heat inactivation of prions, however, is still lacking.

I.3.3 Chemical inactivation

a) Theoretical aspects

Prions are notoriously stabile not only against environmental rigours such as desiccation, thermal extremes, and UV exposure but also against commonly used bactericide and virucide disinfectants. This hallmark is closely connected to special structural features. The tertiary structure of PrP^{Sc} and its tendency to form high molecular mass aggregates are utterly important for the resistance against chemical inactivation. The tendency of PrP^{Sc} to form complexes with other PrP^{Sc} molecules as well as other cell components such as the polyglucose-scaffold (**Dumpitak *et al.*, 2005**) confers an advanced protection. However, it also marks the majority of denaturation processes irreversible. For a successful inactivation of prions, the aggregation-prone nature of PrP^{Sc} has to be destroyed. For that, hydrogen bonds holding together the β-sheet-dominated structure of PrP^{Sc} have to be destabilised. Additionally, salt bridges have to be broken, Van der Waals interactions have to be destabilised, and an intramolecular disulfide bridge between C 179 and C 214 has probably also to be reduced.

b) Practical considerations

The inactivation of prions cannot be achieved with mild disinfectants as they are usually applied for conventional decontamination purposes. This increased resistance against environmental challenges is based on the self-protecting structure, i.e. the existence of amorphous aggregates and amyloid fibrils, and the presence of particularly resistant prion subpopulations,. Only strong alkali such as sodium hydroxide (NaOH), chaotropic salts such as guanidinium hydrochloride (GdnHCl) or guanidinium isothiocyanate (GdnSCN), strong detergents such as sodium dodecylsulfate (SDS), and oxidising agents such as sodium hypochlorite (NaOCl) are suitable to achieve a significant reduction in prion infectivity.

Alkali substances like NaOH at pH values above 12 neutralise the amine and guanidine groups of the amino acids lysine and arginine resulting in an elimination of salt bridges. Alkali substances also convert the amide groups in the amino acids glutamine and asparagine into negatively-charged carboxyl groups. The most efficient disinfection is obtained when the peptide bonds are hydrolysed. A treatment with 2 M NaOH for at least 1 h is recommended (**Brown *et al.*, 1986; Taylor *et al.*, 1994; Lemmer *et al.*, 2004**).

Acids with pH values below 3 neutralise the amino acids glutamate and aspartate resulting in an elimination of salt bridges. The prion inactivation under acid conditions, however, is only effective at elevated temperatures above 60 °C or acid concentrations of at least 5 M. A complete inactivation was obtained by combination of 1 M hydrochloric acid (HCl) and exposure to heat at a temperature of 80 °C (**Appel *et al.*, 2006**).

Chaotropic salts like GdnHCl, GdnSCN, or urea destroy hydrogen bonds within the polypeptide β-sheets leading to an irregularly coiled and a consequently non-infectious polypeptide chain. An inactivation takes place after at least 1 h using 3 M GdnSCN, 6 M GdnHCl, or 8 M urea (**Tateishi *et al.*, 1991; Prusiner *et al.*, 1993; Oesch *et al.*, 1994; Bessen *et al.*, 1995; Manuelidis, 1997; Flechsig *et al.*, 2001**).

Detergents like SDS bind tightly to the peptide backbone thereby melting the hydrophobic centre of PrP^{Sc}. The corollary is a detachment and destabilisation rather than a degradation of PrP^{Sc} (**Lemmer *et al.*, 2004**). Since prion infectivity even remains after heating in SDS, a combination with autoclaving is mandatory (**Taylor *et al.*, 1994; Peretz *et al.*, 2006**). Recommended procedures comprise autoclaving in acidic SDS at a temperature of at least 121 °C for at least 15 min.

Oxidising agents like NaOCl exert an influence on the disulfide bridge of PrP^{Sc} destabilising the protein conformation and facilitating denaturation. NaOCl also appears to lead to numerous covalent modifications in the PrP^{Sc} molecule (**Prusiner *et al.*, 1993**). Treatment with an at least 7.5 % solution corresponding to 17,000 ppm free chloride for at least 1 h is recommended (**Brown *et al.*, 1986; Taylor *et al.*, 1994**).

Although a couple of decontamination methods proved to be useful to eliminate TSE agents, currently published data suggested that under some circumstances these are not completely effective (**Fernie *et al.*, 2004**). Therefore, legislative guidelines require that only a combination of two accepted decontamination methods has to be used. Both decontamination procedures must have proven a significant reduction in prion infectivity. Only autoclaving after or in 1 M NaOH or boiling in 1 M NaOH or NaOCl with at least 17,000 ppm active chlorine appear to eliminate even high titre prion infectivity under worst case conditions (**Taylor, 1994; Taylor *et al.*, 1997; Taylor, 2000; Taylor, 2004**).

Unfortunately, the only methods that currently appear to be effective are aggressive procedures that are harmful to the items processed as well as to the laboratory and hospital personnel. There is an urgent need to develop efficient technologies that are more harmless to products and personnel.

I.4 Impact on industrial requirements

Measures to reduce the potential danger of human infection via cattle products such as the banning of BSE-infected animals as well as of specified risk material from the human food chain and the prohibition of mammalian proteins as protein supplement in meat and bone meal have led to a large decrease in the incidence of BSE. Nevertheless, less obvious routes of human exposure to prion-contaminated material might exist. Bovine tallow is widely used as raw material for the manufacturing of fatty acids, glycerol, alcohols, fatty esters, fatty nitriles, etc. The total world production of oils and fats of animal origin amounts to 20 million metric tons per year (**APAG, 2006**). Derivatives of tallow are converted into detergents, soaps, laundries softeners, shampoos, cosmetics, food emulsifiers, nutritional supplements, pharmaceuticals, candles, waxes, paints and varnishes, printing inks, rubber, textile and leather auxiliaries, plastic stabilisers etc. which will be used in many applications covering pharmaceutical and medicinal, cosmetics, toiletries, resins, cellulose films, paper, tobacco, food and drink, animal feed, and several other chemical uses. Therefore, the finding that the stability of PrP 27-30 against peptide backbone hydrolysis is raised by the presence of lipids (**Appel *et al.*, 2001**) is of particular importance for oleochemical production. There is no evidence that tallow has been a causal factor for BSE (**Taylor et al., 1998**). Nevertheless, it cannot be excluded *a priori* that under those processes prion infectivity might be left within the products representing an efficient challenge to man. The major process for transforming tallow into oleochemicals is hydrolysis, i.e. the splitting of natural triglycerides into crude glycerol and a mixture of crude fatty acids. In the same way, methanolysis is used to produce crude glycerol and methylesters. However, no experimental data on the safety of tallow-derivatives under technically relevant conditions are available.

I.4.1 Hydrolytic fat splitting

The basic procedure of the oleochemical industry is hydrolytic fat splitting which yields bovine tallow derivatives, i.e. fatty acids and glycerol. If these products are used for human consumption and the tallow originates from countries with BSE cases, the potential risk of a BSE contamination of tallow derivatives has to be considered. In the continuous splitting process, fat is injected at the bottom of the splitting column at a temperature of 100 °C. It is heated to reaction temperature by injection of a water steam - saturated atmosphere. Due to differences in density, the fat is transported to the column top whereas water entering at the top is transported in the reverse direction. The products, i.e. fatty acids and a glycerol water mixture, are collected at the top or bottom of the column, respectively. The fatty acid phase is withdrawn at a temperature of 120-180 °C, the glycerol water phase at a temperature of 100-140 °C. In the reaction zone located in the column middle, the hydrolysis occurs routinely at temperatures between 240-260 °C and a pressure of 50-60 bar. The minimum residence time of all particles within the columns is 20 min. (**Wijfels & Rietema, 1972**).

In order to achieve pure glycerol, the crude glycerol water mixture is subsequently subjected to saponification, i.e. a pH 9 treatment with sodium hydroxide at a temperature of at least 120 °C for at least 8 min. The pure glycerol is then washed out of the soap mass and concentrated giving typically another 1.5 h exposure to alkaline conditions at a temperature of at least 120 °C. Managing high amounts of prion infectivity on large scale production units is not acceptable. Therefore, the best feasible simulation of prion reduction in a continuous splitting unit is by considering on a laboratory scale generally valid minimum conditions, i.e. a residence time of 20 min., and simulating separately the industrial conditions prevailing in the upper part (fatty acid regime: 90 % tallow, 10 % water, 200 °C) and the lower part (glycerol regime: 87.75 % water, 9.75 % glycerol, 2.5 % tallow, 140 °C) of fat splitting columns. Regarding prion reduction under industrial conditions of saponification, it is impossible on laboratory scale to produce a pH value of 9 simply by adding sodium hydroxide. Therefore, a buffered solution characterised by a low temperature dependence of its pH value is to be utilised.

I.4.2 Catalytic fat hydrogenation

Hydrolytic fat splitting yields a glycerol water mixture and a crude mixture of different fatty acid types. These are differentiated mainly due to their degree of saturation and to their chain length. Fatty acids appear in chain lengths between 6 and 22 carbon atoms. The most abundant fatty acids are those with 16 and 18 carbon atoms. The melting point of fatty acids increases with the number of carbon atoms and the degree of saturation in the fatty acid chains. In order to increase the melting point and thereby prolong the storage stability and yield non-smelling and tasteless products, unsaturated acids are converted into saturated acids by catalytic hydrogenation. To reduce the level of unsaturation, glycerides of unsaturated fatty acids are subjected to a 12 bar hydrogen atmosphere at a temperature of 160 °C for 45 min. in presence of 0.1 % nickel catalyst or 50 ppm palladium catalyst.

I.4.3 Biodiesel production

In addition to the manufacturing of oleochemicals for use in food, animal feed, and medicinal, cosmetic, and pharmaceutical products, certain tallow-derivatives, namely fatty acid methyl esters, are excellent sources for biodiesel. According to EU legislation, also biodiesel has to be demonstrated free of BSE-contamination. A conventional biodiesel production process out of tallow includes an acidic pre-esterification followed by two alkaline catalysed transesterification steps, and a vacuum distillation under reduced pressure. When fats are used for alcoholysis, the removal of an excessive amount of free fatty acids is vital for the following alkaline catalysis, i.e. the transesterification. Therefore, a preceding acid-catalysed pre-esterification is recommended which is performed at pH 1 for 2 h in boiling methanol, i.e. at a temperature of at least 70 °C. The transesterification is accomplished by potassium methanolate for 20 min. at pH 14 at a temperature of about 40 °C. After transesterification, successive washing steps remove all traces of fatty acid potassium salts, methanol, and soluble by-products and the so-called crude biodiesel is obtained. In order to achieve maximum ester yields, the ester layer is finally purified by vacuum distillation at a temperature of at least 150 °C within 30 min. taking advantage of the fact that methyl esters distil 30 °C to 50 °C lower than free fatty acids.

I.5 Aims of the thesis

An established feature of all prions is their strong resistance to decontamination by thermal or chemical procedures which inactivate conventional pathogens. A lesser inactivation efficiency, as observable in brain tissue in comparison with brain homogenate, is supposed to be the consequence of an unknown kind of fat-attributed protection against heat exposure (**Taylor, 2004; Oberthür, 2001**). Indeed, from studies of conventional microorganisms a lipid-mediated stabilising effect against heat treatment is known (**Sendhaji, 1977; Sendhaji & Loncin, 1977**). The presence of large amounts of external lipids also protects PrP 27-30 against heat degradation (**Appel *et al.*, 2001**). Hence, the lipophilic character of prions and their tendency to form hydrophobic aggregates appear to be closely related to their extraordinary stability. Those findings are also of particular importance for consumer safety, since the fat content of mammalian tissues is considerably high. Consequently, under manufacturing conditions of basic oleochemical processes it cannot be excluded that residual prion infectivity might be left within the products. An earlier study of lipid – prion interactions during heat treatment indicated a protective influence of lipids (**Appel *et al.*, 2001**). However, only the degradation of PrP 27-30 was determined but not the more relevant inactivation of prion infectivity. Since only few conditions were analysed, no experimental data are available on the safety of tallow-derived products under technically relevant conditions which are of utmost interest for the oleochemical industry. On the basis of these considerations, the following two subjects should be addressed during this thesis:

i) A systematic understanding of interactions responsible for thermal prion inactivation should be developed, especially by answering the following questions. Do fats, fatty acid, or glycerol protect against heat inactivation of prions under a variety of conditions? What is the mechanism of thermal prion inactivation, particularly in presence and absence of lipids?

ii) The second purpose was to deliver experimental data for application in industry. Are bovine tallow derivatives, produced under manufacturing conditions of the oleochemical industry, safe for human consumption concerning a potential contamination with prions?

Chapter II

Results & Discussion

II.1 Strategy of experiments

Prion diseases are of outstanding scientific and medical interest but also have an enormous economic impact. To determine prion removal capacities of manufacturing processes on one hand as well as to understand the mechanisms of removal on the other hand, it is indispensable to gain detailed insight into the prion inactivation process on a structural and molecular level. The elucidation of the molecular mechanisms of prion destruction is essential to achieve efficient decontamination protocols in a simple way with low costs, for both scientific laboratories and any manufacturing environment in pharmaceutical, cosmetic, and oleochemical processes.

a) Mechanistic model of prion inactivation

To address this complex issue, a mechanistic model for the inactivation of prions was derived in this thesis based on detailed biophysical studies and under systematic variation of process conditions. Particular attention was directed to the quantitative and qualitative analysis of the influence of fat on thermal prion reduction. In this context, the lower limit of prion inactivation was estimated by determining the yield of peptide backbone degradation under continuous variation of biologically and technically relevant conditions. For degradation experiments, the most purified, concentrated, and stable form of prion infectivity, the so-called PrP 27-30, was used (**Bolton & Bendheim, 1991; Diringer *et al.*,1997; Klein *et al.*,1998; Appel *et al.*, 1999; Fernie *et al.*,2004**). Since PrP 27-30 is the form of prions which is known to be most resistant against inactivation, an analysis of the PrP 27-30 degradation represents the worst case challenge regarding the aspect of product safety. Consequently, the decontamination of prions in laboratory and medical facilities was closely simulated in a worst case manner (**Diener *et al.*, 1982; Prusiner, 1982; Appel *et al.*, 2006**). For more relevant conclusions regarding safety aspects, infectivity studies were performed. Here, the Syrian hamster model infected with the hamster-adapted 263K strain of the scrapie agent was favoured because it is widely accepted as a well validated, highly sensitive model. It is characterised by short incubation periods, high titre of infectivity in the brain, and high resistance to decontamination methods (**Pocchiari, 1991; Pocchiari *et al.*, 1991; Di Martino *et al.*, 1993; Ernst & Race, 1993; Taylor, 1993; Gölker *et al.*, 1996; Brown *et al.*, 2000; Peano *et al.*, 2000**).

The influence of heat exposure to the resistance against digestion with proteinase K (PK) was analysed by thioflavin T (ThT) – assays and Western blots. ThT assays were also applied to examine the reduction of the fibrillar PrP 27-30 structure due to heat treatment. Changes in secondary structure, of aggregation tendencies, and of solubility were characterised by circular dichroism (CD) spectroscopy and differential ultracentrifugation. Structural studies of prions were impaired by the insolubility of PrP aggregates, the heat-caused PrP degradation, and the lacking miscibility of fatty and aqueous phases. Nevertheless, the results obtained allowed to gain detailed insight into the physico-chemical mechanism of prion inactivation.

b) Prion inactivation potential of manufacturing conditions of oleochemical industry

Based on these extensive data for inactivation of prion infectivity and degradation of PrP 27-30 either in presence or absence of fats, fatty acids, and glycerol, the inactivation potentials of oleochemical conditions applied industrially were evaluated.

The basic oleochemical production step is a hydrolytic fat splitting of bovine tallow. The process yields fatty acids and glycerol which are further processed to a huge variety of derivatives. The conditions applied industrially, i.e. 200 °C – 260 °C at corresponding pressure, are generally considered to guarantee the safety of all tallow-derived products. Hitherto, no experimental data were available on the safety of fatty acids and glycerol in case of an unforeseen contamination of tallow with prions under technically relevant conditions. In order to simulate industrial conditions as exact as possible and in a worst case manner, generally valid minimum conditions prevailing in the upper part (90 % tallow, 10 % water, 200 °C, 20 min.) and the lower part (87.75 % water, 9.75 % glycerol, 2.5 % tallow, 140 °C, 20 min.) of industrial fat splitting columns were examined.

Industrial glycerol water mixtures are subsequently exposed to a sodium hydroxide treatment at pH 9, 120 °C for 2 h, to remove remaining fatty acids and achieve pure glycerol. Low-melting glycerides of unsaturated fatty acids are subjected to hydrogenation at a temperature of 160 °C in a 12 bar hydrogen atmosphere, to yield non-smelling and tasteless saturated fatty acids of a long storage stability. Hence, thermal prion reduction curves were also established on

laboratory scale for the pH 9 treatment and under hydrogenation conditions. Since animal fats proved to be excellent sources for biodiesel, i.e. fatty acid methyl esters, prion reduction during the biodiesel production process was also studied.

For all production processes, the lower limit of inactivation was derived from degradation of PrP 27-30. Additionally performed infectivity assays provided more realistic inactivation data. On the basis of both degradation and inactivation data, the inactivation potential of all oleochemical processes mentioned above was evaluated. In summary, unequivocal risk assessment calculations for the industrial conditions of hydrolytic fat splitting, catalytic fat hydrogenation, and biodiesel production could be provided.

II.2 Quantitative analysis of heat-mediated prion destruction

II.2.1 PrP 27-30 degradation is impaired by fat and by glycerol

Under the experimental conditions of this study, prion reduction was achieved as a result of exposure to temperature. Although chemical reactions proceed slowly when the reaction centres are not accessible to the solvent as in polymeric prion rods, destruction rates are expected to increase monotonically with temperature according to the Arrhenius equation. Consequently, increasing degradation efficiencies were obtained after heat exposure at temperatures higher than 40 °C. At temperatures of 100 °C and above, always more than 95 % of the initial PrP amount were degraded. Comparing thermal degradation in presence and absence of fat, fatty acids, and glycerol including industrial conditions of *hydrolytic fat splitting*, it became obvious that lipids and particularly glycerol significantly increase the backbone integrity of PrP 27-30. Reduction factors obtained for heat treatment for 20 min. (RF_{20}) under various conditions are depicted in **Fig. 2.1** in form of graphs $\log_{10}RF_{20}$ as function of reverse temperature corresponding to the Arrhenius equation (**Appel *et al.*, 2001**).

Pure bovine tallow as well as oleic acid exerted a protective effect of one order of magnitude. The enhanced stability is explained by lipids forming micelles enclosing PrP 27-30 aggregates. That results in an increase of the overall structural integrity due to additional hydrophobic interactions. Since hydrophobic interactions between fat molecules and PrP intensify with temperature, the protective effect is maintained even at high temperatures.

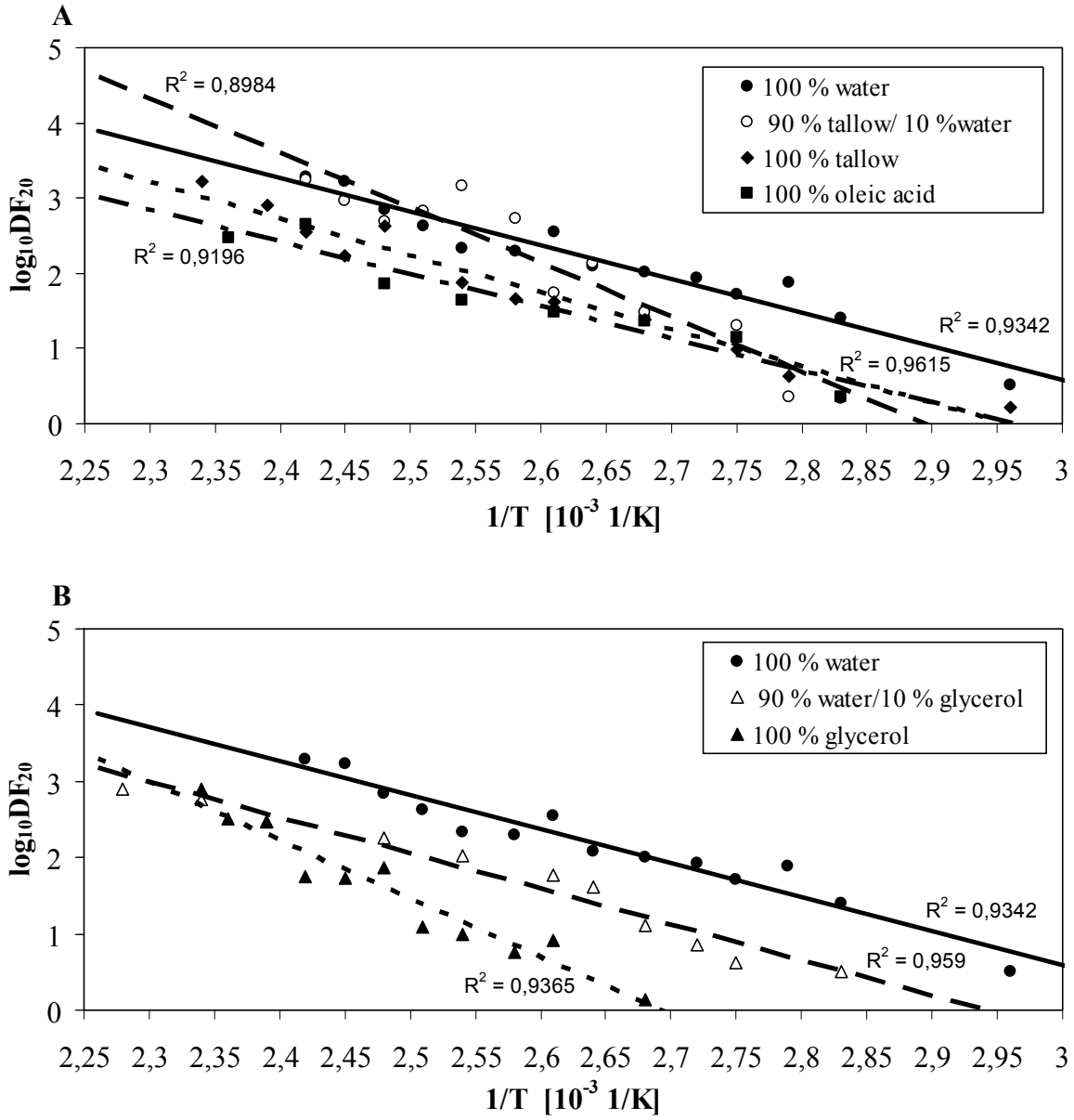

Fig. 2.1: Effect of heat onto degradation of PrP 27-30 in different fat glycerol water mixtures. Logarithmic reduction factors ($\log_{10}$ RF_{20}) for the degradation of PrP 27-30 were determined by Western blots after exposure to heat for 20 min. in (**A**) different fat water mixtures comprising pure water, 90 % fat and 10 % water, pure fat, and pure oleic acid or in (**B**) different glycerol water mixtures comprising pure water, 90 % water and 10 % glycerol, and pure glycerol, respectively, at the temperatures indicated. The data are plotted in form of an Arrhenius plot according to **Appel *et al.*, 2001** with linear regression. Heat treatment in 90 % fat and 10 % water corresponds to industrial conditions of the fatty acid regime of hydrolytic fat splitting, whereas heat exposure in 90 % water and 10 % glycerol simulates industrial conditions of the glycerol regime of hydrolytic fat splitting.

In pure glycerol, the heat stability of PrP 27-30 was even raised by two orders of magnitude. It is suggested that glycerol due to its capability to form hydrogen bonds displaces water at least partially in its structure-forming function. That

leads to an increase in the density of a given solution resulting in a decrease of the unfolding rate constant of proteins (**Hlodan, 1991**).

In aqueous solutions, opposite behaviours were observed. The protective effect of 90 % fat decreased with increasing temperature. At temperatures of 110 °C and above, no significant difference was detected in comparison with pure water. In contrast, the protective effect of as little as 10 % glycerol does not decrease and at high temperatures even extrapolates to about the same stability as in pure glycerol. At medium temperatures, hydrolytic activity is omitted or at least restricted by the exclusion of water molecules due to fat molecules saturating the PrP surface or due to the partial displacement of the PrP 27-30 hydration shell by glycerol. As consequence of this displacement, hydrophobic interactions are stabilised. That results in a suppression of denaturing processes and the establishment of a protective effect. The lipid-mediated dehydrated state is impaired with increasing temperature. At higher temperatures, the non-covalent associations between lipids and polypeptides are increasingly weakened and the lipids are gradually displaced by water molecules. Since PrP 27-30 is inclined to migrate to an aqueous molecular environment with rising temperature (**chapter II.4**), the diminishing of a lipid-mediated protection can be explained by a higher degradation efficiency in the water phase. In contrast, the viscosity-enhancing effect of glycerol appears to dominate hydrolytic denaturation and decelerates unfolding reactions even at high temperatures.

In addition to manufacturing conditions of hydrolytic fat splitting, the experimental set up of PrP 27-30 degradation was further extended to industrial conditions of alkaline treatment of a glycerol water mixture, of catalytic fat hydrogenation, and of biodiesel production.

An alkaline treatment at pH 9 under otherwise identical conditions did not lead to an altered degradation efficiency. In accordance with the literature, only higher pH values can consequently be recommended for decontamination of prions. For a complete prion decontamination, sodium hydroxide concentrations of at least 1 M have to be applied corresponding to pH 14 (**Hörnlimann et al., 2006**).

Under conditions of catalytic fat hydrogenation, i.e. at an overpressure of 12 bar hydrogen, no protective effect of fat was obtained. The PrP 27-30 degradation

was as efficient as in pure water. Probably, the protective presence of fat was overcome by hydrogen molecules hydrogenating not only the double bonds of unsaturated fatty acids but also influencing amino acid residues and achieving a substantial degradation of the PrP 27-30 backbone.

A conventional production process of biodiesel, i.e. fatty acid methyl esters, out of tallow includes an acid preesterification, an alkaline-catalysed transesterification, and a distillation step. Both acid and alkaline conditions at pH 1 or pH 14, respectively, led to an efficient degradation of PrP 27-30. Since prion fibrils are attacked easily by bases but are more stable against acids (**Appel et al., 2006**), an elevated temperature was mandatory for prion decontamination under conditions of preesterification. In contrast, decontamination at pH 12 or above can already be achieved at room temperature (**Taylor, 2000; Taylor, 2004**). Regarding distillation at a temperature of 150 °C, it has to be assumed that proteins are not volatile under these conditions and will remain in the distillation residue. Since PrP was detected neither in the distilled sample nor in the distillation residue, distillation proved to be most effective regarding prion decontamination.

II.2.2 Prion inactivation is impaired by glycerol but not by fat

Degradation affects a chemical property of the prion protein, namely the integrity of the polypeptide backbone. Prion inactivation, however, correlates with a loss of function, i.e. a structural property, which is based on an intact chemical appearance of PrP indeed but also on an infectious conformation (**Riesner, 2004**). Structural and chemical properties of prions can consequently be influenced differently by exposure to a given chemical or physical treatment. After heat treatment in presence of fat, fatty acids, or glycerol an always decreased degradation efficiency of PrP 27-30 was obtained in comparison with pure water conditions (**chapter II.2.1**). Considering prion infectivity, the inactivation achieved also increased with increasing temperature after heat exposure in various fat glycerol water mixtures including manufacturing conditions of hydrolytic fat splitting. As already observable in crude bioassay data, i.e. by means of mean incubation periods of bioassay animals, the relative positions and slopes of inactivation curves, however, varied considerably (**Fig. 2.2**). The determination of reduction factors for inactivation of prion infectivity demonstrated that differences in the destructive potential of nearly up

to five orders of magnitude were achieved (**see II.2.3**). Glycerol did exert a protective effect not only on the backbone integrity of PrP 27-30 but also on prion infectivity. In contrast, pure and aqueous fat only protected against degradation of PrP 27-30. Inactivation of prion infectivity was achieved more efficiently over the whole temperature range analysed.

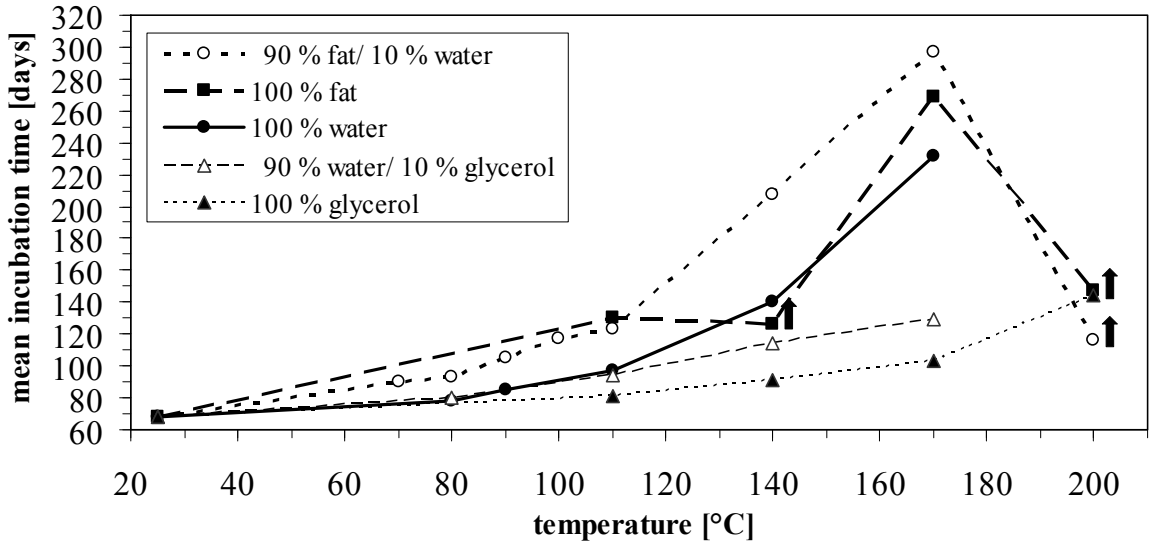

Fig. 2.2: Effect of heat inactivation of prion infectivity in different fat glycerol water mixtures onto mean bioassay incubation periods. Mean incubation periods were obtained after heating PrP 27-30 for 20 min. in 90 % fat/ 10 % water, pure fat, pure water, 90 % water/ 10 % glycerol, and pure glycerol at the temperatures indicated. For the calculation of mean incubation periods only diseased hamsters were utilised. Consequently, when also regarding surviving hamsters the values indicated by arrows (140 °C and 200 °C, 100 % fat; 200 °C, 90 % fat/ 10 % water) would be substantially higher since not all hamsters were clinically affected. For reasons of clarity, standards mean errors were omitted. Heat treatment in 90 % fat and 10 % water corresponds to industrial conditions of the fatty acid regime, 90 % water and 10 % glycerol to industrial conditions of the glycerol regime of hydrolytic fat splitting.

The protective effect of *glycerol* persisted irrespective of temperature. It was even found to be increased at high temperatures. Glycerol is a known and frequently applied reagent for stabilisation of proteins and bacteria. For long term storage, proteins are suspended in 66 % glycerol, whereas 20 % glycerol is added to *Escherichia coli* cells. As outlined in more detail in **chapter II.2.1**, polyoles like glycerol are presumed to unspecifically increase the viscosity of a solution thereby decelerating unfolding reactions (**Hlodan, 1991**). Due to a displacement of the hydration shell, hydrolytic attacks are diminished. The consequence is that not only the polypeptide backbone but also the structural appearance of the PrP 27-30 aggregates, i.e. prion infectivity, is protected. The

fibrillar assembly of prion rods is stabilised by non-covalent interactions. Hydrogen bonds and hydrophobic interactions probably make the strongest contribution to stabilise PrP molecules in an infectious fibril assembly. By means of glycerol, water molecules are excluded. Fibril-stabilising hydrogen bonds are not longer disturbed and hydrophobic interactions are facilitated. The corollary is that denaturing processes are suppressed and prion infectivity is maintained. Even at high temperatures, the glycerol protection dominated the temperature-mediated denaturation.

The presence of *aqueous* or *pure fats* diminished PrP 27-30 degradation at all temperatures analysed but concurrently increased the inactivation of prion infectivity by two orders of magnitude. Sodiumdodecylsulfate (SDS) is known for hydrophobic interactions and a simultaneously decreasing effect onto prion infectivity (**Safar *et al.*, 1993**). Due to the molecular similarity to SDS, fat is also expected to protect PrP 27-30 against degradation by exclusion of water molecules. At the same time, it is presumed to interfere with the secondary and tertiary structure responsible for prion infectivity. Even at high temperature the inactivating effect is maintained. This is probably based on hydrophobic interactions which increase with temperature facilitating the denaturation of PrP 27-30 but concurrently protecting the PrP 27-30 backbone.

The apparently shortened mean incubation periods at temperatures higher than 170 °C are the consequence of the production of appreciable amounts of glycerol due to fat splitting. As already mentioned, glycerol exerts a protective effect on the heat stability of prions and thus lowers the apparent inactivation efficiency. Additionally, after inoculation of those samples, some hamsters did not develop clinical signs of scrapie and were negative for the infectious PrP isoform (PrP^{Sc}). If those surviving hamsters were also included in calculation, the mean values for incubation time and remaining infectivity titre would be higher and the apparent decrease in inactivation efficiency less pronounced.

The finding of an inactivation-enhancing effect of fat was unexpected, since the presence of lipids had often been supposed to be responsible for a low inactivation efficiency, e.g. in brain tissue in comparison with brain homogenate. Analysing the fat-mediated acceleration of prion inactivation at low temperature in more detail, it became obvious that the relative amount of fat, i.e. the presence of 50 %, 75 %, or 90 % fat, did not cause substantial variations in mean incubation times. Probably, all PrP aggregates were

completely saturated by this vast excess of fat molecules and only a further decreased fat content would have resulted in a lower inactivation. After heat treatment for 20 min. at a temperature of as low as 90 °C, reductions in infectivity titre of up to 5 $\log_{10}ID_{50}$ were achieved. After addition of urea and dithiothreitol (DTT), a titre reduction of even 7 $\log_{10}ID_{50}$ was obtained. In pure water, the infectivity titre was reduced by only 3 $\log_{10}ID_{50}$. Due to the concern about the reliability of incubation time interval assays after physical or chemical treatment (**Dickinson & Fraser, 1969; Mould & Dawson, 1970; Taylor, 1986; Taylor & Fernie, 1996**), an endpoint dilution titration was conducted for heat treatment at 90 °C for 20 min. in 90 % fat and 10 % water yielding an infectivity titre of 4.2 log ID_{50}/ml. An incubation time interval assay after heat treatment under identical conditions resulted in an infectivity titre of 3.9 log ID_{50}/ml. Since no significant difference exists between infectivity titres determined by endpoint dilution titration and incubation time interval assay, the calculation of infectivity titres under these and similar conditions on basis of incubation times is reliable.

Industrial conditions of *catalytic fat hydrogenation*, i.e. pure bovine tallow subjected to heat under an overpressure of 12 bar hydrogen, diminished the fat-mediated protection of the PrP 27-30 backbone as presented in **chapter II.2.1**. In accordance, hydrogen did also overcome the inactivation-enhancing effect exerted by fat. The inactivation of prion infectivity under a hydrogen atmosphere was even slightly less efficient than in pure water. As explanation, a less efficient disturbance of the infectious prion structure by saturated fatty acids compared to unsaturated fatty acids might be conceivable.

II.2.3 Inactivation is more efficient than degradation

As outlined in more detail in **chapter II.2.2**, chemical and structural properties of prions can be affected by decontamination methods to different extents. The presence of fat even exerted opposite effects onto PrP 27-30 integrity and prion infectivity. Besides that, the inactivation of prion infectivity was always achieved much more efficiently than the degradation of PrP 27-30 occurred. Under all conditions analysed, differences in reduction of amino acid sequence integrity and structural function of up to four orders of magnitude were observed. In **Fig. 2.3** reduction factors (RF) for heat treatment under various conditions are depicted in form of graphs ln $\log_{10}RF_{20}$ as function of reverse temperature corresponding to the Arrhenius equation (**Appel *et al.*, 2001**).

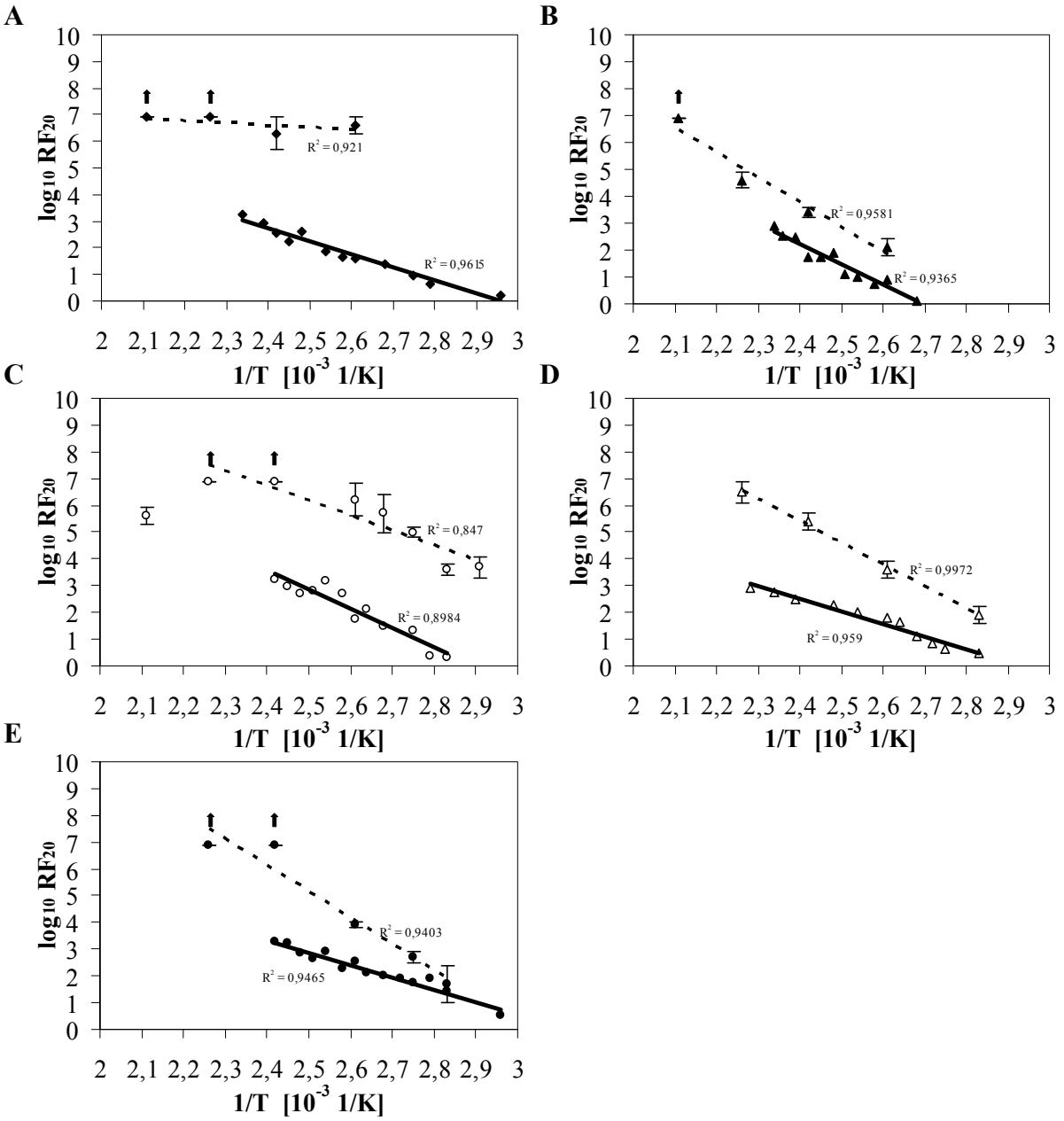

Fig. 2.3: Comparison of inactivation factors and degradation factors after heating PrP 27-30 in different fat glycerol water mixtures as function of temperature. Logarithmic inactivation factors (dot lines, above) determined by bioassays and logarithmic degradation factors (bold lines, below) determined by Western blots were obtained after heating PrP 27-30 in pure fat (**A**), pure glycerol (**B**), a mixture of 90 % fat and 10 % water (**C**), a mixture of 90 % water and 10 % glycerol (**D**), and pure water (**E**). All heat treatments were conducted for 20 min. at the temperatures indicated. The data are plotted in form of an Arrhenius plot according to **Appel *et al.*, 2001** with linear regression. Standard mean errors are indicated for each inactivation data point. Due to the availability of PrP 27-30 with an infectivity titre of 8.9 $\log_{10}ID_{50}$ and a bioassay detection limit of 2 $\log_{10}ID_{50}$, infectivity reduction factors of up to 6.9 $\log_{10}ID_{50}$ could be detected. Therefore, the values indicated by an arrow reflect the detection limit of the bioassay. The actual infectivity reduction factors are most probably higher.

The slopes of the regression curves yield the activation energy of the corresponding degradation or inactivation process, respectively. For inactivation, activation energies of 4 - 19 kJ/mol were obtained. The corresponding values for degradation amounted to 20 – 51 kJ/mol. The deviating kinetics of PrP 27-30 degradation and infectivity reduction suggest a mechanism of prion inactivation consisting of more than one step. At first, infectious prion rods have to be denatured into a form of no or at least lower infectivity. Only at further increased temperature or prolonged incubation time the PrP 27-30 aggregates are probably dissociated and subjected to degradation.

II.3 Mechanistic studies on heat-mediated prion destruction

II.3.1 Heat exposure leads to SDS-resistant aggregates of PrP 27-30

At higher temperatures, an increased tendency to form additional high molecular mass bands was detected. An increase in high molecular mass bands was always paralleled by a decrease in the portion of monomers. Since no PrP bands which are typical for trimers, tetramers, pentamers etc. were detected, a random protein degradation is to be assumed. This random degradation is probably followed by cross-linking reactions among PrP peptides, carbohydrates of the GPI anchor, and the Asn-linked glycosylations. Further contributions to aggregation may be accomplished by transaminations and transesterifications, reduction of disulfide bonds, oxidation of thiol groups, and hydrogenation of double bonds (**Gafni, 1997; Butterfield & Kanski, 2001; Squier, 2001**). Besides monomer bands and random aggregates, signals at 60 kDa indicative of PrP dimers were observed. Weak dimer bands were already present in unheated samples and particularly after exposure to medium temperatures. They disappeared at further increased temperatures in favour of high molecular mass aggregates. Those PrP aggregates remained stable during successive boiling cycles in Western blot loading buffer containing SDS and β-mercaptoethanol. Although no covalent linkage of aggregates was demonstrated in this study, the resistance to boiling in presence of SDS and β-mercaptoethanol points to non-thiol covalent links which were intermolecularly formed between PrP 27-30 molecules. These findings are in agreement with former studies also proposing an increased intensity of higher PrP oligomers due to heat exposure (**Safar *et al.,* 1993; Appel *et al.,* 2001**).

An increase in covalently-linked aggregates should result in a higher sedimentation constant. Therefore, PrP 27-30 was sonicated in presence or absence of SDS and with or without prior heat exposure and then subjected to differential ultracentrifugation to separate soluble proteins from the insoluble pellet fraction (**Fig. 2.4**). Due to the insolubility of PrP 27-30 in water, no conclusions could be drawn after sonication without SDS. After sonication in presence of 0.3 % SDS, about 80 % of the total PrP 27-30 content was soluble. After exposure to temperatures higher than 100 °C for 20 min. prior to sonication, however, about 80 % of the total PrP 27-30 amount remained in the insoluble pellet fraction. Thus, the solubilising effect of SDS was impaired because it was not longer capable to convert PrP 27-30 into a soluble state. In conclusion, both gel electrophoretic analysis as well as differential centrifugation demonstrated that heat exposure induces a further intensified aggregation of PrP 27-30 leading to a significant reduction in solubility.

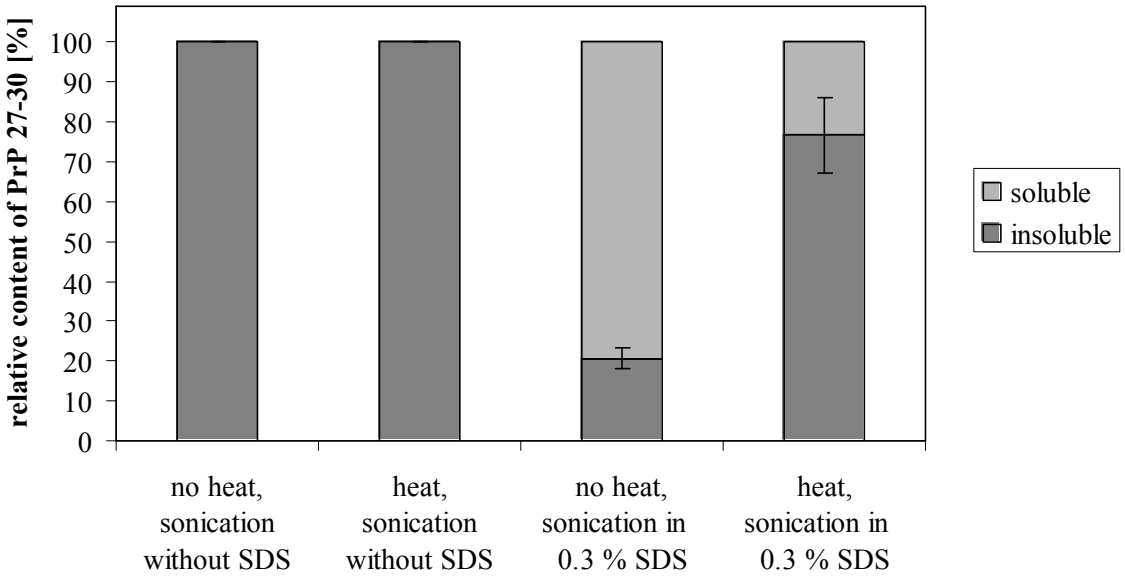

Fig. 2.4: Determination of PrP 27-30 solubility after exposure to heat. The solubility of PrP 27-30 was determined by differential ultracentrifugation after exposure in a 50 ml pressure steel reactor to temperatures of up to 125 °C for 20 min. followed by sonication with a Bandelin HD 2070 sonicator at 65 % for 10 times at 15-sec. bursts in presence or absence of 0.3 % SDS. According to the standards of **Hjelmeland and Chrambach (1984)**, particles remaining in the supernatant after centrifugation at 100,000 x g for 1 h are regarded to be soluble. The amount of PrP 27-30 in supernatant and pellet fractions were determined by Western blotting and densitometric quantification.

II.3.2 β-structured fibrils diminish before PrP 27-30 is degraded

Prion infectivity is based upon intact PrP polypeptide chains as well as on a distinctive fibrillar structure built of aggregates formed by these PrP polypeptides (**Riesner, 2004**). As demonstrated in **chapter II.2.3**, prion infectivity is inactivated more efficiently than PrP 27-30 is degraded. This indicates that a loss in fibrillar structure or even in β-sheet content might occur prior to polypeptide backbone degradation. Therefore, relative depletions of both fibrillar structure and PrP backbone integrity were compared systematically after exposure to heat at temperatures of up to 140 °C. The reduction in fibrillar structure was measured by means of a thioflavin T (ThT) fluorescence assay which specifically indicates the presence of amyloid fibrils. The reduction of intact PrP polypeptide chains was analysed by gelelectrophoresis. At temperatures of 80 °C and above, a substantial reduction in the ThT fluorescence of fibrils occurred before the PrP 27-30 backbone integrity was affected (**Fig. 2.5**). It cannot be excluded that exposure to heat might influence the binding properties of ThT. In this case, no conclusions could be drawn from ThT assays of heat-treated PrP 27-30. It is more probable, however, that heat treatment resulted in the emergence of a new PrP form which is not longer part of a fibrillar structure.

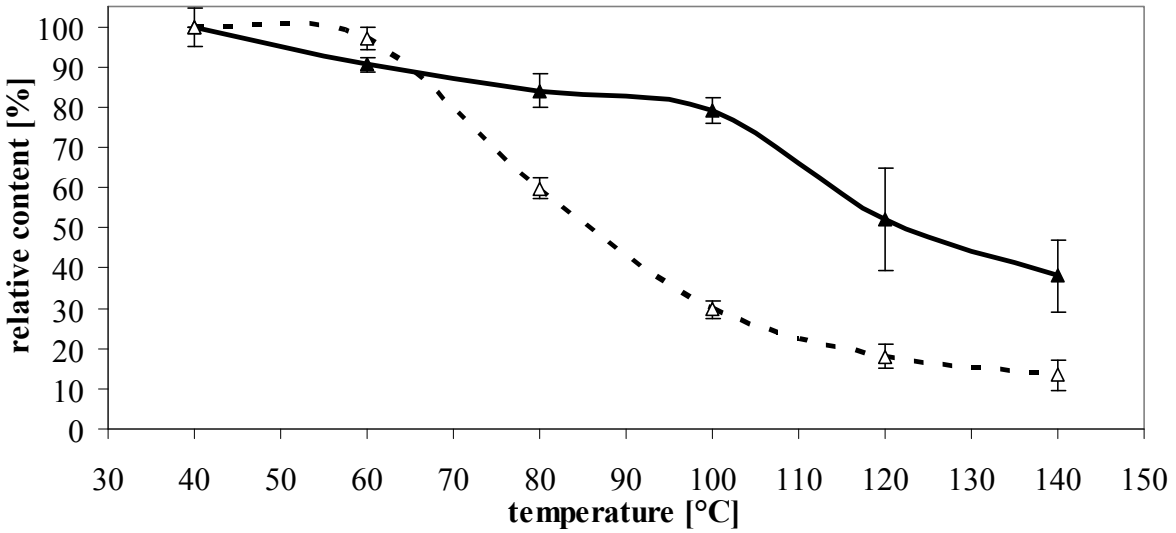

Fig. 2.5: Effect of heat onto PrP degradation and loss of fibrillar structure. The total amount of undegraded PrP (solid line) was determined by Western blots and densitometric analysis and is compared to the proportionate amount of PrP remaining within a fibrillar structure (dot line) which was determined by ThT assays. The PrP 27-30 samples were exposed to heat in 1.5 ml Eppendorf tubes for 20 min. without stirring at the temperatures indicated.

To verify whether the observed loss of fibrillar structure is accompanied by changes on a secondary structural level, circular dichroism (CD) spectra were recorded (**Fig. 2.6**). PrP 27-30 was exposed to temperatures of up to 160 °C in CD buffer with or without 10 % glycerol. To avoid degradation, an incubation time at final temperature of only 30 sec. was chosen. Subsequently, CD measurements were performed at room temperature over the spectral range of 185 nm to 260 nm. It has to be noted, that satisfactory CD spectra were only obtained when PrP 27-30 was sonicated immediately before structural analysis. A possible effect of sonication onto the secondary structure of PrP 27-30 is presumed to be negligible.

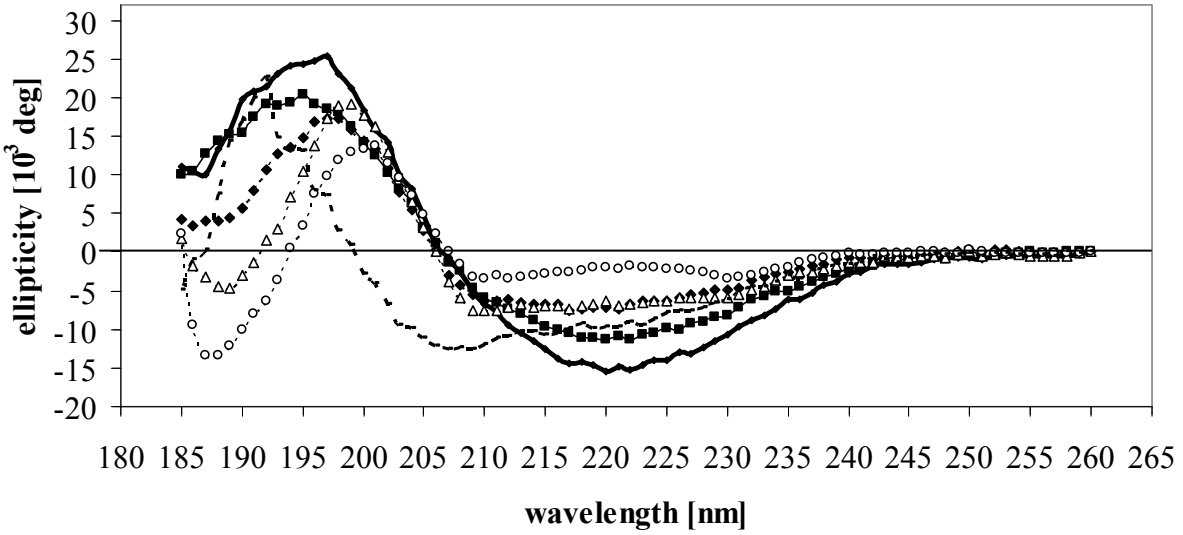

Fig. 2.6: Heat-induced conformational transitions of PrP 27-30 as revealed by circular dichroism (CD) spectroscopy. The CD spectra were recorded in 10 mM sodium phosphate pH 7.2 at room temperature after heat treatment of 1 µg/µl PrP 27-30 in 1.5 ml Eppendorf tubes without stirring for 30 sec. at temperatures of 25 °C (solid line), 100 °C (■), 120 °C (♦), 140 °C (Δ), and 160 °C (○) followed by sonication at 180 W for 5 min. or, for comparison, after sonication at 180 W for 5 min. in presence of 0.3 % SDS without prior heat exposure (dotted line). Below a wavelength of 185 nm an excessive absorption by the buffer impeded further readings.

Exposure to temperatures of up to 100 °C did not change the typical β-sheet spectrum of PrP 27-30 and only diminished ellipticity. As described in **chapters II.2.1** and **II.3.1**, the reduced ellipticity is probable to be the consequence of degradation and a temperature-induced increase in aggregation tendency. After incubation at temperatures of at least 120 °C, a new spectrum appeared comprising two negative peaks at 188 nm and at 210 nm - 212 nm, respectively. The negative peak at 210 nm – 212 nm corresponded to one of the

typical negative peaks of a α–helical secondary structure. Compared to a PrP 27-30 spectrum after α-helix/random coil induction by addition of 0.3 % SDS, however, significant differences were obvious. Most importantly, no change in the crossover point was obtained. Therefore, a sole structural increase in the α-helical content did not explain the experimental results. More likely, the increasing negative peak at 188 nm in addition to a slight red shift of the positive peak advocated the emergence of a β-turn motive. In pure CD buffer but not in presence of 10 % glycerol a negative band at 229 nm - 231 nm was obtained. This peak, however, could not be correlated neither to an increase in α-helices, β-sheets, or β-turns nor to an increase in the random coil content. Since CD spectra consist of a combination of several structural contributions which might even compensate each other, the secondary structure induced by exposure to heat remains to be identified. The results of ThT - assays and CD spectroscopy, however, provided irrevocable evidence for a heat-induced loss of fibrillar structure which is based on an altered PrP 27-30 secondary structure. The CD spectra indicated that the structural alteration in PrP 27-30 might be accompanied by a reduction in the content of β-sheets.

Because proteins normally undergo full unfolding between 40 °C and 80 °C, the CD spectra also substantiated the extraordinary high thermostability of PrP 27-30 which was ascertained in **chapter II.2.1**. Moreover, a comparison with CD spectra recorded in pure buffer demonstrated that in presence of glycerol all structural changes were slightly shifted to higher temperatures (**data not shown**). Consequently, the protective effect of glycerol as determined by a quantitative analysis of prion destruction (**chapters II.2.1** and **II.2.2**) is confirmed by the mechanistic studies presented here.

II.3.3 Heat-altered PrP 27-30 is prone to PK digestion

Exposure to heat led to a decreased solubility of PrP 27-30 due to an increased tendency to form high molecular mass aggregates (**chapter II.3.1**). Heat treatment also reduced the fibrillar structure and induced an alteration in the PrP 27-30 secondary structure towards a probably β-sheet-reduced intermediate PrP form (**chapter II.3.2**). These observations lead to the assumption that the PK resistance of heat-treated PrP 27-30 might also be changed. Upon limited PK digestion, PrP^{Sc} is transformed into its N-terminally truncated but still infectious form of 27 – 30 kDa, denoted PrP 27-30. An accompanying shift in SDS gels of

the PrP^{Sc}-specific bands towards a molecular mass of 27 – 30 kDa confirms the presence of PrP^{Sc}. PrP 27-30 is highly resistant to further digestion but at prolonged exposure to PK can eventually be degraded beyond the typical 27-30 kDa form. After further prolonged PK treatment, a complete digestion was obtained (**data not shown**). PK resistance can consequently be detected by means of the PrP^{Sc} band shift or by means of the band intensity of PrP 27-30. PrP 27-30 was used for all PK digestion experiments because, in contrast to PrP^{Sc}, it represents the most concentrated, stable, and purified form of prion infectivity. The most convincing way to demonstrate that a physical or chemical treatment influences the PK digestibility of prions is therefore by subjecting PrP 27-30 to digestion with PK.

Due to PK digestion of unheated PrP 27-30, both ThT fluorescence intensity and intensity of PrP bands in Western blots decreased evenly. This suggests that fibrillar structure and PrP backbone integrity are concurrently reduced by digestion with PK (**Fig. 2.7**).

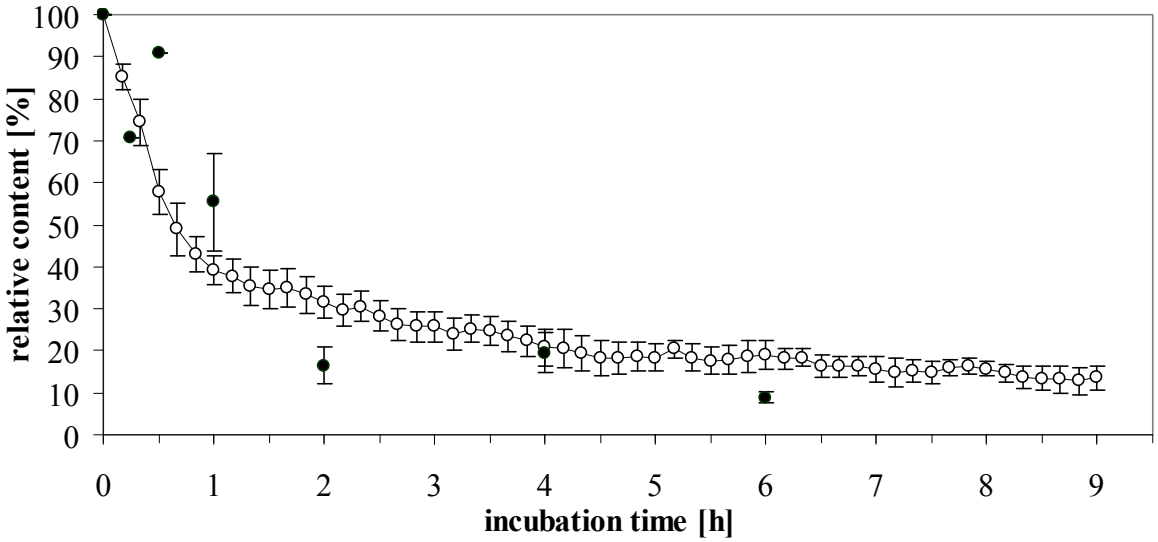

Fig. 2.7: Comparison of reduction in PrP amount and loss of fibrillar structure due to digestion with PK. ThT assay fluorescence (open circles) and Western blot PrP band intensity (filled circles) were determined during PK digestion for the incubation times indicated. Mean contributions (in %) to the initial signals (set as 100 %) before digestion with 50 ng/µl PK at 37 °C in a volume of 50 µl are depicted.

After exposure to temperatures of up to 100 °C, a slightly decreased PK resistance of PrP 27-30 was obtained as detected by Western blots. A reduction in PK-resistant PrP 27-30 of about 20 % was calculated by comparison with unheated PrP 27-30 (**data not shown**). The difference in PK sensitivity is

modest, perhaps because the temperature range inducing a significant increase in PK sensitivity might have not been met experimentally. Nevertheless, the finding is in agreement with **Kuczius *et al.* (2005)** and **Lawson *et al.* (2005)** also claiming that preheated PrP^{Sc} is digested more efficiently. To examine the effect of PK treatment on the fraction of PrP molecules which is still part of a fibrillar structure, PK digestion of heated and unheated PrP 27-30 was compared in ThT – assays. No heat-mediated difference in the PK resistance of PrP 27-30 was observed (**data not shown**). With ThT assays, only the fraction of PrP 27-30 aggregates is measured which is characterised by a fibrillar structure. As demonstrated in **chapter II.3.2**, exposure to heat, however, resulted in the emergence of a new PrP form which is not longer part of fibrils due to an altered secondary structure and a probably reduced content of β-sheets. In contrast to Western blots, this new PrP form is therefore not detectable in ThT assays. Since in Western blots a slightly decreased PK resistance of PrP 27-30 was obtained but not in ThT assays, it is this new PrP form which is concluded to be prone to digestion with PK. All PrP 27-30 molecules which are observable in ThT assays, i.e. all PrP 27-30 molecules with an unaltered secondary structure, remained stable against PK digestion. Consequently, the alteration of the secondary structure of PrP 27-30 due to exposure to heat is probably responsible for the slightly decreased PK resistance. In addition to a causal effect of a reduction in β-sheets, a perhaps weaker binding of structural altered PrP molecules to the prion protein scaffold (**chapter I.1.2**) might also be responsible for the decrease in PK resistance.

II.3.4 Initial extensive decline in prions is followed by slow reduction

If only partial inactivation conditions are applied, a tailing phenomenon, i.e. a rapid decline in prion infectivity followed by a prolonged plateau of slow inactivation, is observed (**Rohwer, 1984; Taylor, 1999; Somerville *et al.*, 2002**). Such a biphasic inactivation curve was obtained when the degradation of PrP 27-30 was analysed for different incubation times under otherwise identical conditions. It became obvious that about 75 % - 95 % of PrP 27-30 were already degraded after reaching the target temperature, i.e. due to the heating and cooling phases. After this initial intensive decline in PrP content, the residual PrP was degraded much more slowly (**Fig. 2.8**).

In accordance with the kinetics of PrP 27-30 degradation, the reduction in prion infectivity achieved for heat treatment at 90 °C in aqueous 90 % fat for

incubation times of 20 min., 60 min., 180 min., and 540 min. did not differ significantly but a residual infectivity titre between 3 $\log_{10}ID_{50}$ and 4 $\log_{10}ID_{50}$ was left. After heat exposure to a temperature of 140 °C in aqueous 90 % fat for 1 min. the infectivity titre was reduced by 4.5 $\log_{10}ID_{50}$. After 20 min. under identical conditions only a further inactivation of 2.4 $\log_{10}ID_{50}$ was obtained. After heat treatment at a temperature of 140 °C in aqueous 10 % glycerol for 1 min. or 20 min., respectively, identical values for inactivation of prion infectivity of 5.4 $\log_{10}ID_{50}$ and 6.2 $\log_{10}ID_{50}$, respectively, were achieved.

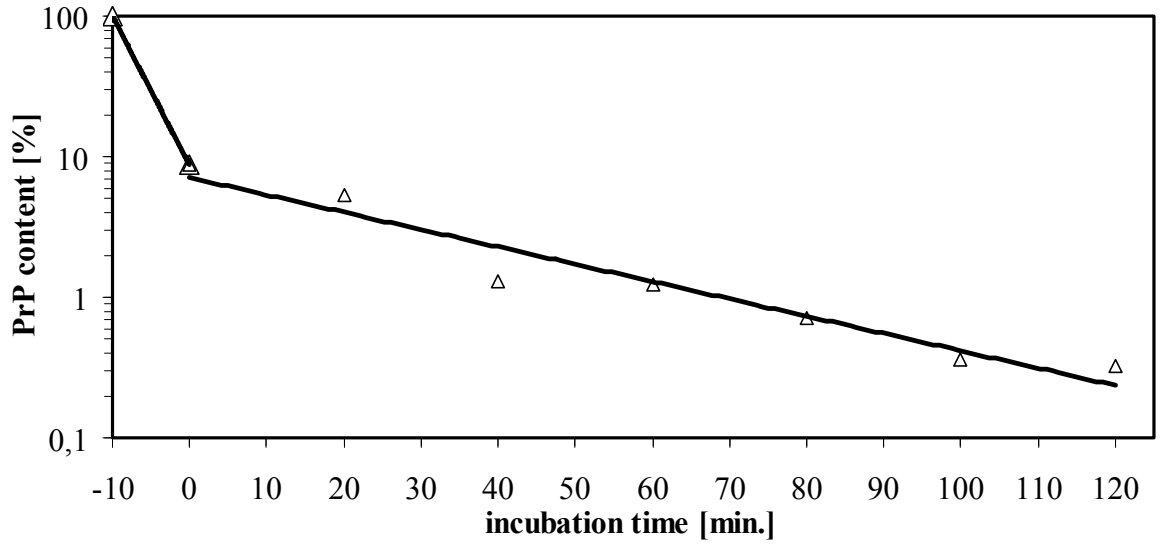

Fig. 2.8: Biphasic degradation curve of PrP 27-30 comprising a fast phase followed by a slow phase. Degradation of PrP 27-30 was determined by Western blots after exposure to heat in a 50 ml pressure steel reactor under stirring at 150 rpm at a temperature of 120 °C in 90 % water and 10 % glycerol for the incubation times indicated. More than 90 % of PrP 27-30 were already degraded due to heating and cooling phases, i.e. after reaching the target temperature (at t = 0).

The results of degradation and inactivation experiments reflect a biphasic destruction curve, i.e. a fast phase during which the majority of prions is destroyed followed by a slow phase of reduced destruction efficiency. Such a tailing phenomenon is explained by a population heterogeneity (**Taylor, 1999**) or by an at least heteromeric structure of the underlying infectious pathogen (**Somerville *et al.*, 2002**). Population heterogeneity implies the existence of at least two subpopulations within one prion sample of different thermostabilities. Although the existence of a small subpopulation of more resistant particles appears to be one reasonable explanation, there are no experimental results to prove this hypothesis. The assumption of an at least heteromeric structure of

prions implies the existence of a thermolabile macromolecular component that is protected by another more resistant macromolecule. The thermolabile macromolecule is believed to be a nucleic acid which is independent of the host and differs in its covalent structure between TSE strains. This virus theory also claims that PrP is only part of a protective nucleocapsid (**Manuelidis, 2004**). Nucleic acids as an essential part of prion infectivity, however, were ruled out by quantitative analyses (**Kellings *et al.*, 1992; Safar *et al.*, 2005**) and excluded finally because of the infectivity of fibrils from pure recombinant PrP (**Legname *et al.*, 2004**). More likely, the presence of a heat-resistant subpopulation results from a heat-induced protective effect due to further intensified aggregation. A rapid decline in prion infectivity is the consequence of a comparatively fast denaturation from the surface resulting in a more resistant aggregate core. The destruction of this core is achieved more slowly and is assumed to be further impaired by intensified aggregation. This assumption is in accordance with the observation that exposure to heat does lead to a decrease in solubility and an increase in the aggregation rate of PrP 27-30 as described in **chapter II.3.1**. As explanation for the presence of a heat-resistant subpopulation of the scrapie agent **Taylor *et al.* (1998)** also stated a protective effect of aggregation which is to occur in homogenates of infected tissue. The stabilisation of a subpopulation may additionally occur due to drying, fixation, or smearing of specimen (**Taylor, 2004**).

II.3.5 Undegraded PrP content is constant regardless of initial PrP amount

Different amounts of prion rods were subjected to heat treatment under otherwise identical conditions. It became obvious that the amount of undegraded PrP was constant regardless of the PrP 27-30 amount initially applied. No degradation was observed when PrP 27-30 amounts smaller than 750 ng were subjected to heat exposure. This result is in accordance with a former study demonstrating that under certain circumstances the amount of BSE or scrapie infectivity that survived inactivation can be relatively constant regardless of the starting titre (**Taylor *et al.*, 1994**). The thermostability acquired is even raised resulting in a decreased further loss of infectivity after heat treatment for a second time (**Taylor *et al.*, 1998**). The survival of prion infectivity is affected by the mode of sample preparation and by the fixation and dehydration state of the

agent but also by surface effects, particularly by smearing on steel surfaces (**Taylor, 1996; Taylor *et al.*, 1998; Taylor, 1999**). Constant infectivity as well as PrP 27-30 titres after thermal treatment may therefore be the consequence of a surface effect and not of an intrinsic property of a prion population. Nevertheless, heat exposure does lead to an increase in aggregation rate and a concomitant decrease in solubility of PrP 27-30 as explained in **chapter II.3.1**. An accompanying acquisition of a more protected state might be feasible. Thus, a type of heat-induced and aggregation-mediated "self-protection" might represent a physico-chemical state more protected against inactivation (**Rohwer, 1996; Taylor *et al.*, 2002; Taylor, 2004**).

II.4 Distribution of prions between fatty and aqueous phases

There is no evidence that BSE infectivity is partitioned preferentially with tallow fractions during rendering. Epidemiological studies failed to find any association between the occurrence of BSE and the feeding of tallow to cattle (**Wilesmith *et al.*, 1991**). An experimental rendering process producing still prion-contaminated meat and bone meal showed no infectivity in the tallow fraction (**Taylor *et al.*, 1997**). Thus, tallow is not regarded to be a causal factor for BSE (**EU, 1992; WHO, 1995; Taylor *et al.*, 1998**). In fact, the prevailing opinion assumes that in spite of the hydrophobic nature of prions and in spite of its notorious insolubility, prion infectivity is inclined to exist rather in a water phase than in a fat phase (**Taylor *et al.*, 1995; SSC, 2003**).

The thermal inactivation of prions depends profoundly on the molecular environment like the fat content of the specimen. Therefore, the distribution coefficient of prions between aqueous and fatty phases is of particular importance. In order to determine the distribution of prions, both the fatty and the aqueous phase of a mixture of equal amounts of bovine tallow and water was analysed for its PrP 27-30 amount at different temperatures. Under all conditions tested, a substantial interphase was obtained. At room temperature, all PrP molecules were found to be restricted to the interphase. Only at increasing temperature, progressively pronounced amounts of PrP 27-30 were enriched in the water phase (**Fig. 2.9**). At all temperatures examined, less than 1.5 % PrP 27-30 was obtained in the fat phase. These findings are in accordance with a former study simulating a solvent extraction process in which also no predilection for scrapie to associate with tallow was found (**Taylor *et al.*, 1998**).

Besides heat, notable amounts of PrP 27-30 were detected within the water phase after addition of sodiumdodecylsulfate (SDS) or after increasing salt concentration. Consequently, at increased temperatures, e.g. in production processes like rendering or hydrolytic fat splitting, prions are expected to accumulate mainly in a hydrophilic environment. On this reason, prion-contaminated meat and bone meal, which represents the hydrophilic phase during rendering, is most probable to have contributed to the emergence of BSE. Since no association between tallow and PrP 27-30 could be found, tallow was rightly put into the lowest category of prion infectivity (**WHO, 1995; SSC, 2003**). In addition, the finding that the presence of fat supports prion inactivation (**chapter II.2.2**) may also have contributed in destroying titres of low infectivity which might have remained within tallow.

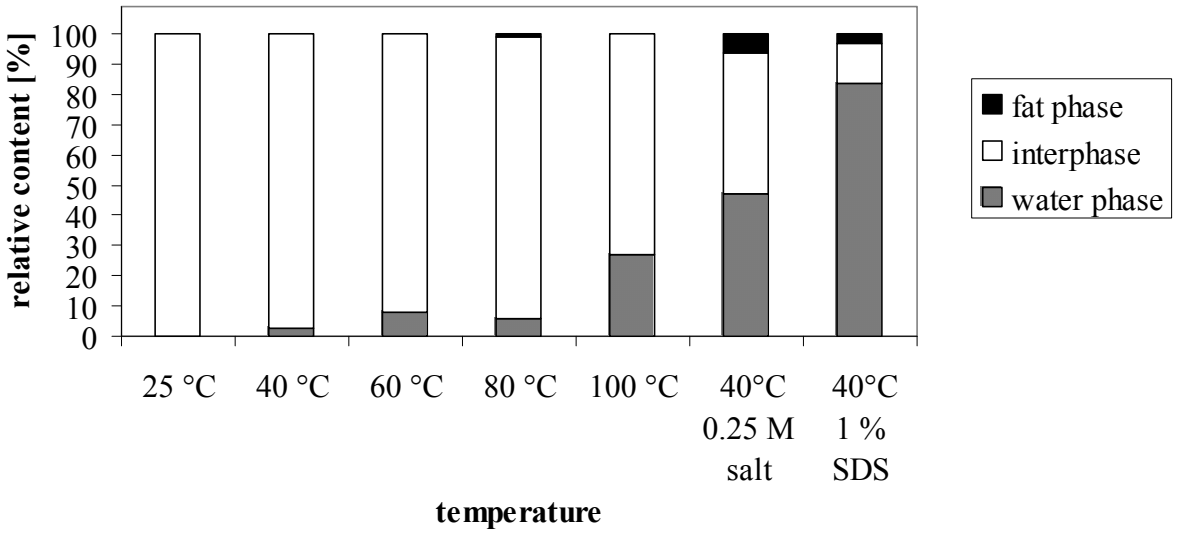

Fig. 2.9: Distribution of PrP 27-30 between fat phase, interphase, and water phase in dependence on temperature or in presence of salt or detergent, respectively. Mean contributions for each phase (in %) to the combined signals (set as 100%) were determined after incubation in 15 ml polymer tubes containing 1:1 mixtures of bovine tallow and water. The specimen were incubated in a water bath until clear phases were achieved (usually for 30 min.) followed by purification of the PrP 27-30 amounts in each phase by methanol chloroform precipitation and analysis by Western blotting and densitometric quantification.

There is no scientific doubt that the use of not properly inactivated meat and bone meal in cattle feeds provoked the BSE epidemic (**Wilesmith *et al.*, 1991; Kimberlin & Wilesmith, 1994; Taylor *et al.*, 1998**). Each animal should have been exposed to the same oral dose of prion-contaminated meat and bone meal. However, only a small number of cattle individuals developed BSE within herds

of hundreds head of cattle. Such a phenomenon is known from end point titrations in which high dilutions of prion infectivity result in disease only in a small fraction of animals inoculated. Accordingly, a small number of BSE-affected cattle is regarded to be the consequence of a low titre of prion infectivity within the meat and bone meal. On the basis of the results presented above it appears reasonable that another factor may have additionally contributed to achieve such a low incidence of BSE cases within a given herd of cattle. During rendering, fat in animal tissues is molten and separated from the remaining solids which are subsequently pulverised to produce meat and bone meal. For melting, elevated temperatures of up to 100 °C are applied. At these temperatures, the majority of prions is restricted to a relatively small interphase between aqueous and fatty phases as demonstrated in **Fig. 2.9**. The corollary is a concentrating effect of prions which might even be further promoted by the tendency of PrP^{Sc} to form high molecular mass aggregates. Due to this heterogeneous distribution of prion infectivity, batches of meat and bone meal were probably produced which did not contain prion infectivity. Other batches, however, contained a concentrated amount of infectivity which represented an effective dietary challenge for cattle. Consequently, only a fraction of all individuals in a herd of cattle became BSE-infected.

Chapter III

Conclusions & Consequences

III.1 Proposed model for prion destruction

The best model of prion structure available at present claims the structural subunit of prions to consist of three monomeric PrP molecules which are attached to each other by short β-sheets (**Fig. I.3**). Two α-helices and the Asn-linked glycosylations are located at the outside. The trimers are stacked on top of each other and are assembled into fibrils (**Govaerts *et al.*, 2004**). The multimeric aggregation is unequivocally associated with a β-sheeted secondary structure causing the partial PK-resistance and insolubility of PrP 27-30. Infectivity has also never been observed with PrP monomers, only with PrP aggregates (*for review:* **Riesner, 2004**). Indeed, loss of infectivity due to the addition of SDS was quantitatively correlated with a decreasing amount of native, β-sheet-like secondary structure (**Safar *et al.*, 1993**). The extraordinary heat stability of prion infectivity is most presumably the consequence of secondary and tertiary structural properties of PrP^{Sc} and its tendency to form aggregates (**Safar *et al.*, 1993; Riesner, 2004; Peretz *et al.*, 2006**).

The experimental data described in detail in the Results and Discussion chapters of this thesis give insights into the molecular mechanism of prion destruction. A systematic interpretation of the unconventional resistance of prions against thermal inactivation will be given on the basis of the molecular interactions involved.

Inactivation of prion infectivity was achieved before PrP backbone integrity was affected (**chapter II.2.3**). This indicated an at least two step mechanism of prion destruction (**Fig. 3.1**).

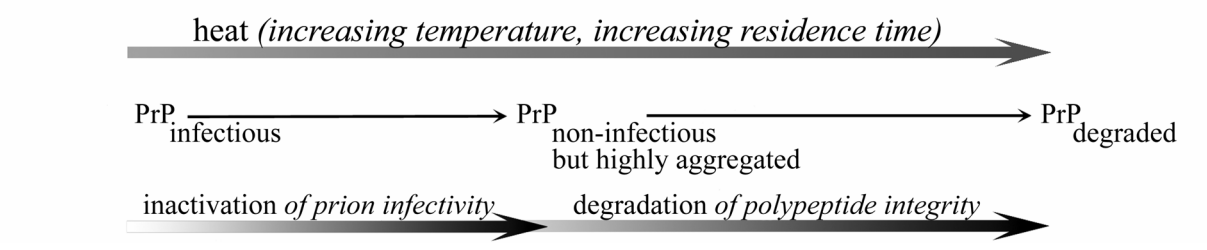

Fig. 3.1: Proposed model for prion destruction.

In a first step, at comparatively low temperatures, infectious PrP aggregates are transformed into a non-infectious form. This form is characterised by an altered secondary structure (**chapter II.3.2**). The intact PrP 27-30 backbone is maintained. In presence of glycerol, the transformation into the non-infectious form is delayed to higher temperatures. In contrast, the presence of fat promotes the inactivation of prion infectivity (**chapters II.2.2**). In the second reaction step at higher temperatures or increased incubation times, the non-infectious PrP form is not longer capable to further resist disassembly. The PrP aggregates are dissociated facilitating the cleavage of the covalent PrP backbone. In presence of fats, fatty acids, or glycerol, the degradation is impaired due to a displacement of water molecules (**chapter II.2.1**).

These findings are reflected by the activation energies for the degradation and inactivation processes (**Fig. 2.3**). The total stability of monomeric proteins amounts to a value of about -15 kJ/mol to -50 kJ/mol which is further increased in case of a polymeric substrate like the fibrillar assembly of PrP 27-30. The activation energies obtained for the first step range from 4.0 ± 1.0 kJ/mol in presence of fat to 19.3 ± 0.1 kJ/mol in pure water or presence of glycerol indicating that only a denaturation process occurs which comprises a loss of mainly non-covalent bonds. The deviating activation energies in presence or absence of fat also illustrate the strong inactivation-supporting effect of fat. High values for the second step ranging from 20 ± 5.3 kJ/mol in pure water over 30.3 ± 3.9 kJ/mol in presence of fat or aqueous glycerol to 51 ± 9.8 kJ/mol in pure glycerol propose that a co-operative reaction occurred after denaturation took place which might comprise a breaking of covalent bonds. An comparison of the activation energies for the degradation processes also reflect the protective effects of fat and particularly of glycerol on the structural integrity of the PrP 27-30 backbone.

From a quantitative comparison of PrP degradation and concurrent loss of fibrillar structure, it became clear that exposure to increasing temperatures induces a reduction in amyloid structure before the amino acid backbone of PrP 27-30 is affected (**chapter II.3.2**). CD spectra demonstrated that reduction in fibrillar structure as detected by ThT assays comprises not only a re-aggregation into unspecified aggregates but also into an intermediate PrP form which is characterised by an altered secondary structure (**chapter II.3.2**). The CD spectra after heat treatment indicated possibly an increase in α-helical

content but more clearly the emergence of a β-turn motive. A clear identification of the heat-induced secondary structure, however, was difficult due to a combination of several structural alterations. Most obviously, the structural alteration proceeds with a reduction in β-sheets.

The alteration in secondary and tertiary structure of PrP 27-30 due to heat exposure has to be introduced by a more or less pronounced partial unfolding reaction leading to a somewhat higher surface proportion of non-polar residues. The molecular rearrangement is enhanced by a dissolving effect of fat which binds tightly to PrP 27-30 backbones on the surface of aggregates (**chapter II.2.1** and **II.2.2**). In contrast, in presence of glycerol a protection of prions was observed because structural alterations are shifted to higher temperatures (**chapter II.3.2**). The transition into a heat-altered and probably β–sheet-poorer state is a reversible equilibrium reaction. Only subsequent chemical modifications or further intensified aggregation of PrP 27-30 shift the chemical equilibrium in favour of the partially unfolded product, i.e. bear the potential to mark the rearrangement process irreversible. From regular intermediate states during protein folding, it is known that aggregation is a frequently experienced hindrance to a correct folding into the native state (**Hlodan, 1991**). Indeed, exposure of PrP 27-30 to heat induces an increased tendency to form high molecular mass aggregates paralleled by a significant reduction in solubility. Although no covalent linkage of aggregates was demonstrated in this study, the resistance of these aggregates to boiling in presence of SDS points to covalent intermolecular interactions (**chapter II.3.1**).

It follows that the reduction in fibrillar PrP 27-30 structure due to limited heat treatment leads to a rearrangement of single PrP 27-30 molecules. This rearrangement results in structurally altered and non-infectious aggregates which are stabilised by hydrophobic interactions and possibly covalent modifications. A protection of still infectious PrP 27-30 molecules by such a rearrangement of more superficial aggregates might provide explanation for a further increased heat stability during a second inactivation cycle and even for the tailing phenomenon, i.e. a trapping of residual prion infectivity (**Rohwer, 1984; Taylor, 1999; Somerville *et al.*, 2002**).

The presence of a tailing phenomenon (**chapter II.3.4**) and of a heat-mediated protective state due to aggregation (**chapter II.3.1**) may lead to the assumption

that the subpopulation of more heat-resistant, re-aggregated particles is also more resistant against digestion with proteinase K. Heat-exposed samples of PrP 27-30, however, are digested more efficiently. All PrP 27-30 molecules with an unaltered secondary structure are still part of fibrils and therefore would remain stable against PK digestion. In contrast, PrP 27-30 molecules with an altered secondary structure were no longer PK-resistant after incubation for 4 h with 50 ng/µl PK (**chapters II.3.3**). For the increase in PK sensitivity due to heat exposure, a reduction in β-sheets or a weaker binding to a ligand such as the prion protein scaffold (**chapter I.1.2**) are conceivable.

A causal correlation between fibrillar structure of PrP aggregates and prion infectivity was demonstrated by **Legname *et al.* (2004)** who created prion infectivity from recombinant PrP by polymerisation into amyloid fibrils. The heat-induced alteration in secondary structure of PrP 27-30 and the reduction in fibrillar structure (**chapter II.3.2**) are accompanied by the major loss of infectivity (**chapter II.2.2**). This suggests that prion infectivity depends on an intact fibrillar structure based on subunit attachment due to β-sheets. It is unproven how prion structure induces disease pathogenesis on molecular level. Several research groups and in more detail **Safar *et al.* (1998)** demonstrated that prion strain properties correlate with different physical properties of PrP^{Sc} like molecular size, glycosylation profile, conformation, stability against denaturation by heat, and stability against degradation by proteinase K. Those studies confirmed that all properties of PrP aggregates such as infectivity, insolubility, PK resistance, and thermostability except a β-sheet–rich secondary structure are restricted to different subsets of PrP^{Sc} molecules. For example, PK resistance could be induced also in other studies without acquiring infectivity (**Post *et al.*, 1998**). Consequently, infectious and non-infectious fibrillar forms of PrP^{Sc} may exist (**Leffers *et al.*, 2005**). On the other hand, it was found that not all forms of cattle-derived infectious PrP^{Sc} are protease-resistant (sen PrP^{BSE}) (**Safar *et al.*, 1998; Birkmann *et al.*, 2006**). In contrast to these properties, a β-sheet–rich secondary structure is regarded to be a hallmark of all PrP^{Sc} fibrils. But even the β-sheet-content seems to differ between PrP aggregates.

After exposure to heat, an intermediate PrP form was identified as outlined in **chapters II.3.2** and **II.3.3**. The biophysical characteristics of this intermediate form are summarised in **Tab. 3.1**. Heat-exposed PrP 27-30 is characterised by low infectivity and an altered and probably β-sheet-reduced secondary structure.

Since secondary structure represents the lowest step in structural hierarchy, an alteration on a secondary structural level is the primary effect which causes changes on higher levels. Due to the altered secondary structure, the tendency of the intermediate PrP form to build fibrillar structures is reduced in favour of unspecified and probably covalently linked aggregates of increased insolubility. Due to the alteration in secondary structure responsible for the loss of fibrillar structure, the PK sensitivity is increased. Consequently, it is reasonable to assume that not only prion infectivity and PK resistance but also thermostability are based on β-sheet-rich aggregates of fibrillar structure.

Tab. 3.1: Biophysical properties of PrP 27-30 in dependence on temperature. The conditions of each analysis are described in the corresponding chapter of the results and discussion section. For comparison, the properties of PrP^C are depicted according to **Riesner *et al.*, 2004.**

	PrP 27-30				PrP^C
temperature [°C]	**below 60**	**80 - 100**	**120 - 140**	**above 160**	
amino acid backbone integrity [%]	100	10-1	0.1-0.01	< 0.01	
prion infectivity [%]	> 10	1-0.1	0.01-0.0001	< 0.00001	not infectious
PK sensitivity	not altered	slightly increased	n.d.	n.d.	not PK-resistant
insolubility in 0.3 % SDS [%]	20	n.d.	75	n.d.	soluble
PrP 27-30 in fibrillar structure [%]	100	60 - 30	20 - 10	< 10	monomeric
secondary structure	β-sheet	β-sheet	reduction in β-sheets, β-turn, α-helix (?)	reduction in β-sheets, β-turn, α-helix (?)	mainly α-helix

n.d. not determined

The relationship between loss of infectivity and alteration towards a probably β-sheet-reduced secondary structure of PrP 27-30 fibrils may provide a simple explanation for prion strains varying in their thermostability. Fibrils are

composed of a wide variety of different proteins but share a common hallmark, namely the β-sheet secondary structure. Nevertheless, fibrils demonstrate a conformational plasticity, i.e. the ability to adopt more than one type of fibrils with conformational differences at the molecular level. While many fibrils described so far are characterised by a parallel β-sheet structure, some have anti-parallel β-sheets (**Meredith, 2005; Anderson *et al.*, 2006**). The existence of several fibril types, i.e. several types of stable tertiary and quaternary folds, implies that they differ in additional or stronger non-covalent bonds. These differences might exclusively be correlated with differences in the β-sheet content or alignment. Accordingly, high thermostability might be associated with a small loss in β-sheet structure and solely the β-sheet content- or alignment-based conformational plasticity accounts for strain differences. Perhaps, different binding capacities of the prion protein scaffold (**chapter I.1.2**) might also contribute to deviating thermostabilities.

It has to be concluded that there is a clear difference between tailing phenomenon, i.e. trapping of prion infectivity, on one side and β–sheet-dependent infectivity , PK resistance, and thermostability on the other side. Exposure to heat induces a structural rearrangement of prions concomitant with a major loss of infectivity and an increase in PK sensitivity leading to an intermediate form of more aggregated and probably covalently-linked, β-sheet-reduced PrP molecules of further reduced solubility which are sufficiently stable to protect still infectious prions. In addition, the efficiencies of PrP 27-30 degradation and inactivation of prion infectivity depend profoundly on the molecular environment, especially on the presence of lipids, fats, or glycerol. A graphical summary of the process of prion destruction in presence and absence of fat and glycerol is given in **Fig. 3.2**.

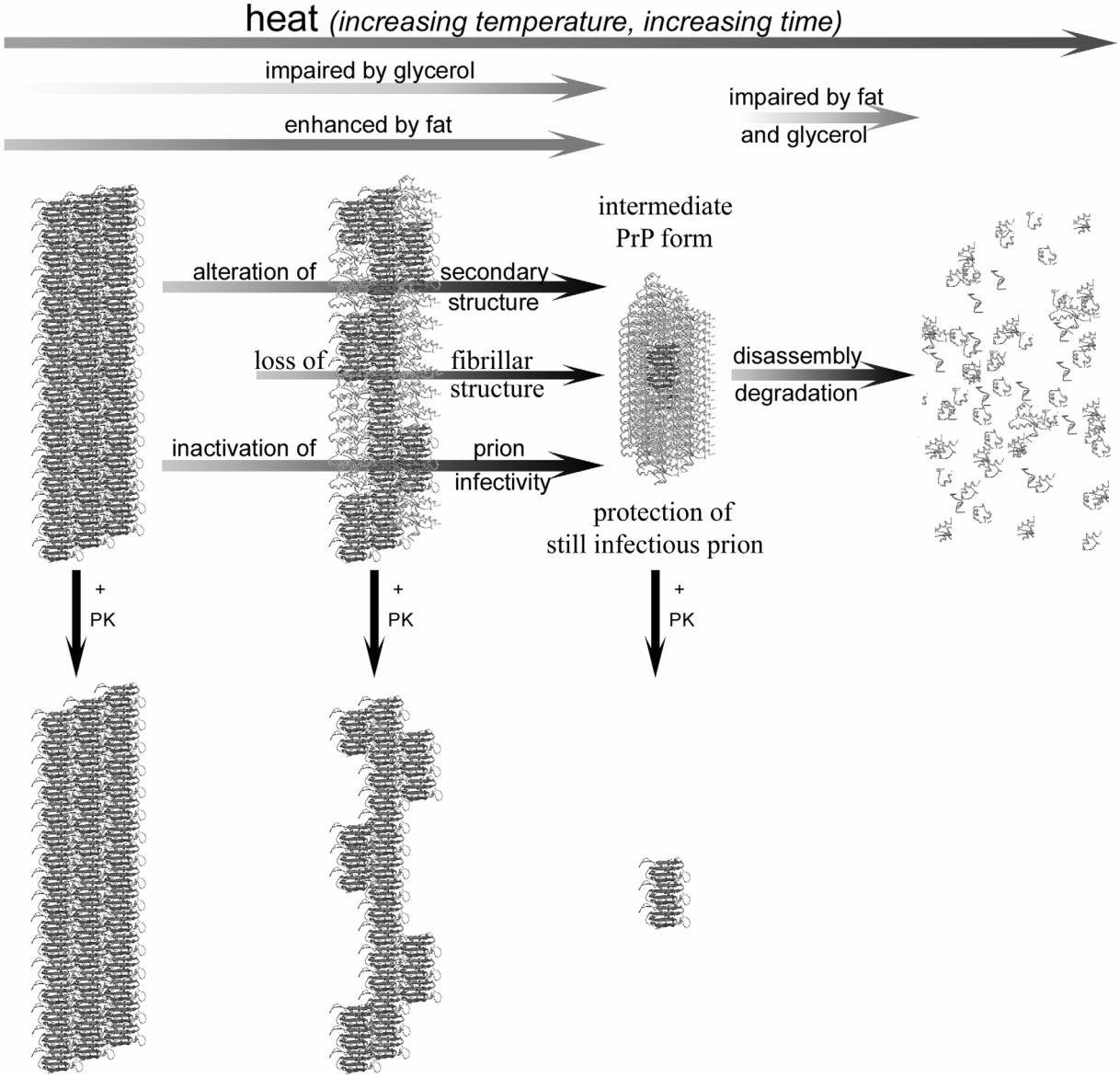

Fig. 3.2: Proposed mechanism for thermal destruction of prions. In a first step, prions are inactivated as consequence of a loss of fibrillar arrangement due to an alteration of secondary structure. In a second step, the polypeptide backbone of the intermediate PrP form is degraded. The biophysical characteristics of the intermediate PrP form are summarised in **Tab. 3.1**.

III.2 Aqueous fat offers a new and mild technology for prion inactivation

Prions are notoriously stable against conventional virucide and bactericide disinfectants and inactivating technologies which are regularly used in laboratories or hospitals. Only strong alkali solutions like sodium hydroxide (NaOH) or oxidising agents like sodium hypochlorite (NaOCl) are capable to achieve a significant elimination of prion infectivity. Nevertheless, for efficient decontamination of potentially high levels of prion infectivity, i.e. to approach or exceed a reduction in infectivity titre of at least six orders of magnitude, a combination with steam sterilisation is recommended (**Taylor, 2000; Fernie *et al.*, 2004; Taylor, 2004**). All suitable treatments for decontamination of prion infectivity, however, are aggressive, with a consequent loss of quality and texture in treated tissues, or not applicable in particular for sterilisation of medical and manufacturing devices of pharmaceutical products. For example, NaOH corrodes aluminium and zinc surfaces, whereas NaOCl corrodes all oxidatively vulnerable metals and thus stainless steel as well. Sophisticated, more expensive equipment such as ophthalmic instruments, fibroscopes, and electrodes is damaged by the recommended methods. In addition to the damaging effect to instruments and equipment, traditional prion disinfectants are mostly hazardous to personnel and clearly not practical for routine decontamination.

In contrast, aqueous and pure fats do unexpectedly offer a mild procedure for prion decontamination. When prion inactivation in presence of fat was analysed at temperatures of 100 °C or less, infectivity titre reductions of up to five orders of magnitude were still obtained. Under standard pure water conditions only a reduction of about three orders of magnitude was achieved. The addition of 0.5 M dithiothreitol (DTT) and 2.25 M urea even further increased the inactivation achieved to seven orders of magnitude (**Fig. 3.3**).

In addition to a significant prion reduction, a simple treatment with aqueous fats at temperatures less than 100 °C offers further advantages such as low energy costs due to low exposure temperatures, a long storage life, no expected health problems for the personnel due to a harmless pH optimum, ecologically beneficial degradability as well as an extensive field of application due to a high compatibility of material. The method can be implemented into existing work

flows simply with minimal additional cost and environmental impact. Thus, due to the necessity of providing alternative sterilisation procedures for TSE risk material, a further assessment of the effects of this mild procedure on prion infectivity is expected to be worthwhile. A patent application was already submitted.

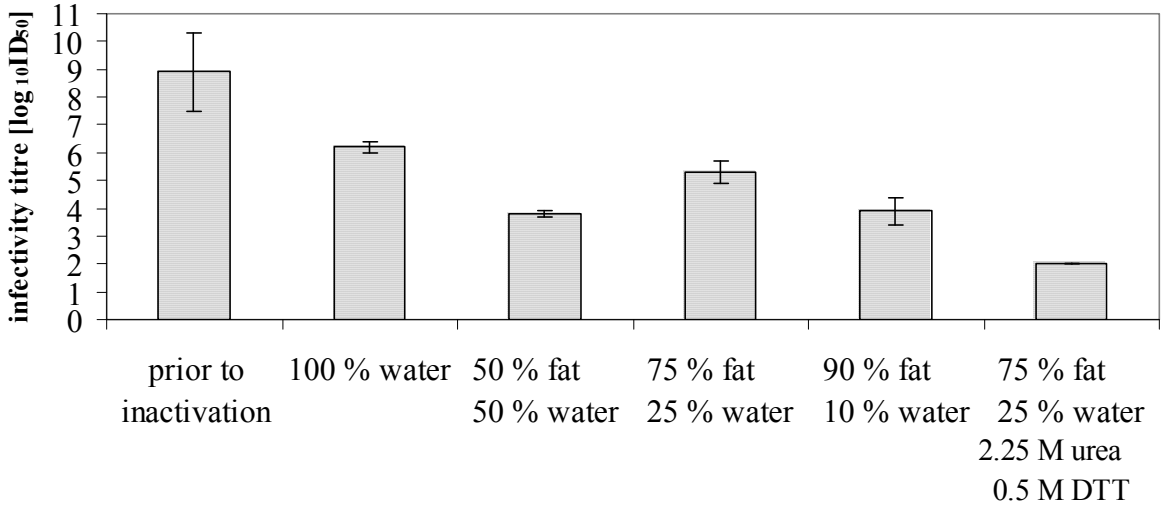

Fig. 3.3: Effect of exposure to heat at a temperature of 90 °C for 20 min. in different fat water mixtures onto inactivation of prion infectivity. Standard mean errors are indicated for all infectivity titres except after inactivation in presence of 2.25 M urea and 0.5 M DTT because in this case the bioassay detection limit of 2 $\log_{10}ID_{50}$ was reached.

III.3 Risk assessment for the industrial processes of hydrolytic fat splitting, catalytic fat hydrogenation, and biodiesel production

As outlined above, the first intention of this study was to derive a systematic understanding of the interactions responsible for heat inactivation of prions which is summarised in **chapter III.1**. The second purpose was to analyse experimentally the safety of basic oleochemical processes regarding an unforeseen contamination with prions.

Since all products of animal origin are potential carriers of prions, the risk of a prion infection as consequence of a given chemical or pharmaceutical product has to be judged on an individual basis (**EU, 1992; Horaud, 1993; Kimberlin, 1996; Rohwer, 1996**). In order to assess the risk of a TSE infection due to a contamination of a given product, the factors below have to be considered with worst case assumptions. The risk can then be deduced in terms of the effective absolute or relative exposure. Since the complete risk assessments are described in detail in **chapters VI.2** and **VI.3**, only a summary is presented here.

(1) The dose of BSE-infected tissue hypothetically contaminating the raw material

Although prion-affected animals as well as specified risk materials are removed from the human and animal food chain (**EU, 1997**), small amounts of residual high-risk material cannot be ruled out in technical processes. The maximum amount of BSE-infected tissue in animal fat, however, cannot be higher than the 0.15 % insolubilities and protein impurities found in Standard European tallow (**APAG, 2005**). From that a dose of 0.0015 g high-risk material per g of animal fat can be deduced.

(2) Prion infectivity within the contaminating tissue

The infectivity titre varies enormously according to the stage of the TSE incubation period and a given tissue of the TSE-affected animal. Therefore, the tissue with the highest BSE titre during the entire incubation period, namely the brain stem, with 1×10^6 i.c. ID_{50} per g brain is used, as obtained from cattle inoculated intracerebrally with bovine BSE-brain homogenate (**Hawkins *et al.*, 2000**).

(3) Prevalence of BSE infection in the source country

Since uniform production batches do not exist in oleochemical industry and

most steps in production are continuous processes, the distribution of prion infectivity within industrial production batches cannot be estimated in a generally valid manner. Instead, a statistically equal distribution of BSE-infected cattle within the industrial production line has to be assumed in a worst case manner. Since the UK is still the country with the highest BSE incidence worldwide, animal fat derived from British cattle represents the worst case-scenario. The prevalence of BSE infection of cattle is supposed to be the sum of all current clinical cases, i.e. 33.4 per million head of cattle as provided by the UK Department of Environment, Food, and Rural Affairs for the time period from November 2005 to October 2006 (**DEFRA, 2005**) and subclinically infected animals, i.e. 3.3 per million head of cattle as estimated by **Arnold & Wilesmith (2003)**. This amounts to a dilution factor of 3.7×10^{-5}.

(4) Prion reduction during industrial processing

The exact industrial conditions of hydrolytic fat splitting, of catalytic fat hydrogenation, and of the biodiesel production process were simulated in the laboratory on a volume-reduced scale. All prion reduction factors calculated are listed in **Tab. 3.2**. Since prion infectivity was inactivated more efficiently under all conditions than the degradation of PrP27-20 occurred (**chapter II.2.3**), the reduction factors for the PrP 27-30 backbone integrity represent the worst case assumption. The reduction factors for inactivation of prion infectivity can be regarded as the most probable assumption.

(5) Dilution during industrial processing

In contrast to hydrolytic fat splitting and catalytic fat hydrogenation, for the production process of biofuels a mixing has to be taken into consideration. According to the European Standard for mineral diesel fuel, biodiesel is only available in a blend of 5 per cent biodiesel and 95 per cent mineral diesel. This amounts to a dilution factor during industrial processing of 5×10^{-2}.

(6) Administration dose

In one production batch might be a single dose of fat, fatty acids, or glycerol (10 g) or a chronic treatment, i.e. a yearly dose (500 g). Due to one accidental transfer of biodiesel, as conservative administration dose a volume of 1 ml might be incorporated. Since consideration of single doses might underestimate the exposure risk whereas an accumulation of single doses to a yearly dose appears to exaggerate the exposure risk (**Kimberlin, 1990; Diringer *et al.*,**

1998; Gravenor *et al.*, 2003; Jacquemot *et al.*, 2005), as conservative measure the larger values are assumed, i.e. a yearly dose of 500 g fats, fatty acids, or glycerol per person and year. This corresponds to one administration of 10 g fat, fatty acids, or glycerol per week. For biodiesel, two events per week are taken into account leading to a conservative administration dose of 100 ml per person and year.

(7) Relative efficiency of a prion infection by the route of administration

For fat, fatty acids, and glycerol an oral uptake represents the worst case assumption. Since the oral exposure of BSE-infected specimen to humans is about 10^5 times less efficient than the intracerebral route of administration (**Kimberlin & Wilesmith, 1994; WHO, 1995; Simon & Pauli, 1998**), on which the data represented in **(2)** are based on, a factor of 10^{-5} is obtained. An oral uptake or even inhaling of prions within biofuels appears to be extremely unlikely. A transfer of an infinitely small fraction of biofuels into eyes, open wounds or cuts, however, cannot be ruled out. As conservative estimation an exposure as efficient as the subcutane route of infection is assumed, yielding a factor of 10^{-4}.

(8) Species barrier

The existence of a species barrier factor of at least one million-fold is established (**Wilesmith *et al.*, 1991; Kimberlin & Wilesmith, 1994; Bruce *et al.*, 1997; EU, 1997; Hill *et al.*, 1997; Anderson *et al.*, 1998; Bons *et al.*, 1999; Lasmezas *et al.*, 2001**). A number of 162 confirmed cases of vCJD in the UK confirmed that the BSE agent is capable to infect humans (**DH, 2005**). For the purpose of risk assessment under worst case assumptions, however, it appears to be reasonable to consider no species barrier.

Final vCJD risk due to prion contamination of fat, fatty acids, glycerol, or biodiesel

The annual probability of contracting a sporadic (s)CJD is one case per million people worldwide. This natural incidence of 1×10^{-6} represents the background risk which has to be compared to the final risks from oleochemical products. In **Tab. 3.2** all risk parameters are listed and final risks are calculated. When risk scenarios are constructed solely from the worst case degradation data, the maximum risk to fall ill with vCJD due to a hypothetical BSE contamination of fat, fatty acids, or glycerol is at least 300 times less than the background risk.

When the infectivity data are taken into consideration, the maximum risks for contracting a vCJD are further decreased to values at least 25,000 times less than the sCJD background risk. For biodiesel, a risk of 2×10^{8} times less is even obtained.

From the risk assessments it can be concluded that the industrial conditions of hydrolytic fat splitting and catalytic fat hydrogenation as well as of the biodiesel production process constitute highly effective means for reducing the risk of a prion contamination to an acceptable minimum. Furthermore, the raw material, i.e. crude tallow, has always undergone several manufacturing steps involving rendering procedures. The manufacturing products are often subjected to further purification steps like distillation. All these additional safety factors are to be multiplied with the final risk factors and thus further add to the safety of tallow derivatives. Consequently, all bovine tallow-derived products can be regarded as absolutely safe, independently of their origin.

Tab. 3.2: Risk assessment for oleochemical products based on reduction factors for the industrial conditions of hydrolytic fat splitting, catalytic fat hydrogenation, and the biodiesel production process.

	hydrolytic fat splitting				**catalytic fat hydrogenation**		**biodiesel production**
	fatty acids		**glycerol**				
	Degra-dation	Inacti-vation	Degra-dation	Inacti-vation	Degra-dation	Inacti-vation	Degra-dation
(1) dose of prion-infected tissue [g per g of fat]	1.5×10^{-3}		1.5×10^{-3}		1.5×10^{-3}		1.5×10^{-3}
(2) prion infectivity [ID_{50} units per g]	1.0×10^{6}		1.0×10^{6}		1.0×10^{6}		1.0×10^{6}
(3) prevalence of TSE infection	3.7×10^{-5}		3.7×10^{-5}		3.7×10^{-5}		3.7×10^{-5}
(4) reduction factors	3.7×10^{-7}	1.3×10^{-7}	1.1×10^{-5}	1.8×10^{-8}	1.7×10^{-5}	1.3×10^{-7}	1.6×10^{-10}
(5) dilution	-		-		-		5.0×10^{-2}
(6) administration dose [g/ml]	500		500		500		100
(7) relative efficiency of prion infection	1.0×10^{-5}		1.0×10^{-5}		1.0×10^{-5}		1.0×10^{-4}
(8) species barrier	-		-		-		-
final risk [ID_{50} units per person and year]	**1.0×10^{-10}**	**3.6×10^{-11}**	**3.1×10^{-9}**	**5.0×10^{-12}**	**4.7×10^{-9}**	**3.6×10^{-11}**	**4.4×10^{-15}**

Chapter IV

Summary

Prions are the causal agents of a series of fatal neurodegenerative diseases in mammals. They are *pro*teinaceous *in*fectious particles, composed primarily of a host-encoded glycoprotein, the so-called prion protein (PrP). In contrast to its non-infectious and mainly α-helical isoform (PrP^{C}), the infectious isoform, designated PrP^{Sc}, forms β-sheet-rich, insoluble, and neurotoxic aggregates which are held together mainly by hydrophobic interactions and exhibit an extraordinary resistance against conventional decontamination methods. To determine prion removal capacities of decontamination procedures in scientific and medicinal facilities and industrial production processes, a detailed understanding of prion destruction on a molecular level is essential.

Within this thesis, a mechanistic model for heat inactivation of prions was established on the basis of detailed biophysical studies and under systematic variation of inactivation conditions. In earlier studies, fat and lipids had been discussed to protect prions against destruction by heat. Therefore, particular attention was directed to the qualitative and quantitative analysis of the influence of fats, fatty acids, and glycerol on thermal prion destruction. Particular emphasis was put on industrial conditions of oleochemical processes. In presence of glycerol, both heat degradation of the PrP^{Sc} core (PrP 27-30) and heat inactivation of prion infectivity were diminished. Fats and fatty acids only exerted a protective effect onto the amino acid backbone integrity of PrP 27-30, whereas prion infectivity was destabilised by addition of fat. For a better mechanistic understanding, the phase distribution of prion particles was analysed, demonstrating that PrP 27-30 accumulates in the interphase of a fat water system but tends to migrate to the aqueous phase at elevated temperatures. The inactivation of prion infectivity by fat water mixtures offers a new and mild technology for prion decontamination.

Under all conditions analysed, a substantial loss of prion infectivity was achieved prior to PrP 27-30 degradation. Systematic studies applying determination of solubility, fibrillisation, secondary structure, PK resistance, and infectivity in dependence upon temperature led to a detailed mechanism of prion destruction. Exposure to heat leads as a first step to stronger aggregated and most probably covalently cross-linked PrP molecules which exhibit a reduced solubility. These heat-induced PrP aggregates are characterised by a secondary structural alteration probably towards a diminished β-sheet content causing a reduction in fibrillisation and PK resistance. It is discussed that rearranged and

non-infectious PrP molecules protect still infectious prions explaining a tailing type of prion inactivation kinetics. At higher temperatures or increased residence times, PrP 27-30 aggregates are not longer capable to resist further disassembly resulting in the degradation of the covalent PrP 27-30 backbone and complete loss of infectivity. It is discussed that the presence of β–sheet-rich fibrils is the key factor not only for prion infectivity but also for the unconventional resistance of prions against decontamination methods.

From the experimental data, the industrially relevant question was addressed if bovine fat derivatives are safe for human consumption with respect to an unforeseen prion contamination. The contribution to prion inactivation by manufacturing conditions of basic oleochemical production processes was discussed within risk assessments concluding that all fat products can be regarded as prion free, independently of their origin.

Chapter V

Zusammenfassung

Prionen sind proteinöse, infektiöse Partikel und stellen die Erreger verschiedener lethaler, neurodegenerativer Erkrankungen von Säugetieren dar. Sie bestehen hauptsächlich aus einem wirtseigenen Glykoprotein, dem sogenannten Prionprotein (PrP). Im Gegensatz zu seiner nicht-infektiösen und hauptsächlich α-helikalen Isoform (PrP^{C}) liegt die infektiöse Isoform des Prionproteins (PrP^{Sc}) in Form β-Faltblatt-reicher, unlöslicher und neurotoxischer Aggregate vor, die hauptsächlich durch hydrophobe Wechselwirkungen stabilisiert werden und sich durch eine außerordentliche Resistenz gegenüber konventionellen Dekontaminierungsmethoden auszeichnen. Um die Effizienz von Inaktivierungsbedingungen zu beurteilen, die in wissenschaftlichen und medizinischen Einrichtungen sowie in industriellen Produktionsprozessen zum Einsatz kommen, ist ein detailliertes Verständnis der Zerstörung von Prionen auf molekularer Ebene von entscheidender Bedeutung.

Auf der Basis detailierter biophysikalischer Experimente und unter systematischer Variation der Inaktivierungsbedingungen wurde im Rahmen dieser Arbeit ein mechanistisches Model zur Hitzeinaktivierung von Prionen etabliert. In früheren Studien wurde diskutiert, daß die Anwesenheit von Fetten und Lipiden einer Inaktivierung von Prionen entgegenwirkt. Deshalb wurde das Hauptaugenmerk dieser Arbeit auf eine qualitative und quantitative Analyse des Einflusses von Fett, Fettsäuren und Glycerol auf die Hitzeinaktivierung von Prionen gerichtet. Besondere Beachtung wurde industriellen Bedingungen oleochemischer Prozesse geschenkt. In Gegenwart von Glycerol war sowohl die Degradation des hoch stabilen PrP^{Sc}-Kerns (PrP 27-30) als auch die Inaktivierung von Prion-Infektiosität verringert. Fett und Fettsäuren dagegen übten einen schützenden Effekt nur auf die Stabilität des Aminosäure-Rückgrats von PrP 27-30 aus, während die Prion-Infektiosität durch die Anwesenheit von Fett destabilisiert wurde. Für ein besseres mechanistisches Verständnis wurde die Verteilung von Prionen in einem Fett-Wasser-System analysiert. Es konnte gezeigt werden, daß Prionen bei Raumtemperatur zwar in einer Fett-Wasser-Interphase akkumulieren, bei erhöhter Temperatur aber eine Tendenz zeigen, in die wäßrige Phase zu wandern. Die Inaktivierung von Prion-Infektiosität durch Fett-Wasser-Mischungen stellt eine neue und vor allem milde Technologie zur Dekontaminierung von Prionen-belasteten Proben, Geräten und Einrichtungen dar.

Unter allen untersuchten Bedingungen wurde eine beträchtliche Verringerung an Prion-Infektiosität erreicht bevor eine Degradation des PrP 27-30-Rückgrats stattfand. Systematische Studien zum Einfluß von Hitze auf Löslichkeit, Fibrillogenese, Sekundarstruktur, PK-Resistenz und Infektiosität führten zu einem detaillierten Mechanismus der Zerstörung von Prionen. Demzufolge resultiert Hitzebehandlung in stärker aggregierten und höchstwahrscheinlich kovalent vernetzten PrP-Molekülen, die sich durch eine reduzierte Löslichkeit auszeichnen. Diese Hitze-induzierten PrP-Aggregate sind durch eine veränderte und wahrscheinlich β-Faltblatt-arme Sekundärstruktur charakterisiert, die ihrerseits eine Verringerung an fibrillärer Struktur und eine Abnahme an PK-Resistenz verursacht. Es wird diskutiert, daß stärker aggregierte und nicht länger infektiöse PrP-Moleküle sogar in der Lage sind, nach wie vor infektiöse Prionen vor weiterer Hitzebehandlung zu schützen, was eine vorgefundene zwei-phasige Inaktivierungkinetik erklärt. Bei höherer Temperatur sind die verbliebenen PrP 27-30-Aggregate nicht länger in der Lage, einer Disassemblierung zu widerstehen. Das führt zur endgültigen Degradation des kovalenten PrP 27-30-Rückgrats und zum kompletten Verlust an Prion-Infektiosität. Es wird diskutiert, daß das Vorhandensein von β-Faltblatt-reichen Fibrillen den Schlüsselfaktor sowohl für Prion-Infektiosität als auch für die außerordentliche Resistenz gegenüber konventionellen Dekontaminierungsmethoden darstellt.

Auf der Basis der experimentellen Daten wurde die Frage erörtert, ob alle Fettderivate bovinen Ursprungs als sicher für den menschliche Verbrauch erachtet werden können. Der Beitrag industrieller Bedingungen grundlegender oleochemischer Prozesse wurde in Form einer umfangreichen Risikoabschätzung diskutiert, die zum dem Schluß kommt, daß alle Fettprodukte unabhängig ihres Ursprungs als Prionen-frei betrachtet werden können.

Chapter VI

Published Results

VI.1 Thermal degradation of prions in presence of fats: implication for oleochemical processes

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1. Summary

Bovine tallow is widely used as raw material for oleochemical processes, i.e. the manufacturing of fatty acids and glycerol and their derivatives. The basic oleochemical process, i.e. the hydrolytic fat splitting with industrial conditions of 200 – 260 °C at corresponding pressure and a minimum residence time of 20 min. is considered to guarantee the safety of all tallow-derived products. As to the present day no experimental data on the safety of fatty acids and glycerol in case of a hypothetical contamination of tallow with TSE agents under technically relevant conditions are available, the intention of this study was to provide quantitative data for the destruction of prions. This short communication reports the first part of research simulating prion inactivation under manufacturing conditions of the upper part of industrial fat splitting columns (fatty acid regime) on a laboratory scale. To establish worst case destruction factors the degradation of prion protein was analysed in dependence upon temperature. The industrial process conditions of the fatty acid regime of hydrolytic fat splitting provide an additional safety factor of at least 1×10^7 confirming that all fatty acids can be regarded as safe irrespective of their origin.

2. Introduction

A variety of fatal neurological diseases of mammals belong to the transmissible spongiform encephalopathies (TSEs) including bovine spongiform encephalopathy (BSE), scrapie in goat and sheep, chronic wasting disease (CWD) in deer and elk, and Creutzfeldt-Jakob disease (CJD) in man. The name TSE indicates that the diseases may be acquired by transmission of an infectious agent. The underlying infective entity is called prion as a short form of 'proteinaceous infectious particle' to indicate that TSE pathogens are composed largely, if not entirely of a protein **[1]**. The only macromolecule demonstrated to be specifically associated with TSE diseases is a host-encoded, hydrophobic glycoprotein called prion protein (PrP). Therefore, TSEs are also named prion diseases. The key event of a TSE infection is that the cellular isoform of PrP (PrP^C) is converted into a disease-associated but chemically identical isoform (PrP^{Sc}) **[2 - 5]**. Upon purification using detergents and limited digestion by proteinase K (PK), PrP^{Sc} is transformed into a N-terminally truncated but still infectious form of 27-30 kDa designated PrP 27-30 which is highly resistant to further PK digestion and forms rod-shaped fibrils, so called prion rods **[6]**.

An established feature of all prions is their strong resistance to decontamination by thermal or chemical procedures which inactivate conventional pathogens **[7].** The unintentional application of inappropriate inactivation methods resulted in the epidemic increase of BSE via consumption of sheep-derived meat and bone meal by dairy cattle **[8, 9]**. Until the late 1970s it was a common practice in UK to additionally subject greaves to a solvent extraction process involving the exposure to hot solvents such as benzene, hexane, heptane and others followed by dry heat and/or steam heat at a temperature of 100 °C before producing meat and bone meal. Although the abandonment of this solvent extraction process by the British rendering industry appeared not to be the key factor for the emergence of BSE **[10]**, it emphasised the importance of suitable decontamination methods. It is known that the efficiency of heat inactivation of conventional microorganisms is impaired by the presence of fat **[11, 12]**. Following Western blot - derived degradation data the heat stability of PrP 27-30 is also raised by the presence of lipids **[13]** which is of particular importance as the fat content of mammalian tissues and particularly of brain is considerably high. Thus, rendering procedures are of particular concern for spreading the disease **[8, 10, 14]**. We will not review rendering procedures in the past concerning the BSE risk. In this study we concentrated on a particular set of reaction conditions applied in the oleochemical industry.

Bovine tallow is widely used as raw material for oleochemical processes, i.e. the manufacturing of fatty acids and glycerol and their derivatives. The total world production of oils and fats amount to 100 million metric tons per year, of which 20 million metric tons are of animal origin **[15]**. The safety of tallow regarding the BSE risk is guaranteed as far as possible by a combination of processing conditions in the rendering process together with the exclusion of high risk tissues comprising specified bovine offal, i.e. skull and spinal cord material **[16]**. So far there is no evidence that BSE infectivity partitions preferentially with tallow during rendering and that tallow has been a causal factor for BSE **[10]**.

The basic oleochemical process is the hydrolytic fat splitting. At industrial scale the hydrolysis of fat yielding crude fatty acids and glycerol is performed in a continuous process, most widely applied in a water-saturated atmosphere. Regarding a hypothetical risk of BSE contamination this industrial process represents an additional processing step beyond a substantial risk reduction by rendering and is considered to lead to tallow derivatives carrying a negligible

risk of BSE transmission. However, to the present day no experimental data on the safety of tallow-derived products – fatty acids and glycerine - in case of a hypothetical contamination of tallow with TSE agents under technically relevant conditions are available. A model study, however, although revealing a significant reduction in PrP showed that lipids are able to protect prions partially against heat treatment **[13]**. Scientific and regulatory publications state that the potential risk associated with a given pharmaceutical or chemical product has to be evaluated individually in the light of the specific circumstances **[17, 18]**.

Therefore, the first intention of this study was to provide quantitative data for the destruction of TSE agents under manufacturing conditions of hydrolytic fat splitting allowing to establish an unequivocal risk assessment for fatty acids, glycerol, and their derivatives. As working with high amounts of infectious TSE material on large scale production units is not acceptable and pilot plants would not reproduce the residence time distribution of an industrial unit, the best possible simulation of prion inactivation in a continuous splitting unit is by separate simulation of the fat and glycerol phases in a laboratory pressure reactor. As large amounts of infectious material would have to be applied to determine the remaining infectivity at high temperatures like 200 °C, all experiments were done at temperatures of up to 140 °C and extrapolated to the actual reaction conditions in the splitting columns. In a first series of experiments the actual process conditions in the upper part of the industrial splitting columns (90 % bovine edible tallow, 10 % water, 200 °C, 20 min.) were simulated and degradation of PrP 27-30 was determined in dependence upon temperature. The lower limit of inactivation i.e. the worst case of inactivation was derived from degradation of PrP corresponding to an approach which was applied earlier **[13]**. This short communication forms the first part of research simulating TSE agent inactivation in an industrial fat splitting column and under hydrogenation conditions as they are applied in industry. This will include bioassays to confirm the destruction of prion infectivity by oleochemical processing.

3. Material and Methods

3.1 Prion protein samples

Prion rods from strain 263K were kindly obtained from Dr. Michael Beekes (Robert Koch-Institut, Berlin, Germany). Their preparation had been described earlier **[19]**.

3.2 Bovine edible tallow

Bovine edible tallow was provided by the European Oleochemicals and Allied Products Group (APAG). The fat had been recovered from adipose tissue and bones obtained at a bovine only slaughterhouse in UK (certified for EU exports). It was produced by dry melting at 100 °C followed by purification of the lipid fraction from tissues and proteinaceous matter by filtration through a diatomaceous earth bed. The free fatty acid content was 0.25 %. Remaining levels of moisture and total insoluble impurities were 0.11 % and 0.018 %, respectively.

3.3 Heat treatment of prions under industrial conditions of hydrolytic fat splitting

In order to mimic the conditions prevailing in the full-scale industrial process as accurate as possible an inactivation system **[13]** was adapted for application of a 50 ml pressure reactor (Parr Instruments Micro Bench Reactor 4591, Moline, Illinois, USA). The reactor vessel was loaded with prion rods in a mixture of 27 ml bovine edible tallow and 3 ml deionised water and heated to a temperature between 40 °C and 140 °C. The start time was taken 3 °C before the target temperature was reached. Irrespective of the reactor content all temperatures were reached within 20 min. After 20 min. at target temperature had expired, the electrical heating was removed and the reactor cooled to 40 °C by partial immersion into cold water. The whole time the reactor content was stirred at 150 rpm to permit a fast-as-possible heating to the target temperature and an even temperature distribution within the reactor vessel.

3.4 Recovery of prions from lipid containing mixtures and quantification by immunoblotting

To quantify the amount of undegraded PrP after heat treatment, a methanol chloroform precipitation based purification method published earlier **[20]** was optimised especially for the quantitative analysis of small amounts of PrP in the presence of a large excess of lipids. The prion protein remaining undegraded after heat treatment was separated in SDS-polyacrylamide gels according to the protocol of **Laemmli [21]**. After electrophoresis the amount of PrP was quantitatively detected by an improved immunoblot comprising staining with a mixture of the monoclonal antibodies 3F4 and R1 and photographic visualisation. The sensitivity threshold of the method was 500 pg. PrP signals were quantified by comparison with at least three standards of known PrP amounts on the same gel which had also been subjected to recovery from an equal lipid-containing mixture. The quantitative evaluation was performed as described by **Appel *et al.* [13]** resulting in degradation factors (DF_{20}), i.e. the ratio between the amount of PrP before heat treatment and the portion of PrP remaining undegraded after heat treatment for 20 min.

4. Results

4.1 Simulation of a large scale industrial process in a small scale laboratory autoclave

In the continuous process of industrial hydrolytic fat splitting the fat is injected at the bottom of the splitting column at a temperature of 100 °C and is heated to reaction temperature by water steam injection. Due to differences in density the fat phase is transported to the column top whereas water entering at the top falls in form of droplets through the slowly rising fat layer in the reverse direction. The products, i.e. fatty acids and a glycerol water mixture, are collected at the column top or column bottom, respectively. The fatty acid phase is withdrawn at a temperature of 120-180 °C, the glycerol water phase at a temperature of 100-140 °C. In the reaction zone located in the column middle the hydrolysis occurs routinely at temperatures between 240-260 °C. To prevent any vaporisation the pressure amounts to 50-60 bar so that air from the outside can be present only in traces. The average residence time of fat in the columns is 105 min. and the minimum time is 20 min. **[22]**.

The continuous counter-current of fat and water phases is simulated in the laboratory autoclave by stirring. The volume to surface ratio in a typical industrial column is about one cubic metre of raw materials to 4.5 square metres of steel surface area. The contact of the reaction mixture with the surface wall is, however, heterogeneous regarding different subvolumes of the reaction mixture. In our laboratory scale autoclave a volume of 30 ml reaction mixture was exposed fairly homogeneously to about 45 square centimetres surface wall. Although the contact was more intensive compared to industrial scale, we did not anticipate a major influence. **Flechsig et al. [25]** demonstrated that a contact time of minutes with scrapie-infected mouse brain suffices to render steel wire highly infectious suggesting that prions are readily bound to stainless steel surfaces. Therefore, in control experiments known amounts of PrP 27-30 were subjected to heat treatment at 40 °C for 20 min. and recovered by the methanol chloroform purification protocol. As shown before **[13]** and confirmed in our study, no loss in the PrP amount was detected demonstrating a quantitative recovery of protein out of the reactor vessel. A minimum PrP recovery rate of 95 % did prove that protein sticking to the inner reactor surface turned out to be of no significance under oleochemical conditions. Additionally, control experiments were carried out using a reactor vessel insert made of teflone demonstrating that the sort of the inner reactor chamber surface material did not influence the PrP degradation (**data not shown**).

As consequence of the continuous character of the industrial splitting process there are no phases of heating up or cooling down for each batch of tallow that is processed. Therefore, for safety considerations only the minimum residence time in an industrial column, i.e. 20 min. was considered for all fat particles. To compensate for the unavoidable degradation during the phases of heating up and cooling down of the laboratory scale reactor vessel, the start time was taken 3 °C before the target temperature was reached. As the time span for increasing the temperature by 3 °C increases the higher the final temperature is but the total residence time of 20 min. was kept constant, degradation during longer phases of heating up was counterbalanced.

It is known that pure water steam conditions as prevailing in industrial units are more effective in achieving a significant reduction in TSE titre compared with a dry heat environment **[23, 24]**. Although industrial columns contain no air other than traces, not the presence or absence of air but the evaporation of a minor

water portion contributes the main factor being responsible for creating a destructive potential as it is also found under standard autoclaving conditions. Nevertheless, for safety considerations it was opted to perform control experiments under an argon only atmosphere of an overpressure of 0.3 bar. As no difference was detected compared with experiments under air atmosphere conditions, the presence of air neither supported nor impeded the degradation of PrP 27-30. As consequence of the air portion remaining within the laboratory scale test chamber, pressure inside the reactor was not identical but similar to the vapour pressure of water at the respective temperature.

4.2 Degradation factors

Heat treatment of prions on the basis of degradation of PrP was analysed for the technical relevant conditions of hydrolytic fat splitting (90 % bovine edible tallow, 10 % water, 200 °C, 20 min.) under worst case assumptions as described in the introduction. Whereas after heating up to 40 °C no loss of PrP was observed, higher temperatures yielded increasing degradation factors. At temperatures of 100 °C and above always less than 5 % of the initial PrP amount remained undegraded. At higher temperatures in addition to the PrP monomer bands an increased tendency to form aggregates of higher molecular mass was observed (**Fig.1**). This finding suggests a random protein splitting accompanied by condensation reactions of PrP peptides with each other, with carbohydrates like the polysaccharide scaffold, the glycosylations, or the glycophosphatidylinositol anchor of PrP, or with components of the bovine edible tallow. As consequence of the purpose to establish worst case degradation factors all PrP-specific signals within a lane were counted. Besides the formation of random aggregates it should be noted that signals at 60 kDa indicative of PrP dimers were detected. Although weak PrP-specific signals at 60 kDa were already present in untreated prion rods and particularly after heat treatment at medium temperatures, at further increased temperatures they disappeared in favour of aggregates at higher molecular mass.

Comparing degradation experiments with and without tallow it became evident that the presence of lipids increases the heat stability of prions by a factor of 10. However, in presence of water the protective effect of lipids decreases with increasing temperature. The obtained degradation factors for heat treatment under conditions of the fatty acid regime are depicted in **Tab. 1**. The data are

also depicted in form of a graph $\log_{10}DF_{20}$ as function of temperature (**Fig.2**). For comparison the new data are presented together with those of **Appel *et al.* [13]** for degradation in 50 ml water and in a mixture containing 50 ml water and 10 g bovine bone fat.

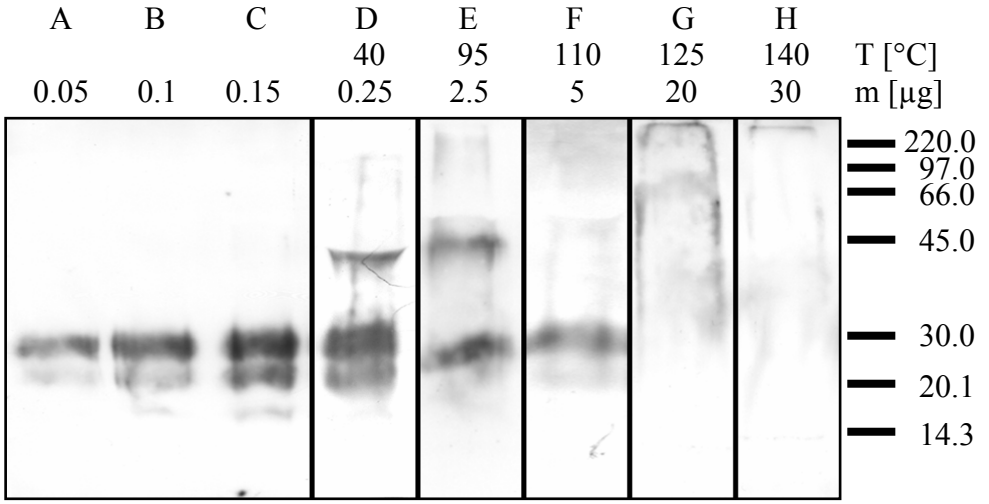

Fig. 1. Thermal stability of prion rods after heat treatment in 90 % bovine edible tallow and 10 % water for 20 min. at different temperatures. On each Western blot membrane the amount of undegraded PrP was quantitatively visualised by a mixture of the monoclonal antibodies 3F4 and R1 and enhanced chemiluminescence. PrP signals were quantified by comparison with three standards of known PrP amounts (**lanes A - C**) on each gel which had also been subjected to recovery from a mixture containing 90 % tallow and 10 % water. It has also to be noted that experiments at higher temperatures were carried out with higher amounts of prion rods in order to determine the higher degradation efficiencies as the detection limit of the immunoblot remained constant (**lanes D - H**). Molecular masses are indicated in kDa.

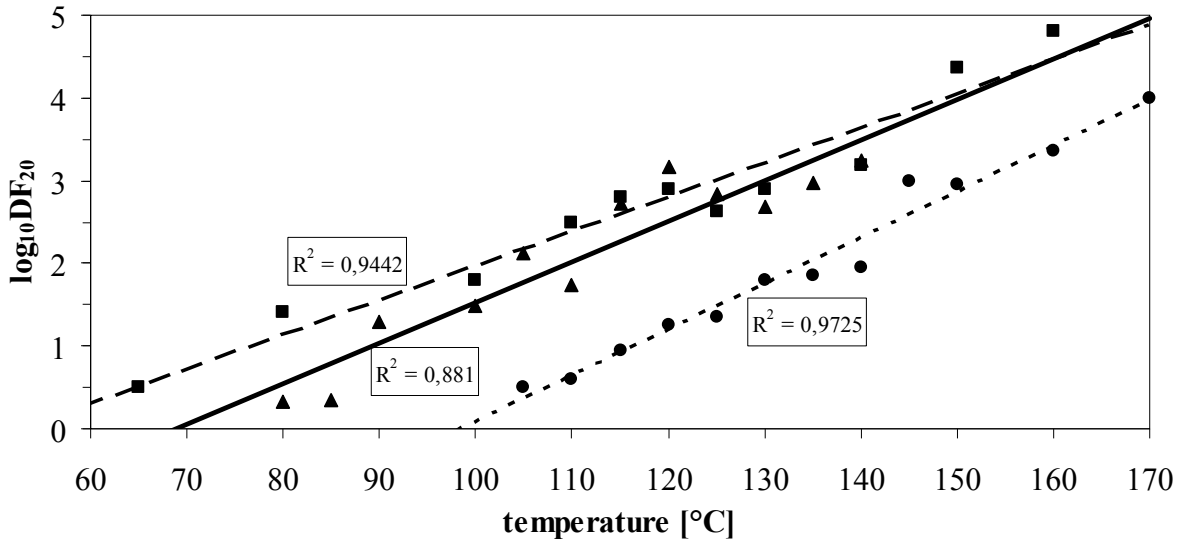

Fig. 2. Logarithmic degradation factors (DF_{20}) obtained after heating prion rods in 90 % bovine edible tallow and 10 % water (▲). The data are plotted with linear regression. For comparison degradation factors for heating prion rods in 100 % water (■, own data and data of **Appel *et al.* [13]**) and in 20 % bovine bone fat and 80 % water (●, data of **Appel *et al.* [13]**) are also depicted.

Tab. 1. Logarithmic values of the degradation factors for prion rods under conditions of the fatty acid regime of hydrolytic fat splitting for 20 min. at different temperatures. The majority of degradation experiments at a certain temperature was repeated up to four times to achieve an accurate degradation curve for extrapolation to higher temperatures. In case of repeated degradation experiments the averaged degradation factors and the mean standard errors are shown.

temperature [°C]	$\log_{10}DF_{20}$
80	0.33 ± 0.04
85	0.35 ± 0.01
90	1.3
95	n.d.
100	1.49 ± 0.28
105	2.13 ± 0.12
110	1.73 ± 0.39
115	2.72 ± 0.17
120	3.16 ± 0.1
125	2.83 ± 0.48
130	2.68
135	2.97
140	3.24

n.d., not determined

5. Discussion

The first part of this study was intended to determine the degree of prion destruction by means of the amount of PrP remaining uncleaved after heat treatment under the industrial conditions of the upper part of fat splitting columns. We decided to use 263K prion rods representing the natural TSE infectivity in a purified and highly-structured aggregated form, since infectivity has never been observed with PrP monomers but is always associated with aggregates of 10,000 - 100,000 PrP molecules per ID_{50} unit **[26]**. Consequently, the real situation, i.e. isolated infectious aggregates embedded in a fat water mixture, was closely simulated. The more customary form of spike material for validation studies has been homogenates or macerates of infected brain tissue **[26 - 30]**. However, **Brown *et al.* [23]** demonstrated that after subjection to steam autoclaving at 134 °C for 30 min. reduction in infectivity titres of fibril preparations were lesser than of whole brain tissue. As prion rods result from purification by extended proteinase K digestion, they represent the most stable fraction of the original infectious material **[19]** allowing to study the absolute worst case challenge with regard to the aspect of product safety. The Syrian Hamster model infected with the hamster-adapted 263K strain of the scrapie agent has been favoured because it is accepted as a well validated, highly sensitive model but which is also highly resistant to decontamination methods **[26 – 32]**.

To demonstrate the efficiency of a decontamination method there are at least two possibilities: at first, a biochemical testing for remaining prions detecting the undegraded PrP as the main if not sole component of the infectious entity; secondly, a direct measuring of scrapie infectivity by detecting clinical symptoms in laboratory animals inoculated with the sample of interest. An assessment of the quantitative relationship between the amount of prion protein and the TSE agent titre in the central nervous system of hamsters orally infected with scrapie revealed a concomitant accumulation of both markers. During the whole course of infection there was a constant ratio of about 10^6 molecules of prion protein per infectious unit **[34]**. In contrast, inactivation conditions of acid hydrolysis do indicate an exponential decay of PrP and of infectivity indeed but prion infectivity was inactivated much more efficiently than PrP was degraded. This suggests a multi-step mechanism of prion inactivation involving a transformation of infectious PrP^{Sc} into a non-infectious form followed by a slower reaction step during which the non-infectious PrP is degraded **[35]**. As an efficient destruction of infectivity is achieved before the integrity of the PrP amino acid sequence is affected, the application of a Western blot assay measuring the PrP degradation made it possible to establish absolute worst case destruction factors. Additionally, an analysis of the industrial conditions of hydrolytic fat splitting by infectivity studies with bioassays is underway.

When bovine edible tallow was heated under industrial conditions we found a significant capacity of tallow to protect prion rods against heat degradation. Whereas after heating up to 40 °C no loss of PrP was observed under all conditions, increasing temperatures yielded increasing extents of degradation and thus increased degradation factors. Within a range of temperatures of up to 140 °C degradation factors of up to $10^{3.2}$ were derived under conditions of the fatty acid regime (**Tab. 1**). The heat degradation data were compared to those obtained in pure water (own data and data of **Appel et al. [13]**) and in a mixture of 20 % bovine bone fat and 80 % water **[13]**. As shown in **Fig. 1** similar tendencies were observed demonstrating that the presence of different kinds of fat protect against heat degradation. However, at higher temperatures the protective effect is always diminished. A quantitative comparison with the data of **Appel *et al.* [13]** is not reasonable because another experimental set up and another type of fat, namely bone fat produced by squeezing bovine bones under mild heating, was applied. Nevertheless, even under varying conditions and with

quite different samples it can be stated that the degradation of PrP is reduced in the presence of lipids.

Extrapolation to real process conditions of 20 min. at 200 °C yielded a degradation factor of about 1×10^7. Consequently, corresponding to our previous results the industrial process conditions of the fatty acid regime of hydrolytic fat splitting represent a huge potential to inactivate prions in the event of a hypothetical contamination. These results provides further confirmation that all fatty acids can be regarded as absolutely safe irrespective of their origin. As prion infectivity is inactivated more efficiently than PrP is degraded **[35]**, our values have to be regarded as absolute minimum factors which even will be further decreased when the results of the infection studies are available. Furthermore, it should be pointed out that the chosen scenario by which tallow has become contaminated with TSE-infected material represents the absolute worse case. Tallow derivatives have always undergone several manufacturing steps involving rendering procedures before hydrolytic fat splitting and several process steps afterwards such as distillation and esterification. All these individual steps further add additional factors to the safety of tallow derivatives.
In addition to the laboratory simulation of hydrolytic fat splitting the hydrogenation of fatty acids will also be studied on the basis of infectivity data in bioassays and PrP degradation data in Western blots.

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VI.2 Risk assessment for fat derivatives in case of contamination with BSE

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1. Summary

Prion diseases are not only of outstanding scientific interest but have also enormous economic impact. In particular the human food and animal feed industry and even oleochemical manufacturing processes are afflicted. In the oleochemical industry bovine edible tallow is widely used as raw material for the production of fatty acids, glycerol, and their derivatives. Although there is no evidence that tallow has been a causal factor for BSE nor that infectivity partitions preferentially with tallow, the potential risk associated with tallow-derived products has to be evaluated in the light of the specific production process. To the present day no experimental data under technically relevant conditions are available on the safety of fatty acids, glycerol, and their derivatives in case of a hypothetical contamination of tallow with prions. A risk assessment calculation is provided here based on quantitative data for the degradation of the pathological prion protein as well as the inactivation of prion infectivity. It can be concluded that the industrial conditions of the basic oleochemical process of hydrolytic fat splitting constitute an effective means for reducing the risk of TSE contamination to an acceptable minimum. All industrial tallow-derived products can be regarded as safe, independently of their origin.

2. Introduction

Bovine spongiform encephalopathy (BSE) belongs to a group of fatal neurodegenerative diseases of mammals known as transmissible spongiform encephalopathies (TSEs). These also include scrapie in goat and sheep, chronic wasting disease (CWD) in deer and elk, and Creutzfeldt-Jakob disease (CJD) in man. The name transmissible spongiform encephalopathy indicates that the diseases are transmissible by an infectious agent. For CJD also a sporadic or genetic etiology is known. In 1982 it was proposed that the infectious entity is not a conventional agent but a proteinaceous infectious particle named prion **[1]**. The proteinaceous character and the complete absence of nucleic acids are the reasons for unforeseen problems in epidemiology, diagnostics, and decontamination of prions **[2]**. These medical problems are not only relevant for medicine but also of economic impact for the human food and animal feed industry and even for oleochemical manufacturing processes. In the oleochemical industry animal fats like bovine edible tallow are utilised as raw material for the worldwide production of fatty acids, glycerol, and their

derivatives. So far, safety restrictions were based on worst case extrapolations but not on quantitatively evaluated experimental data. The purpose of this study is therefore to determine experimentally the inactivation of prions under conditions of the basic oleochemical process of hydrolytic fat splitting.

The infectious agent consists to a major portion, if not entirely of a host-encoded, hydrophobic glycoprotein, the so-called prion protein (PrP). As key event of a prion infection the cellular isoform of PrP (PrP^{C}) is converted into a disease-associated isoform (PrP^{Sc}) thereby amplifying this infectious isoform **[2 - 4]** Both isoforms are chemically identical but differ in their biophysical properties like solubility and secondary and tertiary structure. Upon purification using detergents and limited digestion by proteinase K, PrP^{Sc} is transformed into a N-terminally truncated but still infectious form of 27-30 kDa designated PrP 27-30 which forms rod-shaped fibrils, so-called prion rods **[5]**. Prion rods contain also small amounts (~ 1 %) of specific lipids **[6]** and larger amounts (~ 10 %) of a polyglucose scaffold **[7, 8]**.

A well known feature of prions is their strong resistance to thermal and chemical procedures which inactivate conventional pathogens **[9]**. The survival of prion infectivity is affected further by the mode of sample preparation **[10]**, by the fixation and dehydration state of the agent **[11]**, and by surface effects, particularly on steel surfaces **[12]**. It is known that the heat inactivation of conventional infectious agents is impaired by the presence of fat **[13, 14]**. In a similar way, the stability of PrP 27-30 against hydrolysis is raised remarkably by the presence of lipids **[15]**. This finding is of particular importance for oleochemical production when bovine edible tallow is processed to fatty acids and glycerol at high temperatures. Consequently, it cannot be excluded *a priori* that under those processes residual infectivity from a hypothetical contamination with prions might be left within the products. The safety of tallow regarding the BSE risk is guaranteed as far as possible by a combination of stringent conditions in the rendering process, the exclusion of BSE-affected animals from the food and feed chains **[16]** and the exclusion of high risk tissues comprising skull and spinal cord material **[17]**. There is no evidence that BSE infectivity is partitioned preferentially with tallow fractions during rendering or that tallow has been a causal factor for BSE **[18]**. The hydrolytic fat splitting process represents an additional step and is considered to lead to tallow derivatives being declared fit for human consumption. However, until now no experimental data

on the safety of tallow-derived products from industrial processes in case of a hypothetical contamination with prions under technically relevant conditions are available. Although a model study revealed a significant reduction, the presence of lipids was shown to protect partially the structural integrity of PrP 27-30 against heat treatment **[15]**. The potential risk associated with a given pharmaceutical or chemical product has to be evaluated individually in the light of the specific process conditions **[19, 20]**. Therefore, the first intention of this study is to provide quantitative data for the destruction of prions under manufacturing conditions of hydrolytic fat splitting permitting an unequivocal risk assessment for fatty acids, glycerol, and their derivatives. The aim is not to simulate the infectivity titres within tallow as they could have been prevailing during the BSE epidemic or prevail currently, but to deduce quantitative clearance factors for the industrial process of hydrolytic fat splitting under worst case assumptions regarding a hypothetical contamination with prions.

Whereas in earlier work under related conditions only the lower limit of inactivation was estimated by determining the yield of peptide backbone degradation of PrP **[15, 21]**, we analysed both the degradation of the PrP peptide backbone integrity and the inactivation of prion infectivity under the conditions of the industrial process. In a parallel work **[H. Müller, L. Stitz, D. Riesner, in preparation]** more general studies on the influence of fat components on the heat inactivation of prions were performed. From these extensive data based on inactivation of prion infectivity and degradation of PrP more insight into the molecular mechanism of prion inactivation will be achieved, and the inactivation efficiency might be extrapolated to other processes or physical and chemical treatments.

3. Material and Methods

3.1 Prion protein samples

Prion rods from the scrapie strain 263K were kindly obtained from Dr. S. B. Prusiner (University of California, San Francisco, USA) and were prepared from terminally scrapie-sick Syrian Hamsters as described earlier **[22]**.

3.2 Bovine edible tallow

Standard European bovine edible tallow was provided by the European Oleochemicals and Allied Products Group (APAG). The fat had been recovered from adipose tissue and bones on an industrial scale by dry melting at 100 °C followed by filtration.

3.3 Heat treatment under industrial conditions of hydrolytic fat splitting and recovery of prions from lipid and glycerol containing mixtures

An inactivation system **[15]** was adapted for a 50 ml pressure reactor comprising an external electric heating, temperature control, and magnetic stirrer (Parr Instrument Company reactor 4591 with Parr 4842 controller). The reactor vessel was loaded with prion rods in 27 ml bovine edible tallow and 3 ml water (fatty acid regime), or 26.3 ml water, 2.9 ml glycerol, and 0.75 ml bovine edible tallow (glycerol regime), or 27 ml Tris HCl buffer pH 11.15 and 3 ml glycerol (pH 9 treatment) and heated to a temperature between 40 °C and 200 °C. The start time was taken 3 °C before the final temperature was reached. Dependent upon the reactor content all temperatures were reached within 30 min. The pressure inside the reactor was similar to the vapour pressure of water at the respective temperature. After the residence time had expired, the reactor was cooled to 40 °C by immersion into cold water. To quantify the amount of prions after heat treatment, a purification method being based on a methanol chloroform precipitation **[23]** was optimised especially for the quantitative analysis of small amounts of prions in the presence of a large excess of lipids and glycerol. As described in more detail by **Müller and Riesner [21]** no loss in the amount of PrP was detected demonstrating a quantitative recovery of protein out of the reactor vessel. A minimum PrP recovery rate of 95 % was achieved. The infectivity titre was also not decreased after methanol chloroform precipitation of prion rods subjected to 40 °C for 20 min.

3.4 Quantification of heat-treated prions by immunoblotting and incubation time interval assay

The prion protein remaining undegraded after heat treatment was separated by SDS-polyacrylamide gel electrophoresis according to the protocol of **Laemmli**

[24]. The amount of undegraded PrP was quantitatively detected by an improved immunoblot comprising staining with a mixture of the monoclonal antibodies 3F4 and R1 and enhanced chemiluminescence. The sensitivity threshold of the method was 1 ng PrP determined by dilution series of PrP 27-30. Developed films were digitised at a resolution of 300 d.p.i., 256 grays, and saved as uncompressed TIF files. For densitometric quantification, the analysis software Scion Image 4.0.2 was used (free download from http://www.scioncorp.com) permitting to translate size and density of PrP-specific bands into a distribution function. The integrals of PrP-specific bands were quantified by comparison with at least three standards of known PrP amounts on the same gel which had also been subjected to recovery from an equal lipid-containing mixture.

Bioassays of prion infectivity were performed by inoculation of weanling female Syrian gold hamsters at an age of 34-38 days. After heat treatment of 15 µg prion rods and recovery from the fat glycerol water mixture, each sample was suspended in phosphate-buffered saline (PBS) pH 7.4 to a final volume of 600 µl. Samples were quick-frozen by immersion in liquid nitrogen and stored at –80 °C. For each determination of remaining infectivity, five animals were inoculated intracerebrally with 50 µl of a given specimen and examined for the development of clinical neurological disease at least twice a week. Only coded information was displayed on hamster boxes to avoid observer bias. The bioassays were terminated 390 days after inoculation. Prion titres were calculated by measuring the incubation time intervals from inoculation to onset of clinical symptoms **[25]**. In order to determine worst case reduction factors for the inactivation of prion infectivity the underlying dose response curve was utilised for incubation times up to 140 days. In case of incubation times longer than 140 days, the corresponding animals were not included in the calculation of the inactivation factors. Instead, the bioassay detection limit of log2 ID_{50} was used. This is a conservative treatment to avoid an overestimation of the reduction achieved. The infectivity titre of a specimen that did not infect all animals of a group is at limiting dilution and can therefore be estimated more accurately from the Poisson distribution by the limiting dilution method **[26]**.

The quantitative evaluation was performed as described by **Appel *et al.* [15]** resulting in reduction factors for a residence time of 20 min. at different temperatures (RF_{20}) i.e. the ratios between the amount of PrP or prion infectivity, respectively, before and after heat treatment. The solid lines in **Fig. 2**

and **Fig. 3** represent mean values of all data points which are within the experimental error always in accordance with a linear dependence of $\log_{10} RF_{20}$ on temperature and residence time, respectively.

4. Results

4.1 Simulation of a large scale industrial process in a small scale laboratory autoclave

The basic oleochemical production step is the hydrolytic fat splitting. The continuous process of industrial hydrolytic fat splitting yielding fatty acids and glycerol and its simulation on laboratory scale was described in detail by **Müller & Riesner [21]**. Briefly, the best possible simulation of prion inactivation in a continuous splitting unit is by considering generally valid minimum conditions and simulating separately the industrial conditions prevailing in the upper part (fatty acid regime: 90 % tallow, 10 % water, 200 °C) and the lower part (glycerol regime: 87.75 % water, 9.75 % glycerol, 2.5 % tallow, 140 °C) of fat splitting columns. For safety considerations, the minimum residence time of all particles, i.e. a incubation period of 20 min., was assayed. To compensate for the unavoidable inactivation during the phases of heating up and cooling down of the laboratory scale reactor vessel, the start of the total residence time of 20 min. was taken 3 °C before the target temperature was reached. Since the time span for increasing the temperature by 3 °C is longer, the higher the final temperature is, inactivation during longer phases of heating up was counterbalanced. As shown earlier **[27]**, the lower limit of inactivation, i.e. the worst case of decontamination of prion infectivity, can be derived from degradation of PrP 27-30. When in laboratory small scale experiments the degradation of PrP 27-30 for the upper part of fat splitting columns will be determined, the industrial temperature of 200 °C cannot be met. Due to a constant Western blot detection limit, very high amounts of purified prion rods would be required to detect PrP remaining undegraded after heat treatment under such high temperatures. Unacceptable high numbers of animals would have to be used for those experiments. Consequently, PrP 27-30 reduction factors higher than log 3.5 could not be obtained by means of the Western blot technique and the RF_{20}-value for degradation of PrP 27-30 under industrial conditions of the fatty acid regime of hydrolytic fat splitting at 200 °C was obtained by extrapolation from continuous temperature dependencies measured

at lower temperatures. Due to the availability of high infectivity titre prion rods and a bioassay detection limit of 2 $\log_{10}$ ID_{50}, infectivity assays could be performed even under industrial temperatures as high as 200 °C and infectivity reduction factors of up to 6.9 $\log_{10}$ ID_{50} could be detected. The study presented here does therefore not only analyse the worst case reduction factors obtained by determining the degradation of PrP, but also the biologically more relevant factors which are the reduction factors of prion infectivity determined in bioassays.

4.2 Inactivation of prions in the fatty acid regime and the glycerol regime of hydrolytic fat splitting

In **Fig. 1** representative examples for the determination of PrP 27-30 reduction factors are given. The PrP-specific triplet bands on the Western Blot membranes reflect different levels of glycosylation of PrP 27-30. At increasing temperatures a decrease in the three PrP monomer bands was obtained paralleled by an increasing intensity of random aggregates of higher molecular mass. Besides the formation of random aggregates, PrP-specific bands at 60 kDa represented PrP dimers which were already present in untreated prion rods but were especially prominent after heat treatment at medium temperatures. At higher temperatures they disappeared in favour of aggregates of higher molecular mass. In order to determine the lower limit of degradation all PrP-specific signals within one lane were added up. Since the error limits of the Western blot densitometry amounted to 10 %-15 %, the majority of degradation experiments at a certain temperature was repeated until reproducible PrP 27-30 degradation factors were achieved which allowed us an extrapolation to higher temperatures. The mean standard error of all degradation data points amounted to a value of $\log_{10}$ 0.048.

In **Fig. 2** reduction factors after heat treatment of prion rods under the conditions of industrial hydrolytic fat splitting are shown. The data are depicted in form of a plot of logarithmic reduction factors ($\log_{10} RF_{20}$) as function of temperature. As shown earlier **[15]**, the thermodynamically exact linear interpolation has the form $\ln \log_{10} RF_{20}$ as function of 1/T if a single activation energy is assumed. However, this assumption is not guaranteed and within the limits of error the data can also be interpolated by a straight line in the way of **Fig. 2**. Thus, that interpolation has to be regarded as an empirical relationship with sufficient correlation which is easy to manage. The original data from the titration

experiments, i.e. mortality rates and mean incubation times, are presented in **Tab. 1**.

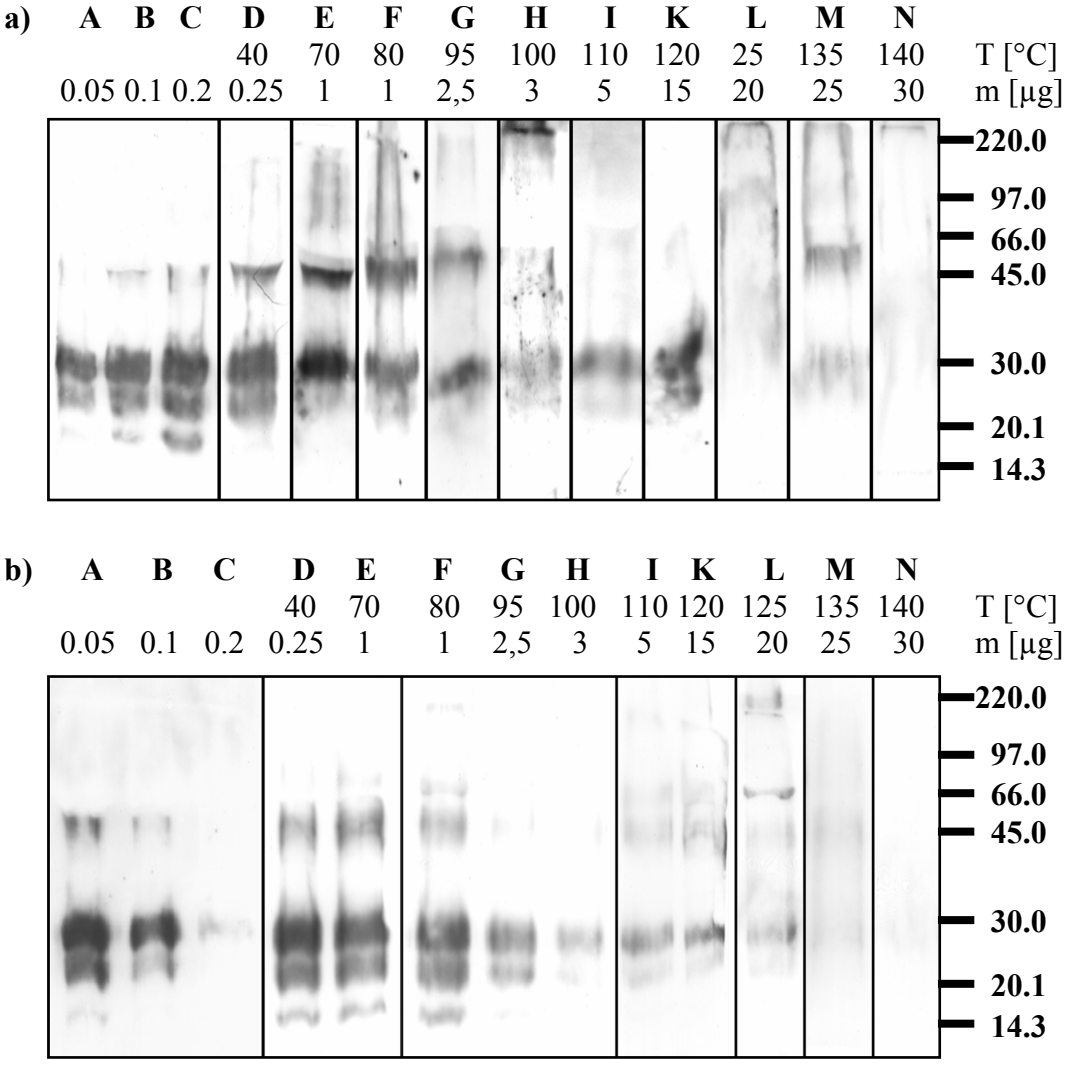

Fig. 1: Thermal stability of prion rods after heat treatment under conditions of the fatty acid regime (a) and the glycerol regime (b) of hydrolytic fat splitting at the temperatures indicated. Experiments at higher temperatures were carried out with higher amounts of prion rods in order to bring the amount of remaining PrP into the calibration range of the PrP standards (lanes **A - C**) and to determine the higher degradation efficiencies (lanes **D - N**) as the detection limit of the immunoblot remained constant. The standards comprise known amounts of prion rods which have also been subjected to a methanol chloroform precipitation from an equal fat glycerol mixture. Molecular masses are indicated in kDa..

The degradation and inactivation efficiencies are increasing with increasing temperature. The temperature dependence of both parameters, however, is quite different. Within the temperature range analysed the inactivation of prion infectivity is always achieved much more effectively than the degradation of PrP 27-30 occurs. Comparing the reduction factors of both regimes, the glycerol regime is characterised by a much lower potential to destruct prions by heat

treatment, especially when inactivation is analysed. All data points (exception at 200 °C, cf. below) can be interpolated by a linear dependence. The nearly temperature independent inactivation in the fatty acid regime is a consequence of the limit of the titre determination by incubation time assay. The incubation times increase the higher the temperature is demonstrating enhanced inactivation.

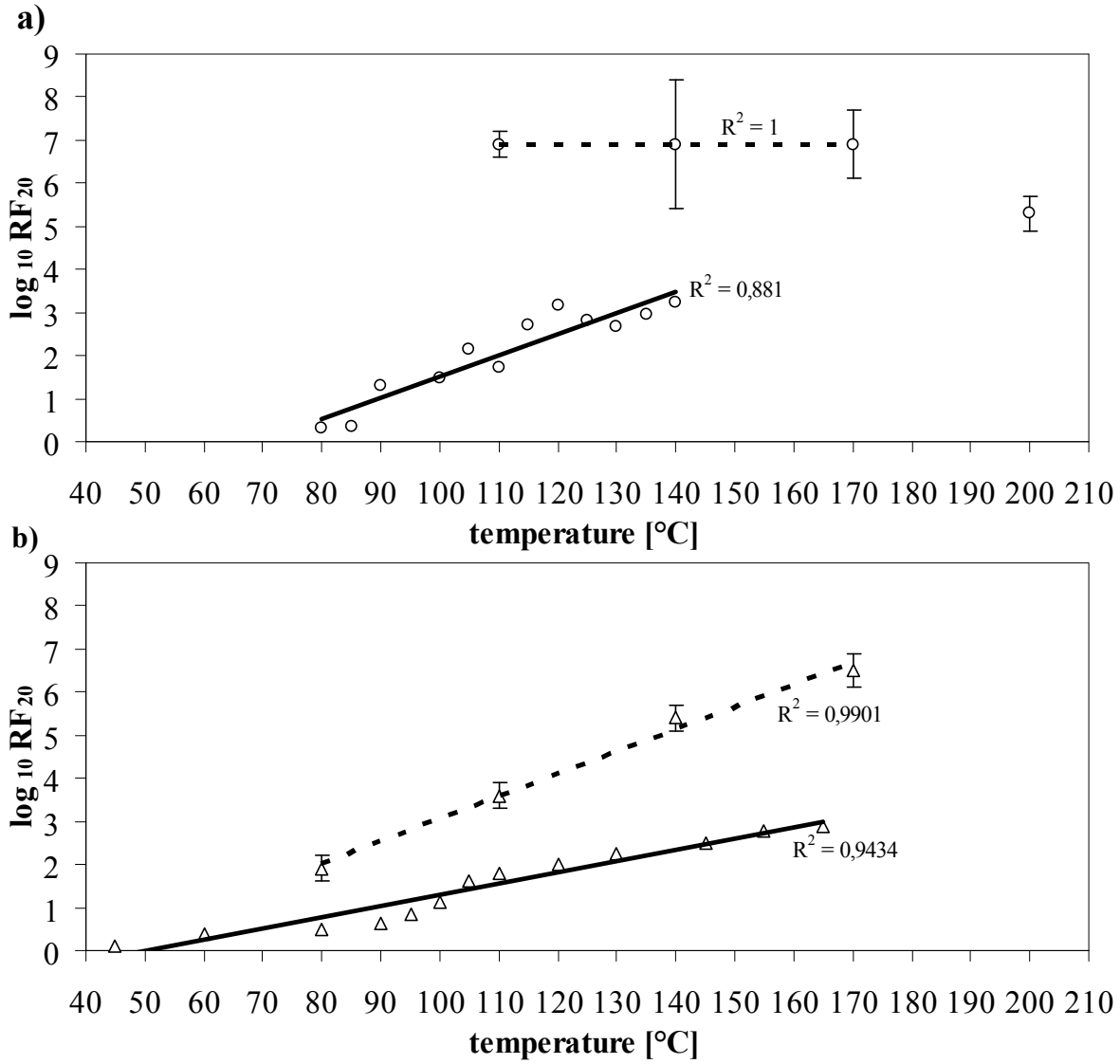

Fig. 2: Logarithmic reduction factors (RF) comparing degradation of PrP 27-30 and inactivation of prion infectivity obtained after heating prion rods under conditions of both the fatty acid regime (a) and the glycerol regime (b) of hydrolytic fat splitting at the temperatures indicated. The degradation data (solid lines) as well as the inactivation data (dotted lines) are plotted with linear regression. Standard mean errors are indicated for each inactivation data point. For the calculation of mean incubation times only diseased hamsters were utilised. Consequently, when also regarding surviving hamsters the reduction factor after heat treatment under industrial conditions of the fatty acid regime at a temperature of 140 °C and 200 °C would be substantially higher since not all hamsters were clinically affected. The reduction factor after heat treatment at 200 °C represents the mean of both bioassays (**Tab. 1**). The degradation data in (a) are taken from **Müller & Riesner, 2005**.

Whereas all reduction factors increase with increasing temperature, the factor for reduction of infectivity at 200 °C is not in accordance with a monotonic behaviour. Under all other conditions tested **[H. Müller, L. Stitz, D. Riesner, in preparation]**, the inactivation at higher temperatures was in agreement with a linear interpolation from lower temperatures. The literature does also not indicate a decreasing inactivation rate with increasing temperatures. A repetition of the measurement at 200 °C in a completely independent experiment confirmed the first result. Nevertheless, this data point is considered seriously in the discussion chapter.

Tab. 1: Effect on the 263K strain of scrapie agent of heat treatment at the temperatures indicated under industrial conditions of the fatty acid regime and the glycerol regime of hydrolytic fat splitting. For calculation of mean incubation periods, infectivity titres, and standard errors only diseased animals were utilised.

treatment	No. infected/ No. injected	mean incubation period ± SE [days]	infectivity titre ± SE [log ID_{50} per sample]
negative control (50 % tallow/ 50 % glycerol, 200 °C, 20 min.)	0/5		-
positive controls			
starting titre	5/5	75 ± 0	7.7 ± 0.0
after sonification	5/5	73 ± 3	8.0 ± 1.1
after MeOH/$CHCl_3$ precipitation and sonification	5/5	68 ± 3	8.9 ± 1.4
fatty acid regime/ 20 min.			
110 °C	5/5	146 ± 7	<2.0
140 °C	2/5(†)	208 ± 67	<2.0
170 °C	5/5(*)	297 ± 54	<2.0
200 °C	5/5	117 ± 6	3.2 ± 0.4
200 °C	4/5(‡)	115 ± 5	<3.4 ± 0.4
glycerol regime/ 20 min.			
80 °C	5/5	80 ± 2	7.0 ± 0.3
110 °C	5/5	94 ± 2	5.3 ± 0.3
140 °C	5/5	114 ± 4	3.5 ± 0.3
170 °C	5/5	130 ± 7	2.4 ± 0.4

(†) The three hamsters remaining healthy after 390 days were negative for Western blot PrP after digestion with proteinase K.

(*) One hamster although developing clinical disease was negative for Western blot PrP after digestion with proteinase K.

(‡) As outlined in more detail in the text, the measurement at 200 °C was repeated. After 200 days post inoculation only four of five hamsters were clinically affected.

4.3 Degradation of prions at pH 9 in a glycerol water mixture

In the industrial process the products of hydrolytic fat splitting, i.e. fatty acids and a mixture of 90 % water and 10 % glycerol, are separately collected and the glycerol water mixture is subjected to a sodium hydroxide treatment at pH 9 and 120 °C for 120 min. The processing conditions of this pH 9 treatment were also simulated on laboratory scale by measuring the PrP degradation efficiency in dependence on residence time. It is impossible on laboratory scale to produce a pH 9 solution by adding sodium hydroxide to a glycerol water mixture because in a non-buffered aqueous solution probably due to fluctuations in temperature no constant pH value could be adjusted. Thus, a mixture of 10 % glycerol and 90 % Tris HCl buffer, characterised by a known temperature dependence of its pH value, was adjusted at room temperature to pH 11.15, so that after heating to 120 °C a pH 9 was achieved. The time course of the thermal degradation of PrP 27-30 at 120 °C at pH 9 is depicted in **Fig. 3** in form of a plot of logarithmic reduction factors as function of residence time showing that the degradation process follows an exponential decay.

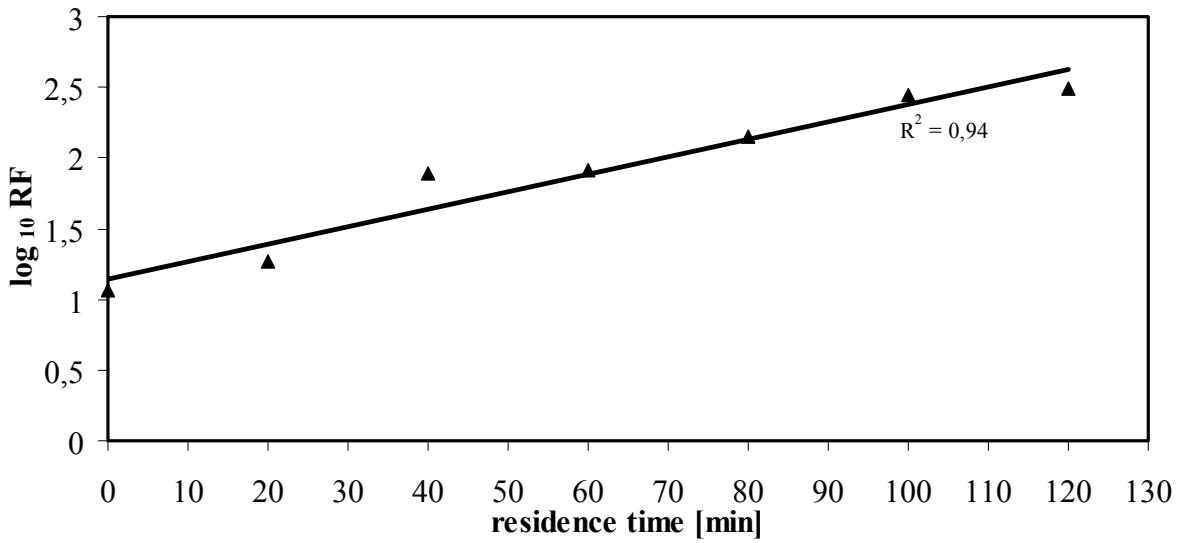

Fig. 3: Logarithmic reduction factors (RF) for the degradation of prion rods after heat treatment in a mixture of 90 % water and 10 % glycerol at pH 9 and 120 °C for the residence times indicated. The regression curve represents mean values of all data points.

5. Discussion

5.1 Scope of the study

Commonly used disinfection methods like irradiation, autoclaving at 121 °C or most chemicals are unsuitable to eliminate prion infectivity. Only a combination of strong decontamination methods like autoclaving at 134 °C and treatment with 2 M NaOH is sufficient to eliminate completely even high titres of prion infectivity [9]. We reported that the peptide integrity in PrP 27-30 aggregates was protected by lipids against hydrolysis **[15, 21]**. Although this earlier study represented a warning for hypothetical hazards in oleochemical processes, they had to be extended in several aspects in order to arrive to a reliable risk assessment. Firstly, not the mere presence of lipids but the exact process conditions had to be simulated in the laboratory study. Secondly, under a variety of conditions prion infectivity is inactivated more efficiently than PrP is degraded **[27; H. Müller, L. Stitz, D. Riesner, in preparation]**. Consequently, PrP degradation is an extrapolation to worst case condition but a more realistic risk assessment has to rely on infectivity studies. As both types of studies were carried out in the present work, a reliable argumentation could be given now.

5.2 Hamster scrapie strain 263K prion rods as a suitable model system

The Syrian hamster model infected with the hamster-adapted 263K strain of the scrapie agent has been favoured because it is accepted as a well validated, highly sensitive model. Apart from a short incubation period (60 to 80 days) and a high titre of infectivity in the brain (up to 10^{11} ID_{50} g^{-1}), the 263K strain is also appreciated because of its high resistance to decontamination methods **[28 - 35]**. Prion strains may differ to specific inactivation procedures. For example, **Peretz *et al.* [36]** reported that sCJD prions were dramatically more resistant to inactivation by acidic SDS than Sc237 scrapie. It can therefore only be concluded that 263K scrapie provides one of the most resistant forms of prion infectivity for the conditions analysed. Considering BSE and scrapie strain inactivation properties only little comparative data exist. The wet heat treatment of 263K brain tissue, brain homogenate, or macerate at temperatures between 132 °C and 138 °C for 9 minutes to 60 minutes never resulted in complete inactivation of prion infectivity **[10, 12, 31, 35, 37 - 39]**. Remaining prion titres

of between 0.7 ID_{50}/g brain to 5.0 ID_{50}/g brain were measured. BSE brain homogenates or macerates subjected to heat treatment at temperatures between 126 °C and 138 °C for 3 minutes to 60 minutes resulted in infectivity titres of 1.1 ID_{50}/g brain to 5.8 ID_{50}/g brain **[12, 37, 40, 41]**. Therefore, we conclude on the basis of the present knowledge that the 263K scrapie strain behaves similarly to BSE during inactivation processes. Additionally, a potentially smaller titre loss of the BSE agent compared with 263K cannot necessarily be regarded as evidence for a higher thermostability. It is known that the 263K infectivity titre is reduced rapidly during sub-lethal autoclaving but a more resistant subpopulation survives which therefore renders the 263K strain more stable against complete inactivation than BSE or mouse scrapie **[15, 37]**. In order to circumvent or at least attenuate the aspect of strain dependencies we opted to utilise the most purified, concentrated, and stable form of TSE infectivity known so far. The highly-structured prion rods were formed by detergent extraction and extensive proteolysis of brains of terminally sick Syrian golden hamsters **[6, 7, 42, 43]** allowing us to study the absolute worst case challenge with regard to the aspect of product safety. The most customary form of prion material for validation studies have been macerates or homogenates of infected brain tissue **[12, 31, 44, 45]**. However, **Brown *et al.* [38]** clearly demonstrated that after subjection to autoclaving at 134 °C for 30 min., i.e. under similar conditions as analysed in this work, reduction in infectivity titres of prion rods preparations were by nearly two orders of magnitude lesser than of brain tissue. Thus, by application of prion rods the real situation, i.e. isolated infectious aggregates embedded in a fat water mixture, is closely simulated, and the obtained reduction factors are definitively valid for all prion-containing substrates conceivable.

5.3 Degradation and inactivation efficiencies

The intention of this study was not to prove that the primary material, i.e. tallow, is free of infectivity nor to reproduce the potential state of residual prions in tallow but to provide reduction factors for the basic oleochemical process of hydrolytic fat splitting using the most stable form of TSE infectivity known so far. The reduction factors extrapolated to the conditions of industrial hydrolytic fat splitting including the additional pH 9 treatment of the glycerol water mixture are listed in **Tab. 2.** The experimental data of the fatty acid regime

degradation experiments have already been published in a preliminary report **[21]** and have to be interpreted here in the context with the new inactivation data. The factors for reduction of PrP 27-30 peptide integrity have to be regarded as absolute minimum values for the reduction of prion infectivity. As shown in **Fig. 2** and described in the literature **[27; H. Müller, L. Stitz, D. Riesner, in preparation]**, prion infectivity is inactivated always more efficiently than the degradation of the peptide backbone in PrP 27-30 occurs. The reduction factors for prion infectivity are also listed in **Tab. 2**. In case of the fatty acid regime within the limits of error similar reduction factors were obtained at 110 °C, 140 °C and 170 °C which is however the consequence of the detection limit of log 2 ID_{50} in the incubation time assay (cf. Material and Methods). Considering directly the incubation times, a more effective inactivation was obtained at 170 °C as compared to 110 °C and 140 °C, respectively. In the literature, the heat inactivation of 263K scrapie for at least 30 min. at 121 °C resulted in an about log 4.5–fold titre reduction **[1]**. Only after subjecting 263K to heat treatment at a temperature of 121 °C for at least 60 min. an infectivity titre reduction of log 5.5 **[31]**, log 7.5 **[46]**, or log 8.3 **[47]** was obtained. It was therefore somewhat surprising that with the fatty acid regime a reduction factor for prion rods infectivity of log 6.9 (**Fig. 2 a**) was already observed at a temperature of only 110 °C. This effect is under investigation in a further infectivity study.

Tab. 2: Reduction factors for the inactivation of prion infectivity and the degradation of PrP 27-30 backbone intactness after hydrolytic fat splitting.

treatment	reduction factor for degradation	reduction factor (†) for inactivation
fatty acid regime, 200 °C	2.7×10^6	7.9×10^6
glycerol regime, 140 °C	2.2×10^2	1.3×10^5
pH 9 treatment of glycerol water mixture	4.2×10^2	not applied industrially

(†) In case of the fatty acid regime extrapolated from the data points up to 170 °C; the data points at 200 °C are not taken into account here.

It should also be noted, that physical and chemical treatment of prions appears to extend incubation periods beyond the end of the dose response curve of untreated agent compromising the estimation of titres on the basis of incubation times **[48 – 51]**. According to **Prusiner *et al.* [25]** and **Lax *et al.* [52]** the treatment does not reduce the infectivity titre but can lengthen the incubation

period by about 10 days equivalent to a discrepancy of one log ID_{50}. An endpoint dilution titration of a specimen subjected to fatty acid regime conditions at 90 °C for 20 min. gave a titre of 4.2 log ID_{50}/ml. An incubation time interval assay of identical conditions resulted also in an infectivity titre of 3.9 log ID_{50}/ml (**H. Müller, L. Stitz, D. Riesner, in preparation**). Although the degree to which infectivity titres calculated by endpoint dilution titration compared to incubation time titration appear to vary according to treatment, in our hands and in accordance with the literature a discrepancy of smaller than 1 log ID_{50} was obtained. Also the finding that some fatty acid regime incubation periods were substantially longer than the end of the dose response curve were taken into account by assuming an infectivity titre not lower than log 2 ID_{50}. It is known that at those low concentrations of infectivity, the incubation times within one bioassay group vary randomly by up to hundreds of days **[53]**. Since under fatty acid regime conditions at temperatures of 140 °C and 170 °C, standard deviations of 94 days and 76 days, respectively, were obtained, these specimen should be presumed to have similarly low titres. Consequently, log 2 ID_{50} is an upper limit for 140 days.

The more customary but also more animal and time consuming mean of estimating infectivity titres is the endpoint titration. The addition of detergent to the diluent used in titration, however, also resulted in an increase in the infectivity estimate suggesting that the prion titre in a specimen may be underestimated as well, depending on the biochemical milieu of the inoculum **[54]**. Three of the animals inoculated with the specimen after fatty acid regime conditions at 140 °C did not develop disease, whereas one hamster in the 170 °C cohort although demonstrating clinical signs was negative for Western blot PrP after digestion with proteinase K. This indicates that the specimen inoculated were, by definition, at limiting dilution enabling a much more precise calculation of residual titre **[26]**. Since in both cohorts were at least 3 uninfected animals of 10 inoculated, the probability to escape infection was 3 in 10. The corresponding titre calculated from the Poisson distribution was 1.5 log ID_{50}/ml.

All arguments above indicate titres for the fatty acid regime at 140 °C and 170 °C lower than log 2 ID_{50}. Nevertheless, in order to completely minimise the risk of an overestimation of the reduction achieved, the bioassay detection limit was used as conservative treatment for all incubation times longer than 140 days.

The logarithmic scale reduction of the degradation of PrP 27-30 and the inactivation of prion infectivity always depended linearly or at least monotonically on temperature. Only after heat treatment at 200 °C under fatty acid regime conditions as well as in pure bovine edible tallow **[H. Müller, L. Stitz, D. Riesner, in preparation]** reduction factors for prion infectivity were obtained suggesting a lower decontamination potential at higher temperatures (**Tab. 1, Fig. 2**). All the other data points, however, came out in a consistent and reproducible performance within the limits of error indicating that the inactivation rate under oleochemical conditions does not decrease with increasing temperatures. However, one has to take into account that during hydrolytic fat splitting bovine edible tallow is split into crude glycerol and a mixture of crude fatty acids. In order to obtain a high degree of splitting the temperature applied industrially exceeds 200 °C. Nevertheless, even at temperatures above 160 °C appreciable amounts of glycerol are produced since the rate of fat splitting compared to 100 °C is already raised by factor 60. At 200 °C a rise of velocity by factor 460 is achieved **[55].** Glycerol, however, in accordance with **Fig. 2 b** exerts a protective effect on the heat stability of prions and lower the apparent inactivation efficiency of the fatty acid regime. Under laboratory scale fatty acid regime conditions, temperatures of 140 °C or 170 °C were reached within 11 min. or 19 min., respectively, whereas the heating phase up to a temperature of 200 °C was extended to 27 min. Consequently, at target temperatures higher than 160 °C the fat splitting reaction yielding prion-protective glycerol was allowed for nearly doubled reaction times. Both, increasing target temperature and increasing heating time are probable to have contributed to produce significant amounts of glycerol. In contrast to the volume-reduced laboratory scale fat splitting, in industrial columns differences in density establish a constant separation of glycerol water phase and fat phase. The fat phase in industrial columns is transported to the column top whereas water entering at the top falls in form of droplets through the slowly rising fat layer in the reverse direction. In the reaction zone located in the column middle the hydrolysis occurs yielding fatty acids and glycerol. Due to differences in density nascent glycerol is immediately separated from the fatty phase. A separation of glycerol phases and fat phases, however, cannot be achieved in the laboratory autoclave and is even hindered by stirring. Whereas the upper part of industrial fat splitting columns does not contain glycerol, the laboratory scale fatty acid regime at sufficiently high temperatures does contain a substantial

amount of glycerol. Since even the presence of only 10 % of glycerol significantly reduces the inactivation efficiency, artificially low inactivation factors are obtained at temperatures above 170 °C, i.e. at 200 °C in our experiments (**Tab. 1, Fig. 2**). Therefore, it is justified to use for the risk assessment (**Tab. 3**) the extrapolated infectivity reduction of 7.9×10^6. Even if the experimental value at 200 °C of 2×10^5 were used, it would be within the error limits of the corresponding reduction of peptide backbone integrity of 2.7×10^6.

5.4 Risk assessment for the industrial process of hydrolytic fat splitting assuming a hypothetical contamination with BSE or scrapie

The risk of a TSE-infection as consequence of a given chemical or pharmaceutical product has to be judged on a individual basis **[19, 56, 43]**. In order to assess the BSE risk regarding a hypothetical contamination of tallow derivatives the following factors are considered with worst case assumptions **(1)** the dose of BSE-infected tissue (in g) hypothetically contaminating the industrial tallow, **(2)** the prion infectivity (expressed in ID_{50} units) per g of contaminating tissue, **(3)** the prevalence of BSE infection in the source country, **(4)** the prion inactivation during the industrial process, **(5)** the administration dose (in g) in a maximum treatment regime, **(6)** the relative efficiency of a TSE-infection by the route of administration, and **(7)** a species barrier **[57 – 59]**. The risk can then be deduced in terms of the effective absolute or relative exposure.

5.4.1 The dose of BSE-infected specimen (in g) hypothetically contaminating the industrial tallow

The risk assessment will be performed for the hypothetical case that the raw material is contaminated by a worst case assumed accident. Under normal conditions pure tallow is free of TSE-infectivity for the following reasons **[19, 60]**. Epidemiological studies failed to find any association between the occurrence of BSE and the consumption of tallow by cattle **[61]**. In spiking studies with scrapie material, no infectivity remained in the tallow fraction from a rendering process that produced still prion-contaminated meat and bone meal **[60]**. Thus, tallow was put into the lowest category of TSE infectivity **[62, 63]**.
Although animals affected by BSE as well as specified risk materials are

removed from the human and animal food chain **[17]**, small amounts of residual high-risk material cannot be ruled out in technical processes. It is, however, quite difficult to estimate the most likely but generally valid amount of infectivity contaminating products due to inaccuracies in industrial slaughtering. When a reasonable numerical assessment has to be attempted, the production process of a given slaughtering or rendering plant has to be investigated in detail. We therefore consider the absolute worst case assuming that the whole 0.15 % insolubilities and protein impurities found in Standard European tallow **[64]** are exclusively BSE-infected tissue. Then the theoretical maximum amount of BSE-infectivity in industrial tallow would be a dose of 0.0015 g high-risk material per g of tallow.

5.4.2 Prion infectivity (expressed in ID_{50} units) per g of contaminating tissue

The infectivity titre of the tissue with the highest BSE titre during the entire incubation period, namely the brain stem, with 1 x 10^6 ID_{50} per g brain is used as obtained from cattle inoculated intracerebrally with bovine BSE-brain homogenate **[65]**. A titration experiment with the same isolate was also conducted by **Buschmann & Groschup [66]** resulting in a titre of $10^{3.27}$ i.c. ID_{50} per g brain for wild type mice and $10^{7.67}$ i.c. ID_{50} per g brain for transgenic mice overexpressing bovine PrP^C and even exceeding the sensitivity of cattle. In case of challenging wild type mice, the cattle/mouse species barrier is probably responsible for the comparatively low titre. In contrast, transgenic animal models artificially overexpressing PrP^C are necessary for highly sensitive bioassays. It is generally assumed that the estimated infectivity titre is higher when PrP^C is overexpressed. Such an animal model is useful when as little as possible numbers of infectious particles have to be determined. In a risk assessment, however, we have to stick with the definition of infectious dose not regarding the number of infectious particles contributing to one infectious dose. Therefore, infectious dose determination without assuming a species barrier is the most appropriate and at the same time most conservative estimation of infectivity. Thus, the infectious dose is 1 x 10^6 i.c. ID_{50} per g brain stem tissue. Since the infectivity titre varies enormously according to the stage of the TSE incubation period as well as a given tissue of the TSE-infected animal, an infectious dose of 1 x 10^6 i.c. ID_{50} per g specimen is again a worst case

assumption. The most likely value for an average infectious dose would be substantially reduced.

5.4.3. Prevalence of BSE infection in the source country

If material from a BSE-infected cattle would have entered the industrial production line, the dilution of that source has to be estimated. It would be easy to assume that contamination from one cattle would have entered one production batch. But uniform production batches do not exist and most steps in the production are continuous processes. Therefore, the distribution of prion infectivity within industrial production batches cannot be estimated in a generally valid manner. A statistically equal distribution of BSE-infected cattle has to be assumed instead. In contrast to natural scrapie in sheep, genetic variations in cattle appear not to be important for BSE-susceptibility. Consequently, all breeds of cattle are equally susceptible to BSE **[68 – 70]**. Since the UK is still the country with the highest BSE incidence worldwide, industrial tallow derived exclusively from British cattle represents the worst case-scenario. In the time period from April 2005 until March 2006 44.0 per million head of cattle above the age of 24 months suffered from BSE **[71]**. The majority of cattle, however, is slaughtered at an age below 24 months. In order to estimate the number of infected animals not yet clinically affected but in the late stage of the incubation period which are likely to enter the human food chain, **Arnold & Wilesmith [72]** provided a risk assessment assuming that the sensitivity of currently approved EU diagnostic tests decreases as the time before onset of the disease increases and that at least all animals older than 30 months are tested. The total number of infected animals in the last year of the incubation period expected to enter the food chain for the calendar years 2005-2010 is presumed to be always below one tenth of the total number of clinical cases, i.e. the sum of reported clinical cases and rapid post mortem test positives. The fact that in Great Britain only animals under 30 months were to be processed into tallow until the end of 2005, would reduce the number of subclinical carriers applied here but is not considered for worst case reasons. The prevalence of BSE infection of cattle is consequently supposed to be the sum of all clinical cases, i.e. 44.0 per million head of cattle, and subclinically infected animals, i.e. 4.4 per million head of cattle. This amounts to a dilution factor of 4.8×10^{-5}. Since the BSE incidence outside UK is substantially lower,

the average prevalence of BSE infection in all tallow-producing countries represents the most likely assumption. The number of reported European cases of BSE during the years 2004 and 2005 in farmed cattle excluding the United Kingdom amounts to 529 and 292 cases, respectively **[73]** of a total of 150,000,000 European cattle **[74]**, leading to dilution factors of 3.9×10^{-6} and 2.1×10^{-6}, respectively. Nevertheless, for worst case risk assessment the dilution factor for industrial tallow derived exclusively from British cattle is utilised.

5.4.4 Prion inactivation during the industrial process

The capacity of the hydrolytic fat splitting to reduce BSE infectivity depends on its efficiency to inactivate or to physically separate prions form the product. A physical removal of prions is not considered here but certainly plays a role in case of further purifying fatty acids and glycerol by filtration, chromatography, absorption, extraction, precipitation, or other mechanical means. Prions do also not replicate during the industrial process because PrP is not newly synthesised. The reduction factors due to the industrial conditions of hydrolytic fat splitting are listed in **Tab. 2**. They are in accordance with an earlier study yielding a reduction factor of 10^7, but the dependence upon the exact tallow water ratio or the additional presence of glycerol was not analysed **[59]**. In the present study, however, the exact industrial conditions were simulated on a reduced scale as accurately as possible. The reduction factors for degradation of the PrP 27-30 backbone intactness represent the worst case assumption, whereas the reduction factors for inactivation of prion infectivity can be regarded as the most probable assumption.

5.4.5 Administration dose (in g) in a maximum treatment regime

In one production batch might be a single dose of fatty acids or glycerol (10 g) or a chronic treatment, i.e. a yearly dose (500 g). Early studies of experimental scrapie suggested that infection occurs as a “all-or-none” event **[75]**. A high incidence of scrapie, however, was obtained in mice receiving multiple challenges of low prion infectivity by intraperitoneal injections, whereas mice challenged once with the same total dose did not develop disease **[76, 77]**. Moreover, the risk of infection was significantly higher when the intervals between the repeated injections of the subinfectious prion doses were shorter. The survival time was also shorter when the number of inoculations was high

[76]. In contrast, **Diringer *et al.* [78]** found that a total fixed dose had a reduced probability of causing oral infection when the material was presented as multiple challenges. It was concluded, however, that although the risk of oral infection also increases with multiple challenges, it does so to a lesser degree than would be expected if challenges combine in a cumulative way **[77]**. Consequently, an accumulation of single or daily doses to a large yearly dose would exaggerate the exposure risk. Since consideration of a single/daily dose might underestimate the exposure risk, we assume as conservative measure the larger value, i.e. a yearly dose of 500 g fatty acids or glycerol, respectively, per person and year corresponding to one administration of 10 g fatty acids or glycerol per week. Although an incorporation of 500 g of pure substance is unrealistic and most likely reduced to a minor fraction, it represents the worst case scenario.

5.4.6 Relative efficiency of a TSE-infection by the route of administration

The total world production of oils and fats amounts to 100 million metric tons per year, of which 20 million metric tons are of animal origin. More than 80 % of the total oils and fats are used for human nutrition. The worldwide consumption of basic oleochemicals (fatty acids, fatty alcohols, and glycerol) is estimated to 3.5 million metric tons per year **[64]**. These oleochemical feedstocks are converted into a wide range of chemical products for use in lubricants, soaps and detergents, cosmetics, pharmaceuticals, food additives, leather, paints and coatings, printing inks, rubber, plastics, metal-working and many other industries. Regarding the way in which these products will be potentially incorporated by man only the oral and subcutane routes of infection are conceivable.

The relative efficiencies of different routes of infection have been obtained from titration studies of scrapie in mice **[79]**. Assuming similar differences across a species barrier, the oral exposure of BSE-infected specimen to humans is about 10^5 times less efficient than the intracerebral route of administration **[62, 80]** on which the data represented in **(2)** are based on. Concerning the uptake of prions by infiltration of the skin no experimental data exist. Considering that intact human skin cannot be penetrated by prions and only an infinitely small fraction of cosmetic products may be transferred into eyes, open wounds or cuts, a conservative estimation would assume a cosmetic exposure as efficient as the

subcutane route. It would be unrealistic to calculate the final risk on the basis of a subcutane transfer of even 10 times less than 500 g fatty acids or glycerol (see **(5)**) into eyes or open wounds, even in the case that for worst case risk assessment several events per week would be taken into account. Rather an oral uptake of a yearly dose of 500 g substance represents the worst case assumption.

5.4.7 Species barrier

Epidemiological evidence suggests that the occurrence of CJD is not associated with scrapie in sheep, although a large number of people must have been exposed to scrapie during the centuries. An estimation of the species barrier on the basis of infectivity titres within animal brain and the detection limit of bioassays might indicate a species barrier factor of at least one million-fold. There is no scientific doubt that the BSE epidemic is the result from feeding of insufficiently inactivated meat and bone meal to cattle **[17, 61, 79]**. Whereas a bovine-borne infection of man with BSE is the most likely hypothesis for the origin of the new variant of CJD (vCJD), there is no evidence for a transmission of scrapie to man **[81 – 85]**. A number of 158 confirmed cases of vCJD in the UK confirmed that the BSE agent is capable to infect humans **[86]**. Although the existence of a species barrier factor of several orders of magnitude is established **[17, 61, 79, 81 - 85]**, for a worst case risk assessment it is reasonable to consider no species barrier. Moreover, neither the age nor a genetic susceptibility of humans to vCJD which definitely exists with respect to the polymorphism at codon 129 of the Prnp gene are taken into account because a risk assessment has to apply to a general population and not to individuals.

5.5 Summary risk from prion contamination in fatty acids and glycerol

The annual probability of contracting a sporadic (s)CJD is one case per million people worldwide. This natural incidence of 1×10^{-6} represents the background risk which has to be compared to the final risk from oleochemical products which, of course, should be orders of magnitude lower than the natural risk to achieve a sufficient margin of safety. In **Tab. 3** all risk parameters are listed for both regimes and final risks (in ID_{50} units) are calculated. Although one ID_{50} unit represents a 50 % probability of infection, for worst case reasons the values are not divided by two. As shown, the major contribution for the

reduction of the final risk is accomplished by the manufacturing process emphasising the huge potential of the hydrolytic fat splitting to inactivate a hypothetical contamination with prions. If a worst case scenario is constructed solely from the degradation data of the infectious prion protein, the maximum risk to fall ill with vCJD as consequence of a hypothetical BSE contamination of tallow is at least 800 times (glycerol regime, **Tab. 3**) less than the background risk. This distance appears to be rather small but when the infectivity data obtained from the bioassays are taken into consideration (**Tab. 3**), the maximum risks for contracting a vCJD are further decreased to values at least 65,000 times (fatty acid regime, **Tab. 3**) less than the sCJD background risk.

Tab. 3: Risk assessment for oleochemical products based on reduction factors for the industrial conditions of hydrolytic fat splitting.

	fatty acids		**glycerol**	
	degradation of PrP27-30	inactivation of prion infectivity	degradation of PrP27-30	inactivation of prion infectivity
(1) dose of BSE-infected tissue [g per g of tallow]	1.5×10^{-3}		1.5×10^{-3}	
(2) BSE infectivity [ID_{50} units per g bovine brain stem]	1.0×10^{6}		1.0×10^{6}	
(3) prevalence of BSE infection	4.8×10^{-5}		4.8×10^{-5}	
(4) reduction factors (†)				
hydrolytic fat splitting	3.7×10^{-7}	1.3×10^{-7}	4.5×10^{-3}	7.4×10^{-6}
pH 9 treatment	-		2.4×10^{-3}	
(5) administration dose [g]	500		500	
(6) relative efficiency of BSE infection	1.0×10^{-5}		1.0×10^{-5}	
(7) species barrier	-		-	
final risk [ID_{50} units/person and year]	**4.1×10^{-11}**	**1.5×10^{-11}**	**1.2×10^{-9}**	**2.0×10^{-12}**

(†) As the prion titre is reduced due to the conditions of industrial hydrolytic fat splitting, the reduction is presented as reciprocals of the reduction factors.

The risk estimate might be too high because of several too pessimistic worst case assumptions. In case of applying more likely values for all risk factors as described in detail in each section presented above, the final risk would be further reduced by several orders of magnitude. However, it is not possible to give an estimate for an "overall relative" risk. Additionally, crude tallow has always undergone several manufacturing steps involving rendering procedures.

After hydrolytic fat splitting further safety factors have also to be considered if a subsequent distillation is used to purify glycerol and fatty acids from remaining impurities and to separate the mixture of fatty acids into fractions of a more specific chain length. Furthermore, fats are subjected frequently to a catalytic fat hydrogenation. All these individual steps are to be multiplied with the final risk factors established in the present study and thus further add to the safety of tallow derivatives.

From the risk assessment presented here it can be concluded that the industrial process conditions of hydrolytic fat splitting constitute a highly effective means for reducing the risk of TSE contamination to an acceptable minimum. All industrial tallow-derived products can be regarded as safe, independently of their origin.

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VI.3 Influence of water, fat, and glycerol on the mechanism of thermal prion inactivation

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1. Summary

Extending the recent analysis of the safety of industrial bovine fat-derived products for human consumption **[1]**, we investigated systematically the effects of fat, fatty acids, and glycerol on the heat destruction of prions. Prion destruction was qualitatively and quantitatively evaluated in PrP 27-30, or prion rods, by the inactivation of infectivity as well as by the degradation of the polypeptide backbone. Under all conditions analysed, inactivation of prion infectivity was achieved more efficiently than backbone degradation by several orders of magnitude. The presence of fat enhanced prion inactivation and offers a mild treatment for prion decontamination. In contrast, the presence of fat, fatty acids, and especially glycerol protected the PrP 27-30 backbone against heat-induced degradation. Glycerol also protected against heat-induced inactivation of prion infectivity. A phase-distribution analysis demonstrated that prions migrated to the interphase of a fat-water mixture at room temperature and accumulated in the water phase at higher temperatures. In a systematic study of the mechanism of prion destruction we found an intermediate structure of PrP that has fewer fibrils in β-sheet formation, lower resistance to protease digestion, greater aggregation, and reduced solubility compared to PrP 27-30, but retains residual infectivity. These findings suggest that prion infectivity depends on β-sheet–rich, fibrillar structure, and that inactivation proceeds in a stepwise manner, which explains the tailing effect frequently observed during inactivation.

2. Introduction

The prion diseases include bovine spongiform encephalopathy (BSE), scrapie in goat and sheep, chronic wasting disease (CWD) in deer and elk, and Creutzfeldt-Jakob disease (CJD) in humans. These diseases can be acquired by transmission of an infectious agent, occur sporadically, or be inherited. In contrast, other fatal neurodegenerative disorders like Alzheimer's disease, Parkinson's disease, and Huntington's disease are exclusively characterised by a sporadic or genetic etiology.

The entity causing these diseases is called a prion, derived from "*pro*teinaceous *in*fectious particle" **[2]**. Prions are composed primarily, if not exclusively, of an abnormal isoform, denoted PrP^{Sc}, of the normal, cellular prion protein (PrP^{C}),

which is a hydrophobic glycoprotein expressed in all mammals studied to date. During disease pathogenesis, PrP^{C} is converted into PrP^{Sc} with the assistance of one or more cofactors **[3–5]**. Although the mechanism of conversion remains unclear, the "protein-only" hypothesis posits that PrP^{Sc} acts as template for the posttranslational conversion of PrP^{C} into PrP^{Sc}. The recent demonstration that infectious PrP^{Sc} fibrils can be formed solely from recombinant PrP offers strong support for the "protein-only" hypothesis **[6]**.

The conformational transition of PrP^{C} to PrP^{Sc} changes the physicochemical properties of the protein. PrP^{C} is soluble in mild detergents, exhibits a mainly α-helical secondary structure, and is sensitive to degradation by proteases, e.g., proteinase K (PK). In contrast, PrP^{Sc} is partially PK-resistant and forms insoluble, β-sheet–rich, multimeric aggregates that accumulate, most prominently in the central nervous system. Upon purification using detergents and limited digestion by PK, the N-terminus of PrP^{Sc} is truncated to yield a still infectious fragment of 27-30 kDa, designated PrP 27-30. As a result of hydrophobic interactions, PrP 27-30 forms rod-shaped fibrils, also called prion rods **[7]**. Small amounts of two sphingolipids attach to PrP in prion rods **[8]** and 10 % (w/w) of a polyglucose scaffold has been associated with PrP 27-30 **[9]**.

Prions are extraordinarily resistant to decontamination by physical or chemical procedures that inactivate conventional pathogens **[10]**. The unintentional application of inappropriate inactivation methods during rendering of offal to prepare meat and bone meal may have selected for the most resistant prions, which may have perpetuated the BSE epidemic in Europe as dairy cattle consumed this infected dietary supplement **[11, 12]**. Compelling evidence indicates that CJD can be transmitted by improperly decontaminated surgical equipment **[13]**. Even though the chemical nature of prions is not fully characterised, effective methods for a significant reduction of prion infectivity titres have been described. Most widely used are treatments with 1 M sodium hydroxide (NaOH) or 2.5 % sodium hypochlorite (NaOCl). Partial inactivation can also be achieved by heat, i.e. steam autoclaving at 134 °C for 20 min. For complete inactivation, either steam autoclaving at ≥121 °C after treatment with 1 M NaOH or boiling in 1 M NaOH is required **[10]**.

Many factors affect prion inactivation, including the prion strain; method of sample preparation (e.g. undiluted tissue, tissue homogenate, tissue macerate

[14]); the fixation and dehydration state of the agent **[12]**; and surface effects (smearing, particularly on steel surfaces **[15]**). For instance, for 263K prions, a Syrian hamster-adapted scrapie strain, reductions of 3.2 $\log_{10}$ to 7.5 $\log_{10}$ **[16–17]** in the infectious dose (ID_{50}) have been reported for a 30-min incubation at 134 °C under comparable conditions. Prion inactivation is less efficient in brain tissue compared to brain homogenate, which was suggested to be due to fat molecules protecting against heat inactivation **[10]**. Indeed, lipid-mediated stabilisation against heat treatment has been reported for bacteria **[18, 19]**. The presence of large amounts of external lipids also protects PrP 27-30 against heat degradation **[20]**, which is of particular importance since the fat content of mammalian tissues and particularly of brain is considerable. For example, greaves of ovine and porcine origin contain 19.5 % fat, constituting a potent environment for the heat protection of PrP 27-30. The lipophilic character of prions and their tendency to form hydrophobic aggregates appear to be closely connected to their extraordinary stability.

When prions are completely inactivated by autoclaving, destruction over time proceeds in an exponential fashion. However, when milder, partially inactivating conditions are applied, a tailing phenomenon is observed **[15, 21, 22]**. In this case, infectivity declines rapidly followed by slow inactivation with increasing treatment time. When conventional microorganisms present such a tailing inactivation, it reflects a protective effect of aggregation or population heterogeneity, i.e., a small subpopulation of more resistant particles **[15]**. In the case of prions, biphasic inactivation curves were explained by a heteromeric structure that enciphers informational and macromolecular components **[23]**.

The intention of this study was to extend the analysis of oleochemical processes to a systematic understanding of interactions responsible for thermal prion destruction. A risk assessment calculation for all tallow-derived products was already provided **[1]**, confirming that industrial conditions for the basic oleochemical process of hydrolytic fat splitting constitute an effective means for reducing the risk of prion contamination to an acceptable minimum. To establish a mechanistic model for heat destruction of prions, we provide qualitative and quantitative data on the inactivation of infectivity as well as the degradation of the PrP 27-30 polypeptide backbone under continuous variation of conditions, particularly in presence of different concentrations of fat, fatty acids, and glycerol.

3. Experimental Procedures

3.1 Prion protein samples

PrP 27-30 from Syrian hamsters infected with scrapie strain 263K was prepared as described earlier **[24]**. Centrifugation of a 50 % sucrose gradient at 4 °C for 1 h at 100,000 × *g* in a TL45 rotor yielded the final PrP 27-30 pellet, which was stored at -70 °C until use.

3.2 Bovine edible tallow, oleic acid, and glycerol

Bovine edible tallow was provided by the European Oleochemicals and Allied Products Group (APAG). The fat had been recovered from adipose tissue and bones by dry melting at 100 °C followed by purification of the lipid fraction from tissues and proteinaceous matter by filtration through a diatomaceous earth bed. Remaining levels of moisture and total insoluble impurities were 0.11 % and 0.018 %, respectively. Since the most abundant natural fatty acids are those with 16 and 18 carbon atoms, pure oleic acid of 18 carbon atoms was used, kindly provided by Bernd Pokits and Martin Mittelbach (University of Graz, Graz, Austria). Glycerol of highest purity was purchased from Roth (Karlsruhe, Germany).

3.3 Heat treatment of prions and recovery from mixtures containing fat, fatty acids, and glycerol

As described in detail earlier **[1, 25]**, an inactivation system was utilised comprising a pressure reactor with external electric heating, digital pressure and temperature displays, and magnetic stirrer (Parr Instruments Micro Bench Reactor 4591). PrP 27-30 was loaded into the reactor vessel containing 30-ml mixtures of raw materials (**Tab. 1**). Depending upon the reactor content, temperatures of up to 200 °C were reached within 47 min (**Tab. 1**). For the duration of the reaction, the reactor content was stirred at 150 rpm to permit an even temperature distribution within the reactor vessel as well as rapid heating and cooling.

To quantify the amount of prions remaining undegraded after heat treatment in a large excess of fat and glycerol, a quantitative purification method based on a methanol chloroform precipitation was applied **[1, 25, 26]**. A minimum PrP

recovery rate of 95 % was achieved. Methanol chloroform precipitation of PrP 27-30 subjected to 40 °C for 20 min did not decrease infectivity titres.

Tab. 1: Summary of experimental set-ups for testing the influence of heat on the stability of PrP 27-30 in the presence of water, fat, and glycerol.

experimental set up	A	B	C	D	E	F(§)
deionised water [%]	100	-	10	-	-	87.75
tallow [%]	-	100	90	-	-	2.5
oleic acid [%]	-	-	-	100	-	-
glycerol [%]	-	-	-	-	100	9.75
highest temperature examined [°C]	140	155	140	150	155	165
(Western blot/bioassay)	170	200	200	n.d.	200	170
time to reach highest temperature [min]	21	24	11	22	22	27
(Western blot/bioassay)	28	45	30	n.d.	47	28

n.d. not determined.

(§) In the presence of glycerol, a relative content of as low as 2.5 % tallow did not influence the degradation and inactivation experiments. For reasons of clarity, this experimental set-up is henceforward referred to as 10 % glycerol and 90 % water.

3.4 Quantification of heat-treated prions by immunoblotting and incubation time interval assay

After heat treatment, undegraded PrP was separated by SDS-polyacrylamide gel electrophoresis (PAGE) **[27]**. The amount of undegraded PrP was quantitatively detected by an improved immunoblot comprising staining with a mixture of the monoclonal antibodies 3F4 and R1, enhanced chemiluminescence, and densitometry as described earlier **[1, 25]**. The sensitivity threshold of the method was 1 ng PrP as determined by a dilution series of PrP 27-30.

Bioassays of prion infectivity were performed by inoculation of weanling female Syrian gold hamsters **[1]**. Bioassays were terminated 390 days after inoculation. Prion titres were calculated by measuring the time intervals from inoculation to onset of clinical symptoms **[2]**. The dose-response curve from these titres was utilised for incubation times up to 140 days. For incubation times longer than 140 days, a bioassay detection limit of 2 $\log_{10}$ ID_{50} was used. For hamsters remaining healthy after 390 days, brain homogenates were prepared and subjected to PK digestion followed by Western blotting.

Quantification of prion levels was performed as described **[20].** Briefly, the heat destruction of prions can be described by first-order decay kinetics with a rate

constant k **[20, 28]**. Therefore, the reduction factor $RF_{(t)}$ can be defined as the ratio between the concentration of PrP or prion infectivity, respectively, before and after heat treatment at a certain temperature for time t as shown in Equation 1,

$$\ln [C_{PrP}(t)/C_{PrP}(0)] = -k \times t \quad \text{(Eq. 1)}$$

or with the reduction factor after heating for 20 min. as shown in Equation 2

$$\log_{10} RF_{20} = k \times 1200/2.303 \quad \text{(Eq. 2)}$$

The rate constant depends on the temperature according to the Arrhenius Equation 3,

$$\delta \ln k/\delta\, 1/T = -E_A/R \quad \text{(Eq. 3)}$$

with the activation energy E_A and the gas constant R. Combining both equations leads to a linear relationship of $\ln \log_{10} RF_{20}$ as a function of 1/T. The mean values of all data points fall within the experimental error and are in accordance with the linear dependence **(Fig. 1)**. Since the error limits of the Western blot densitometry were 10 %-15 %, the majority of degradation experiments was repeated until reproducible. The mean standard error of all degradation data points in **Fig. 1** was $\log_{10} 0.24$.

3.5 Distribution of PrP 27-30 between fat and water phases

Different amounts of PrP 27-30 were suspended in 15-ml polymer tubes containing 1:1 mixtures of bovine edible tallow and water preheated to a temperature of 40 °C. After agitation for 30 sec, the specimens were incubated in a water bath at temperatures of 20 °C, 40 °C, 60 °C, 80 °C, or 100 °C until clear phases were achieved (usually for 30 min). The still-liquid fat phase was carefully separated using a needle attached to a glass syringe. Subsequently, the polymer tube was broached at the bottom and the water phase was extracted. The protein in all three phases, i.e. fat, water, and remaining interphase of about 2.5-ml solution, was purified by methanol chloroform precipitation as described above. The amount of PrP 27-30 in each phase was determined by Western blotting and densitometric quantification as described above.

3.6 PK assay

PK digestions were performed in 1.5-ml Eppendorf tubes or 96-well, black-bottom plates (Nunc, Wiesbaden, Germany). Specimens were incubated in 50 µl of 50 mM Tris HCl (pH 8.0), 0.1 mM NaCl, and 2.5 mM EDTA with PK at a final concentration of 50 µg/ml at 37 °C for incubation times between 15 min and 60 h. The reactions were terminated by the addition of SDS-PAGE loading buffer and boiling for 6 min. The degree of digestion was determined by real-time fluorescence (thioflavin T assay) or by comparison with an undigested control on the same SDS gel (Western blotting).

3.7 Determination of solubility by differential centrifugation

According to the standards of Hjelmeland and Chrambach **[29]**, particles remaining in the supernatant after centrifugation at 100,000 × *g* for 1 h are regarded as soluble. To test whether heat treatment affected solubility, PrP 27-30 was or was not subjected to temperatures of up to 125 °C for 20 min as described above. Solubilised PrP 27-30 was obtained by sonication with a Bandelin HD 2070 sonicator (Bandelin Electronic, Berlin, Germany) in 10 mM sodium phosphate buffer (pH 7.2) and 0.3 % SDS. The specimens were sonicated 10 times at 15-sec bursts at 65 % power on ice followed by a 15-sec cooling pause. Subsequent centrifugation in a TL-100 ultracentrifuge (Beckman Instruments, Fullerton, USA) with a TLA-45 rotor for 1 h at 100,000 × *g* and 4 °C separated soluble PrP 27-30 from the insoluble pellet fraction. The amounts of PrP 27-30 in the supernatant and pellet fractions were determined by Western blotting and densitometric quantification as described above.

3.8 Negative staining and electron microscopy

Negative staining was performed as previously described **[33]**. In brief, negative staining was done on formvar/carbon-coated, 200-mesh copper grids (Ted Pella, Inc.; Redding, USA), which were glow-discharged prior to staining. Five-µl samples were adsorbed for approximately 30 sec, then the grids were washed with 50 µl of each 0.1 M and 0.01 M ammonium acetate (pH 7.4). Afterwards, the grids were stained with 50 µl of freshly filtered stain, 2 % ammonium molybdate. After drying, the samples were viewed in a FEI Tecnai F20 electron microscope (Eindhoven, The Netherlands) at 80 kV and a standard

magnification of 25,000. Electron micrographs were recorded with a Gatan Ultrascan CCD camera. The magnification was calibrated using negatively stained catalase crystals and ferritin.

3.9 Congo red birefringence

Protein solutions diluted to 150 µg/ml were air dried on a glass microscope slide and incubated for 10 min in 0.5 % Congo red, 80 % ethanol. After successive washing with 90 % ethanol and 96 % ethanol, the specimens were viewed at 400× magnification with a Zeiss III polarising microscope.

3.10 Thioflavin T (ThT) assay

Thioflavin T binds specifically to amyloid fibrils such that its long axis is parallel to the long axis of the fibril, i.e. in neat rows running along the length of the β-sheets between the amino acid side chains, perpendicular to the strands **[30]**. In the absence of amyloid fibrils, ThT exhibits fluorescence at the excitation and emission maxima of 342 nm and 430 nm, respectively. Binding to PrP 27-30, however, results in new excitation and emission maxima of 442 nm and 482 nm, respectively. This change depends on the aggregated state of PrP **[31, 32]**. Due to the irregular arrangement of β-sheets within amorphous aggregates, the ThT assay is capable of discriminating between amorphous and fibrillar structures.

In order to test for fibril formation, fluorescence measurements were performed in 96-well, black-bottom plates (Nunc, Wiesbaden, Germany) in a microplate reader Safire (Tecan Trading AG, Switzerland). All measurements were carried out at 37 °C in 50 µl of 50 mM Tris HCl (pH 8.0), 0.1 mM NaCl, 2.5 mM ethylenediaminetetraacetate (EDTA), and 5 µM ThT. The recording parameters were: excitation wavelength of 445 nm, spectral range of 470 nm to 550 nm, step resolution of 2 nm, bandwidth of 2.5 nm, gain of 180, z-position of 4400 ± 400 µm. Kinetics were recorded for up to 240 cycles with intervals of 15 min. For analysis, the integral of the intensity measured from 470 nm to 550 nm was calculated.

3.11 Circular dichroism

Circular dichroism (CD) spectra were recorded with a Jasco spectropolarimeter, model 715 (Jasco Inc.) and are depicted as ellipticity [θ]. All measurements were performed at room temperature in 10 mM sodium phosphate (pH 7.2) with or without addition of 10 % glycerol. Ten spectra were recorded over the spectral range of 185 nm to 260 nm at a scan speed of 50 nm/min and a step resolution of 1 nm. Below a wavelength of 185 nm, excessive absorption by the buffer impeded further readings. The presence of glycerol limited the spectral range to wavelengths higher than 191 nm. A blank spectrum for cuvette and buffer was subtracted from each spectrum. Satisfactory CD spectra were only obtained with sonicated PrP 27-30 at a concentration of 1 µg/µl. Sonication was carried out with a Labsonic U cup sonicator (B. Braun Diessel Biotech) at 180 W for 5 min at <25 °C. The specimens were contained in a screw-cap tube immersed 1 cm into the water bath of the sonicator. Thermostating of the sonication bath was established by a water cooling system.

4. Results

All experiments were carried out with prion rods purified from hamster brains infected with the hamster-adapted scrapie strain 263K. Heat treatment of prion samples was performed in a lab-scale autoclave appropriate for high pressure **[1]** and analysis was accomplished by gel electrophoresis followed by Western blotting as well as by bioassays in Syrian golden hamsters.

4.1 Degradation of PrP 27-30

From the quantitative analysis of Western blots, reduction factors (RF_{20}) for degradation, indicated by hydrolysis of the peptide bonds, of PrP 27-30 in different fat-water mixtures were determined after heat treatment for 20 min at temperatures up to 165 °C (**Fig. 1A**; corresponds to Fig. 2.1 A, p.33, Chapter II, Results and Discussion). No degradation experiments were conducted at temperatures >165 °C because unacceptably large amounts of PrP 27-30 would have been required. Comparing degradation experiments with and without tallow, the presence of fat increases the backbone stability of PrP 27-30 by one order of magnitude. However, in the presence of water, the protective effect of fat decreases with increasing temperature (**Fig. 1A**; corresponds to Fig. 2.1 A,

p. 33, Chapter II, Results and Discussion**)**. At temperatures ≥110 °C, no significant difference was detected when degradation in 90 % fat and 10 % water was compared to that in pure water. Heat treatment in pure tallow in comparison with pure oleic acid did not exhibit significant differences over the whole temperature range.

The slopes of the regression curves yield the activation energy of the degradation processes. The activation energy of heat-mediated degradation in the presence of fat or fatty acids was ~31 (± 4.4) kJ/mol, whereas in pure water, an activation energy of 20 (± 5.3) kJ/mol was obtained.

Compared to the presence of fat, the presence of glycerol resulted in greater stability of PrP 27-30 against heat degradation **(Fig. 1B**; corresponds to Fig. 2.1 B, p. 33, Chapter II, Results and Discussion**)**. The heat stability of PrP 27-30 in pure glycerol was raised by two orders of magnitude compared to that of PrP 27-30 in pure water. In contrast to fat, the presence of water in the mixtures did not affect the protective effect of glycerol; we found that 10 % glycerol offered as much protection against degradation as 100 % glycerol at high temperatures. The activation energies in 100 % and 10 % glycerol were 51 (± 9.8) and 29 (±2.2) kJ/mol, respectively. The latter value should be considered with some care because the data suggest a biphasic degradation.

At higher temperatures, in addition to the PrP triplet bands reflecting different levels of glycosylation, increased amounts of aggregate mixtures of higher molecular mass were noticed. Besides the formation of those large aggregates, signals at 60 kDa indicative of PrP dimers were detected as described earlier **[20, 34]**. Weak PrP-specific signals at 60 kDa were already present in untreated PrP 27-30 and particularly noticeable after heat treatment at medium temperatures. At further increased temperatures they disappeared in favour of aggregates of higher molecular mass. Higher amounts of oligomers after heat exposure were always paralleled by lower concentrations of monomer bands (**data not shown**).

4.2 Inactivation of prion infectivity

In addition to PrP 27-30 degradation, inactivation of prion infectivity was studied after heat treatment at temperatures of up to 200 °C under conditions otherwise identical to the degradation experiments. Compared to untreated

samples, infectivity of heat-treated samples in the presence of different fat-glycerol-water mixtures varied considerably **(Tab. 2, Fig. 2**; corresponds to Fig. 2.2, p. 36, Chapter II, Results and Discussion**)**. The different molecular environments induced differences in inactivation of up to five orders of magnitude. Assuming that the curves relating incubation time and titre also apply to heat inactivation in the presence of fat and glycerol (cf. below), $\log_{10}$ RF_{20} values can also be derived for inactivation of infectivity **(Fig. 3**; corresponds to Fig. 2.3, p. 39, Chapter II, Results and Discussion**)**. Comparing degradation and inactivation data, inactivation of prion infectivity was much more efficient than degradation of PrP 27-30. Under all conditions examined, we found a roughly linear temperature dependence on the inactivation efficiency **(Fig. 3**; corresponds to Fig. 2.3, p. 39, Chapter II, Results and Discussion**)**. In some experiments, such as those in fat-containing mixtures, not all inoculated animals became sick. In these cases, mean values could not be calculated, resulting in imprecise interpolation, and significant values for activation energy could not be determined. The inactivation energy values ranged from 18 kJ/mol to 21 kJ/mol for pure glycerol, aqueous glycerol, and pure water.

In contrast to its protective role in PrP degradation, the presence of fat helped to destroy PrP infectivity. However, heat treatment at 200 °C in 100 % or 90 % fat resulted in shorter incubation times, reflecting decreased prion inactivation, compared to similar treatment at lower temperatures **(Fig. 2**; corresponds to Fig. 2.2, p. 36, Chapter II, Results and Discussion**)**. This phenomenon is most likely a consequence of the production of appreciable amounts of glycerol due to fat hydrolysis at 200 °C. According to this assumption, values for inactivation in fat and in glycerol converge for experiments run at 200 °C. Consequently, the protective effect of glycerol seems to dominate not only the influence of water but also that of fat. Glycerol shows qualitatively similar protection against PrP degradation and inactivation as notable at temperatures ≥170 °C **(Fig. 1B, 3B and 3D**; corresponds to Fig. 2.1 B, p. 30, Fig. 2.3 3B, p. 39, Fig. 2.3 3D, p. 39 Chapter II, Results and Discussion**)**.

Due to concerns about the reliability of incubation-time interval assays after physical or chemical treatment **[35–38]**, we conducted an endpoint dilution titration for heat treatment at 90 °C for 20 min in a 90 % fat and 10 % water mixture, which yielded an infectivity titre of 4.2 log ID_{50}/ml **(Tab. 2)**. An incubation-time interval assay after heat treatment under identical conditions

resulted in an infectivity titre of 3.9 log ID_{50}/ml. Since no significant difference exists between these two titres, we conclude that the calculation of infectivity titres under these or similar conditions from incubation times is reliable.

4.3 Optimisation of low-temperature inactivation of prions by fat-water mixtures and other ingredients

From our observations on the enhancing effect of fat on prion inactivation, we wished to determine whether different amounts of fat would yield the same results. We studied inactivation in the presence of 50 %, 75 %, or 90 % fat, but did not find substantial variations in mean incubation times **(Tab. 2)**. After heat treatment for 20 min at ≤90 °C, reductions in infectivity titre of up to 5 $\log_{10} ID_{50}$ were achieved in presence of different amounts of fat. In pure water, the infectivity titre was reduced by 3 $\log_{10} ID_{50}$ only. When we combined the inactivating effect of fat with denaturants, we found that an aqueous mixture of fat, urea, and dithiothreitol (DTT) reduced the infectivity titre by up to 7 $\log_{10}$ ID_{50} **(Tab. 2)**.

Tab. 2: Effect of heat treatment in different fat-glycerol-water mixtures on infectivity. For calculation of mean incubation periods, infectivity titres, and standard errors, only diseased animals were utilised.

treatment	No. infected/ No. injected	mean incubation period ± SEM [days]	infectivity titre ± SEM [log ID_{50} per sample]
negative control[4]			
(50 % tallow/ 50 % glycerol, 200 °C, 20 min.)	0/5	-	-
positive controls[4]			
starting titre	10/10	74 ± 1	7.9 ± 0.5
after MeOH/$CHCl_3$ precipitation	5/5	68 ± 3	8.9 ± 1.4
100 % fat, 20 min			
110 °C	5/5	130 ± 4	2.3 ± 0.3
140 °C	5/5	126 ± 8	2.6 ± 0.6
170 °C	4/4[1]	270 ± 31	< 2.0
200 °C	8/10[2]	148 ± 32	< 2.0
90 % fat/ 10 % water, 90 °C			
60 min.	5/5	104 ± 1	4.2 ± 0.1
180 min.	5/5	120 ± 6	3.0 ± 0.4
540 min.	4/4[1]	108 ± 4	3.9 ± 0.3

90 % fat/ 10 % water, 20 min			
70 °C	5/5	94 ± 4	5.2 ± 0.4
80 °C	4/4[1]	93 ± 1	5.3 ± 0.2
90 °C	9/9[1]	108 ± 2	3.9 ± 0.2
100 °C	5/5	118 ± 10	3.2 ± 0.7
110 °C[4]	10/10	124 ± 8	2.7 ± 0.6
140 °C[4]	2/5[2]	208 ± 67	< 2.0
170 °C[4]	5/5	297 ± 38	< 2.0
200 °C[4]	9/10[2]	116 ± 6	3.3 ± 0.3
90 % fat/ 10 % water, 140 °C, 1 min	5/5	102 ± 40	4.4 ± 0.4
90 % fat/ 10 % water, 90 °C, 20 min			
10^{-1}	3/4	151 ± 16	
10^{-2}	3/5	182 ± 23	
10^{-3}	0/5	-	4.2 ± 0.0
10^{-4}	2/5	195 ± 40	
10^{-5}	0/5	-	
10^{-6}	1/5	150 ± 0	
75 % fat/ 25 % water, 90 °C, 20 min			
-	5/5	93 ± 3	5.3 ± 0.4
2.25 M Urea/ 0.5 M DTT	5/5	141 ± 3	< 2.0
50 % fat/ 50 % water, 90 °C, 20 min	4/4[1]	110 ± 1	3.8 ± 0.1
100 % water, 20 min			
80 °C	5/5	78 ± 4	7.2 ± 0.7
90 °C	5/5	85 ± 1	6.2 ± 0.2
110 °C	5/5	97 ± 1	5.0 ± 0.1
140 °C	5/5	141 ± 3	< 2.0
170 °C	5/5	231 ± 33	< 2.0
90 % water/ 10 % glycerol, 20 min[4]			
80 °C	5/5	80 ± 2	7.0 ± 0.3
110 °C	5/5	94 ± 2	5.3 ± 0.3
140 °C	5/5	114 ± 4	3.5 ± 0.3
170 °C	5/5	130 ± 7	2.4 ± 0.4
90 % water/ 10 % glycerol, 140 °C, 1 min	5/5	124 ± 70	2.7 ± 0.5
100 % glycerol, 20 min			
110 °C	5/5	81 ± 2	6.8 ± 0.3
140 °C	5/5	91 ± 2	5.5 ± 0.2
170 °C	5/5	103 ± 3	4.3 ± 0.3
200 °C	4/5[3]	145 ± 11	< 2.0

[1] One hamster found dead without clinical signs or PK-resistant PrP.

[2] Hamsters remaining healthy after 390 days were negative for PK-resistant PrP^{Sc} in Western blots.

[3] One hamster remaining healthy after 390 days was positive for PK-resistant PrP^{Sc} in Western blots and has therefore to be considered to be diseased.

[4] Data partly from **Müller *et al.* [1]**

4.4 Kinetics of PrP 27-30 degradation and prion inactivation

To determine the kinetics of prion degradation and inactivation, we measured PrP levels at different time points during heat treatment. For degradation experiments, we treated samples at 120 °C in the presence of 90 % glycerol and 10 % water. We found that the amount of PrP declined rapidly in the first 20 min of incubation, which was followed by gradual degradation over the remaining time **(**60, 80 and 540 min; **Fig. 4)**. Of the initial PrP levels, 75 %–95 % was degraded during heating and cooling phases **(Fig. 4)**.

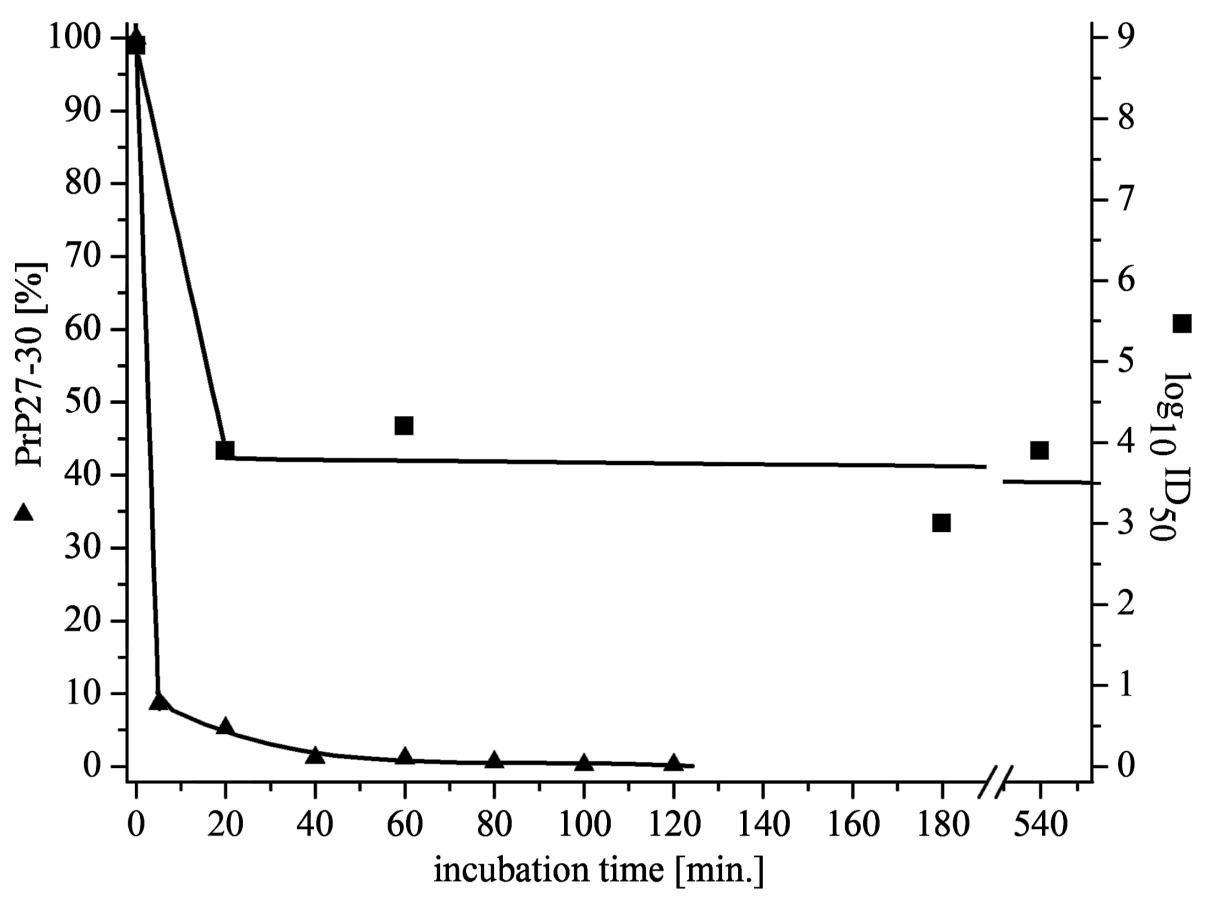

Fig. 4: Inactivation of prion infectivity and degradation of PrP 27-30 are biphasic processes. Degradation of PrP 27-30 was determined by gel electrophoretic analysis after exposure to 120 °C in 90 % water and 10 % glycerol for the incubation times indicated (▲). The value for degradation after heating and cooling without remaining at target temperature is depicted at 1 min. Inactivation of prion infectivity was determined by bioassays after exposure to 90 °C in 90 % fat and 10 % water for the incubation times indicated (■).

For prion inactivation studies, we treated samples at 90 °C in the presence of 90 % fat and 10 % water, with incubation times of 20, 60, 180, or 540 min. Similar to the kinetics of degradation, we found a biphasic inactivation:

infectivity rapidly decreased after 20 min, with residual infectivity diminishing very gradually over the remaining time points **(Fig. 4)**. The results from other solution conditions also exhibited similar biphasic kinetics **(Tab. 2)**. These findings indicate that heat destruction of prions comprises two distinguishable phases: a fast phase during which the majority of prions is destroyed followed by a slow phase of reduction.

4.5 Distribution of PrP 27-30 between fatty and aqueous phases

Since fat and water are immiscible and both affect degradation of PrP 27-30 as well as inactivation of prion infectivity, the distribution of PrP between the phases is of particular relevance. The amount of PrP 27-30 in each phase of a thoroughly stirred mixture of equal volumes of bovine edible tallow and water was analysed at different temperatures after phase separation. Between both phases, an interphase of insoluble protein was detected, which was analysed separately. At room temperature, nearly all PrP 27-30 was detected within the interphase **(Tab. 3)**. Only at higher temperatures were progressively increased amounts of PrP 27-30 found within the water phase. At 100 °C, about 25 % of the total PrP 27-30 content was enriched in the water phase. In contrast, less than 1 % of PrP 27-30 was detected within the fat phase at all temperatures examined. We conclude that PrP 27-30 tends to accumulate in the interphase rather than in either the lipophilic or the aqueous phase. Notable amounts of PrP 27-30 were found in the water phase only when salt or SDS was added **(Tab. 3)**.

Tab. 3: Distribution of PrP 27-30 in fat, water, and the fat/water interphase. The phase distribution of PrP 27-30 was measured after treatment under the conditions indicated. PrP 27-30 found in each phase is given as a mean percentage of the total signal.

treatment	fat phase	fat-water interphase	water phase
25 °C	0	100	0
40 °C	0	97.5 ± 0.5	2.5 ± 0.5
60 °C	0	92.0 ± 0.5	8.0 ± 0.5
80 °C	1.1 ± 1.0	93.1 ± 2.8	5.8 ± 2.4
100 °C	0	73.2 ± 1.1	26.8 ± 1.1
40 °C, 250 mM NaCl	6.1 ± 5.2	46.6 ± 3.0	47.3 ± 3.1
40 °C, 1 % SDS	3.4 ± 3.4	13.0 ± 9.4	83.6 ± 8.4

4.5 Solubility and aggregation of PrP 27-30 after heat treatment

To determine the effect of heat treatment on the solubility of PrP 27-30, differential ultracentrifugation was performed. Samples sonicated in the presence of 0.3% SDS yielded approximately 80 % of total PrP in the supernatant after ultracentrifugation, which reflects soluble PrP **(Tab. 4)**. However, an additional heat treatment (100–125 °C for 20 min) prior to sonication in 0.3 % SDS resulted in a majority of insoluble PrP 27-30. Heat treatment impaired the solubilising effect of SDS and increased the insolubility of PrP.

Tab. 4: Effect of heat on the solubility of PrP 27-30. The solubility of PrP 27-30 after heat treatment was determined by differential centrifugation at 100,000 × *g* for 1 h followed by gel electrophoresis, Western blotting, and densitometric quantification. Heat treatment was performed at <125 °C for 20 min. Insoluble and soluble PrP were determined as the percentages of total PrP found in the pellet and supernatant, respectively, after ultracentrifugation **[29]**.

treatment	insoluble PrP [%]	soluble PrP [%]
none	100	0
heat exposure	100	0
sonication without SDS	100	0
heat exposure, sonication without SDS	100	0
sonication in 0.3 % SDS	20.7 ± 2.8	79.3 ± 2.7
heat exposure, sonication in 0.3 % SDS	76.7 ± 9.4	23.3 ± 9.4

As described above, heat exposure induced the formation of aggregates, indicated by the high molecular mass bands in SDS gels. To confirm this observation, we used negative-stain electron microscopy to characterise the ultrastructure of samples after heat treatment in the presence of pure water, 10 % aqueous glycerol, and 90 % aqueous fat **(Fig. 5)**. In all cases, we observed that the fibrillar ultrastructure of PrP 27-30 was converted to amorphous aggregates. The loss of ordered, fibrillar substructure correlated with increasing temperature, indicating that the transition was temperature dependent. In the presence of 90% fat, the ultrastructure of PrP 27-30 was more readily converted to amorphous aggregates **(Fig. 5K)**, compared to samples treated at the same temperature in pure water **(Fig. 5D)**. Additionally, lower temperatures were sufficient to form amorphous aggregates in the presence of fat **(Fig. 5J)**. In contrast, glycerol appeared to protect partially the ultrastructure of prion rods against heat denaturation **(Fig. 5G and 5H)**, confirming the electrophoresis and solubility results.

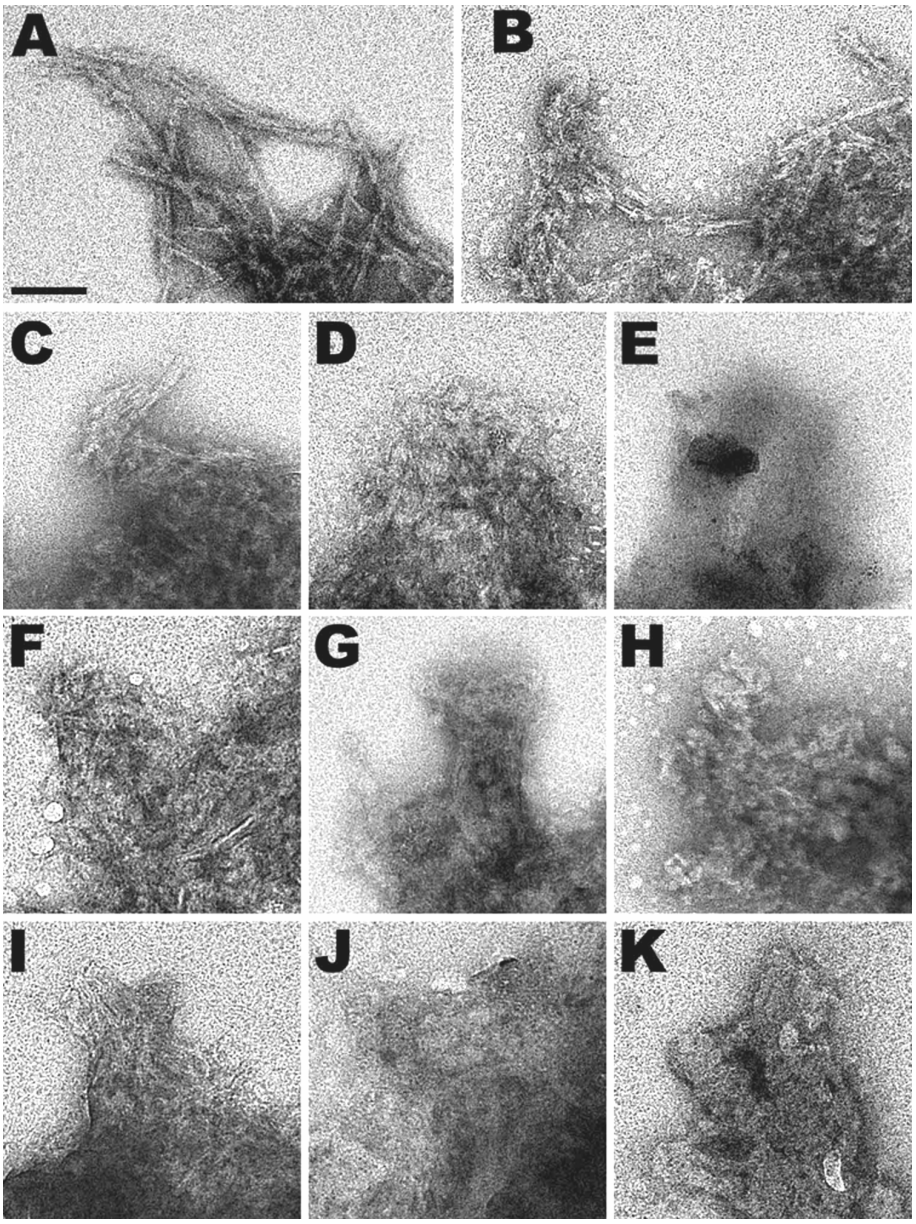

Fig. 5: Electron micrographs of PrP 27-30 before and after heat treatment in the presence of fat, glycerol, and aqueous buffer. (**A**) PrP 27-30 in the original preparations in the presence of about 60% sucrose. (**B**) PrP 27-30 after removal of the sucrose via ultracentrifugation. The rods are noticeable aggregated and fragmented. PrP 27-30 in aqueous buffer (50 mM Na HEPES, pH 7.2) before heat treatment (**C**), after treatment for at 100 °C (**D**) and 160 °C (**E**). PrP 27-30 in 90 % buffer/10 % glycerol before heat treatment (**F**), after treatment for at 100 °C (**G**) and 160 °C (**H**). PrP 27-30 in 90 % fat/10 % water before heat treatment (**I**), after treatment for at 80 °C (**J**) and 100 °C (**K**). All heat treatments were conducted for 30 sec. For some panels, the contrast was adjusted in a non-linear fashion (via Fourier filtering) to reduce the optical density of the negative stain. The bar in (**A**) represents 100 nm and applies to all panels.

In another method to visualise the ultrastructure of the fibrils, we stained samples with Congo red dye, which binds to amyloid and exhibits green-orange

birefringence under cross-polarised light. For samples treated at <100 °C, we observed amyloid structure **(Fig. 6)**. At higher temperatures (≥100 °C), we observed protein areas with dark appearance in cross-polarised light, i.e. without birefringence, indicating β-sheet-poor regions, which coincided with increased aggregate size **(Fig. 6)**. Heat treatment at 160 °C resulted in only a few small aggregates and no birefringence. From these observations, we conclude that heat treatment induced a transition from highly structured prion rods to amorphous aggregates with a less ordered substructure.

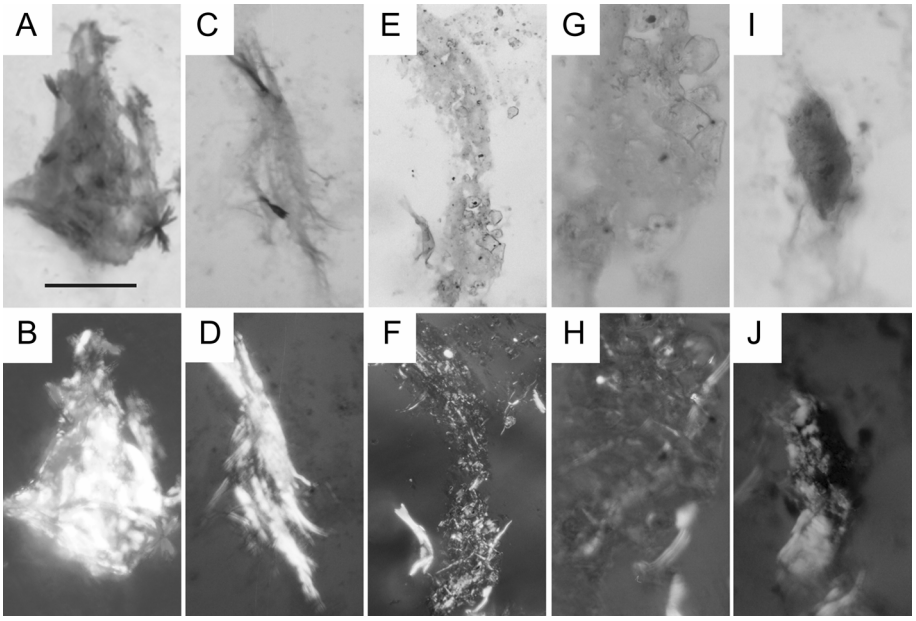

Fig. 6: Congo red staining of PrP 27-30 after heat treatment in aqueous buffer. PrP 27-30 (in 10 mM sodium phosphate, pH 7.2) was treated at RT (**A, B**), 100 °C (**C, D**), 120 °C (**E, F**), 140 °C (**G, H**), and 160 °C (**I, J**), then stained with Congo red and observed at 40× magnification under bright field (top row) or by polarisation microscopy (bottom row). Prion rods in the original preparations are shown as a control (RT). The bar in the top left panel represents 50 µm and applies to all panels.

Using another technique to analyse the fibrillar structure of PrP 27-30, we monitored the presence of amyloid fibrils using the ThT fluorescence assay **(Fig. 7**; corresponds to Fig. 2.5, p.42, Chapter II, Results and Discussion**)**. At temperatures of ≥80 °C, a significant reduction in the ThT fluorescence of fibrils was observed. In contrast, the amount of undegraded PrP, indicating an intact PrP 27-30 backbone, remained stable until temperatures exceeded 100 °C. This difference between the fibrillar structure and PrP backbone integrity confirmed the presence of an intermediate PrP 27-30 form (i.e., amorphous aggregate), which has less fibrillar structure but an intact protein backbone.

4.6 High temperatures decrease β-sheet–like secondary structure

To verify whether the heat-induced changes of fibrillar substructure are accompanied by alterations in secondary structure, circular dichroism (CD) spectra were recorded. PrP 27-30 samples were heated in 10 mM sodium phosphate, pH 7.2, to temperatures of 25 °C, 100 °C, 120 °C, 140 °C, or 160 °C (**Fig. 8**; corresponds to Fig. 2.6, p.43, Chapter II, Results and Discussion). To avoid degradation, samples were incubated for only 30 sec at the final temperature. The CD spectrum recorded at 25 °C showed a typical β-sheet pattern. Incubation at temperatures of up to 100 °C led to a decrease in ellipticity but no qualitative changes in spectral maxima and minima or in crossover points were obtained. At temperatures of ≥120 °C, we observed a further overall decrease in ellipticity and three negative peaks at 188 nm, at 210–212 nm, and at 229–231 nm. The crossover points remained identical.

The influence of fat could not be studied by CD spectroscopy due to high absorption. In the presence of glycerol, all structural changes were observed at slightly higher temperatures, reflecting the protective effect of glycerol as described above (data not shown). The spectral minimum at 229–231 nm was not detected. For reasons of comparison, a PrP 27-30 spectrum after α-helix/random coil induction by addition of 0.3 % SDS is also presented in **Fig. 8** (corresponds to Fig. 2.6, p.43, Chapter II, Results and Discussion).

4.7 Heat treatment decreases PK resistance

To determine the effect of heat treatment on the protease resistance of PrP 27-30, we compared the effect of PK digestion on the intensities of SDS gel bands and ThT fluorescence with and without prior heat treatment. For unheated PrP 27-30, the intensity of PrP bands and the ThT fluorescence intensity decreased simultaneously upon PK digestion, suggesting that fibrillar substructure and PrP backbone integrity are reduced concurrently. After incubation for several hours, both the PrP band intensity in SDS gels and the ThT fluorescence intensity were reduced to less than 25 % of the initial signal. During digestion for further prolonged incubation times, the intensities decreased much more slowly, reflecting the tailing phenomenon of prion destruction as described above **(data not shown)**.

After exposure to heat up to 100 °C, SDS gel band intensities demonstrated that the PK resistance of PrP 27-30 was reduced by 20 %–30 % in comparison with unheated samples. In contrast, ThT fluorescence intensities of PrP 27-30 during PK digestion did not differ in heated and not heated samples (**data not shown**). From these observations, we conclude that the PrP 27-30 fraction in fibrillar structure is also the PK-resistant portion.

5. Discussion

More insight into the molecular mechanism of *in vivo* prion amplification has become available only recently **[6, 39, 40]** and the components of the living cell involved in this process are unknown. Also, methods of prion decontamination are based on empirical grounds rather than on a molecular interpretation. Prions are unusually resistant to heat, irradiation, chemical disinfectants, and enzymatic digestion. Furthermore, the inactivation process is heterogeneous and depends upon the hydration of prions. Prion strains, the existence of which is difficult to interpret, have been characterised by different resistances to inactivation **[22, 41]**. Additionally, particularly resistant subpopulations of prions exist. The molecular environment, such as the presence of lipids or a polyglucose scaffold, can have a profound influence on inactivation **[9, 20]**.

We initiated the present study to analyse prion inactivation during hydrolytic fat splitting, a basic industrial process, and to deliver a risk assessment for BSE contamination **[1]**. Towards this end, we characterised systematically the thermal inactivation of prions and assessed the effect of fat, fatty acids, and glycerol in this process in order to understand the mechanism of inactivation.

5.1 Methodological aspects

We chose to use PrP 27-30 for our studies because it is the most stable fraction of infectious PrP^{Sc} **[8, 42–45]** and therefore represents the worst-case scenario with respect to decontamination of prions. We isolated PrP 27-30 from Syrian golden hamsters infected with the hamster-adapted, 263K scrapie strain **[7, 24, 42]**. These animals yield short incubation times and high infectivity titres in the brain, which make them a widely accepted, well validated, and highly sensitive model for prion infection. Most importantly, PrP 27-30 isolated from these animals is highly resistant to decontamination methods **[46–52]**.

5.2 Fat stabilises PrP 27-30 against degradation but destabilises prion infectivity against inactivation

At least four types of interactions compete during heat treatment, including hydrophobic intra- and intermolecular interactions of PrP aggregates, hydrophobic PrP-fat interactions, hydrophilic intra- and intermolecular interactions of PrP aggregates, and hydrophilic PrP-water interactions including hydrolytic activity. These interactions are characterised by different temperature dependencies. The enhanced stability of the PrP 27-30 backbone in the presence of fat may be due to additional hydrophobic interactions saturating the PrP surface. The hydrophobic interactions between PrP and fat molecules increase with temperature, which maintains the protective effect even after structural denaturation. The hydrolytic activity of water exhibits the strongest increase with temperature. Fat restricts hydrolytic activity by excluding water molecules. However, with increasing temperature, hydrolytic activity overcomes the protective effect of fat in water-fat mixtures.

In contrast to its stabilising effect against PrP 27-30 degradation, fat destabilises prion infectivity over the whole temperature range analysed **(Fig. 2**; corresponds to Fig. 2.2, p. 36, Chapter II, Results and Discussion**)**. Consequently, fat appears to compete with the intra- and intermolecular hydrophobic interactions of prions, which are responsible for the infectious structure. The effect might be similar to that of SDS, which is known to impose hydrophobic interactions and, at the same time, to reduce infectivity **[53]**. Since hydrophobic interactions increase with temperature, denaturation of PrP 27-30 by fat molecules is gradually facilitated, whereas the PrP 27-30 backbone is concurrently protected, at least at temperatures below 110 °C. At temperatures ≥110 °C, the hydrolytic activity of water dominates the backbone-protecting effect of fat, which results in degradation efficiencies similar to those from pure water or a fat-water mixture.

The competition between hydrophobic and hydrophilic interactions, particularly at room temperature, became obvious from the finding that all PrP 27-30 molecules were concentrated in the fat-water interphase and tend to migrate to the water phase only at higher temperatures **(Tab. 4)**. The PrP 27-30 fraction in the water phase increased also in the presence of salt or detergents, which is probably due to denaturation of the PrP molecule. The phase distribution

observed is in accordance with water molecules displacing fat molecules at higher temperature as well as with findings reported by others, assuming that prion infectivity exists in a water phase rather than in a fat phase **[54, 55]**.

5.3 Fat as a disinfectant

Unexpectedly, pure fat and fat-water mixtures offer mild prion inactivation. When prion inactivation was determined at temperatures below 100 °C, fat-water mixtures reduced prion infectivity by 5.1 $\log_{10}$ ID_{50}. In contrast, an inactivation factor of only 2.7 $\log_{10}$ ID_{50} was achieved for water. The addition of 2.25 M urea and 0.5 M DTT further inactivated prion infectivity, resulting in a reduction of 6.9 $\log_{10}$ ID_{50} **(Tab. 2)**. These observations can be explained by structural denaturation rather than degradation. In contrast to standard prion disinfectants, a mixture of water, fat, and urea at temperatures below 100 °C can be used as a mild prion disinfectant, which can be used to sterilise medical and manufacturing equipment. Due to the need for alternative sterilisation procedures, further assessment of the effects of this mild protocol on prion infectivity will be worthwhile.

5.4 Glycerol stabilises PrP 27-30 against both degradation and inactivation

Glycerol exerts a protective effect on both the polypeptide backbone integrity of PrP 27-30 as well as prion infectivity, which was observed at all temperatures examined. Glycerol is a known reagent for stabilisation of bacteria and proteins. For long-term storage at low temperatures, 20 % and 66 % glycerol are added to *Escherichia coli* cells and proteins, respectively. Due to its ability to act as both a hydrogen bond acceptor and donor, glycerol replaces water at least partially in its structure-forming function. It drastically increases viscosity and thus decelerates unfolding reactions, whereas its effect on the folding rate constant of proteins is small **[53]**. Furthermore, hydrolysis of the peptide bonds is diminished in the presence of glycerol. Glycerol might replace water also in the cage structure or destroy the cage on hydrophobic areas of the protein surface, leading to a stabilising entropy gain. In addition to protection against thermal impact, glycerol has been shown to protect PrP 27-30 infectivity against inactivation by hexafluoroisopropanol, a known α-helix–inducing solvent **[33]**.

5.5 Mechanism of heat inactivation

Partial PK resistance is used as surrogate marker for prion infectivity. Both PK resistance and insolubility of PrP 27-30 result from multimeric aggregation, which is associated with a increased β-sheet–rich secondary structure. In prion rods, a fibrillar substructure based on β-sheets correlates with prion infectivity **[5]**. Treatments altering the content of β-sheet–rich structure and multimeric aggregation ought to be accompanied by a reduction of infectivity **[53]**. In complete accordance with this expectation, our experiments demonstrate prion destruction occurring in at least of two steps: (i) denaturation of PrP to an intermediate form, which is characterised by a loss of structure responsible for prion infectivity and (ii) the complete loss of structure and backbone degradation of PrP **(Fig. 9**; corresponds to Fig. 3.2, p. 60, Chapter III, Conclusions and Consequences**)**. The first step occurs at lower temperatures, while the second step is observed at higher temperatures. Most but not all prion infectivity is destroyed during the first step.

For prion destruction, three effects have to be considered: (i) displacement of solvent molecules, which stabilise or destabilise prions; (ii) denaturation of prion structure, and (iii) degradation of the PrP backbone. Each effect adds to an activation energy that must be overcome for complete prion destruction. According to the literature, the total stability of monomeric proteins amounts to a ΔG^0 value of -15 kJ/mol to -50 kJ/mol. This stability increases for a polymeric substrate, such as with the fibrillar assembly of PrP 27-30, adding to the activation energy barrier for prion destruction. All activation energies obtained during the inactivation of the majority of prion infectivity, i.e. the first step, were <21 kJ/mol **(Fig. 3**; corresponds to Fig. 2.3, p. 39, Chapter II, Results and Discussion**)**. Such a low value indicates that a denaturation process featuring a loss of mainly non-covalent bonds has occurred. During the second step, we obtained higher values from 20 ± 5 kJ/mol to 51 ± 10 kJ/mol, which suggests that a cooperative reaction featuring the breakage of covalent bonds has occurred. It should be noted that a superimposition of several effects contributes to the activation enthalpies obtained.

A quantitative comparison of the loss of β-sheet–based fibrillar structure with the degradation of the PrP backbone indicated clearly that heat treatment alters the structure of PrP first **(Figs. 3 and 7**; corresponds to Fig. 2.3, p. 39 and

Fig. 2.5, p.42, Chapter II, Results and Discussion**)**. This analysis demonstrated a temperature difference between β-sheet reduction and backbone degradation of about 30 K. This loss of fibrillar structure was characterised by additional biophysical techniques. Gel electrophoresis and differential ultracentrifugation after sonication in SDS demonstrated that solubilisation of PrP aggregates became less effective. Electron microscopy confirmed a tendency to form larger aggregates. In correlation with electron microscopy, Congo red birefringence and ThT fluorescence confirmed the loss of fibrillar structure during the first transition.

From CD spectra, we conclude that the secondary structure was altered drastically during the first transition **(Fig. 8**; corresponds to Fig. 2.6, p.43, Chapter II, Results and Discussion**)**. At temperatures >100 °C, a strong negative peak at 188 nm and two smaller negative peaks at 210–212 nm and 229–231 nm, respectively, appeared. The spectra cannot be interpreted by superimposition of known spectra from the literature. The negative peak at 188 nm in addition to a slight red shift of the positive peak by 2.5 nm might indicate the emergence of one or several β-turn motifs. The additional negative bands at 210–212 nm and 229–231 nm, however, cannot be correlated to known secondary structure elements. Since CD spectroscopy can be evaluated quantitatively only for soluble proteins, an experimental artefact due to light scattering cannot be excluded for our highly insoluble samples. Artefacts, however, should be more pronounced in the range below 210 nm because scattering increases with decreasing wavelength and protein absorption is much higher below 210 nm. Consequently, we conclude that the appearance of the two negative peaks at 210–212 nm and 229–231 nm indicates a new, as-yet unknown structure.

Concomitant to the loss of fibrillar structure, prion infectivity is reduced drastically but not completely abolished; residual infectivity remains. This clearly suggests that infectivity of prion rods depends on an intact fibrillar structure containing β-rich subunits. At higher temperatures, PrP 27-30 aggregates dissociate, which facilitates the cleavage of the covalent PrP backbone and inactivates prion infectivity completely **(Fig. 9**; corresponds to Fig. 3.2, p. 60, Chapter III, Conclusions and Consequences**)**. In the presence of glycerol, degradation is impaired over the whole temperature range due to a displacement of water molecules that are necessary for hydrolysis.

5.6 Multi-step inactivation or subpopulations with different stabilities?

The prion inactivation curve **(Fig. 4)** exhibits a rapid decline in prion titre followed by an extremely subtle and slow decline. Similar tailing effects have been observed earlier **[14]**, which have been suggested to result from a population of infectious agents with heterogeneous properties, in particular, a subpopulation of higher heat resistance. While the existence of *in vivo* heterogeneity cannot be excluded, we consider it more likely that the heat-resistant infectivity results from the first transition as described above. The rapid decline in prion infectivity appears to result from a comparatively fast denaturation during the first step, which yields an intermediate state with a more highly aggregated and therefore even more heat-resistant core. Thus, partially denatured and more highly aggregated PrP molecules are assumed to protect core prion particles responsible for residual infectivity. In Western blots, PK resistance was reduced slightly after exposure to heat. This finding is in accordance with **Kuczius *et al.* [56]** and **Lawson *et al.* [57]**, who claim that preheated PrP^{Sc} is digested more efficiently. When infectivity and PK resistance were related to the degree of fibrillisation, we found that the loss of fibrillar organisation, based on β-sheet–rich secondary structure, decreased PK resistance. However, amorphous aggregates also exhibited substantial residual PK resistance, and β-sheet structure and PK resistance have been induced in other studies without acquiring infectivity **[58]**. Thus, infectious and non-infectious fibrillar forms of PrP^{Sc} clearly exist **[59]**.

In summary, heat treatment induced an intermediate form of amorphous PrP particles that are characterised by reduced solubility, increased aggregation, reduction in β-sheet and fibril contents, lower PK resistance, and diminished but residual infectivity. This residual infectivity was destroyed by increased treatment temperatures, which caused the disassembly of aggregates and PrP backbone hydrolysis **(Fig. 9**; corresponds to Fig. 3.2, p. 60, Chapter III, Conclusions and Consequences**)**. This intermediate state observed in our studies might explain the phenomenon of resistant subpopulations.

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Chapter VII

Appendix

VII.1 References

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VII.3 Abbreviations

263K	hamster-adapted scrapie strain
AA	amino acids
BSA	bovine serum albumine
BSE	bovine spongiform encephalopathy
°C	degrees Celsius
CD	circular dichroism
$CHCl_3$	chloroform
CJD	Creutzfeldt-Jakob disease
cm	centimetre
CWD	chronic wasting disease
Da	Dalton
e.g.	for example
et al.	and others (lat. *et alteri*)
EUE	exotic ungulate encephalopathy
fCJD	familial CJD
FFI	fatal familial insomnia
Fig.	figure
FSE	feline spongiform encephalopathy
g	gram
x g	average acceleration due to gravity of earth surface
GPI	glycosyl-phosphatidyl-inositol
GSS	Gerstmann-Sträußler-Scheinker Syndrome
h	hour
iCJD	iatrogenic CJD
ID_{50}	amount of infectivity that 50 % of prion-exposed animals become infected
i.e.	in detail (lat. *it est*)
kDa	kilodalton
log	logarithm
M	molar (mol/l)
MeOH	methanol
min.	minute
ml	millilitre
mM	millimolar
MW	molecular weight
µg	microgram
µl	microliter
µM	micromolar
ng	nanogram
µg	microgram
NMR	nuclear magnetic resonance

PAGE	polyacrylamide-gelelectrophoresis
PBS	phosphate buffered saline
PK	proteinase K
PrP	prion protein
PrP 27-30	N-terminally truncated PrP with MW = 27-30 kDa from prion rods
PrP^{C}	cellular prion protein
PrP^{Sc}	PrP Scrapie
rpm	revolutions per minute
RT	room temperature
sec	seconds
sCJD	sporadic CJD
SDS	sodium dodecyl sulfate
SHaPrP	PrP of Syrian gold hamster (*Carassius auratus*)
Tab.	table
TBST	Tris buffered saline, tween
ThT	thioflavin T
TME	transmissible mink encephalopathy
Tris	Tris-[hydroxymethyl-]aminomethane
TSE	transmissible spongiform encephalopathy
vCJD	variant CJD

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