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研究報告の概要

英国健康保護局は，手術器具の汚染除去に適すると考えられる新規のプリオン不活化法を開発する試みとして，Bacillus lentus，サブチリシン遺伝子を変異させて得られたアルカリプロテアーゼである MC3（または PrionzymeTM）の使用について説明している。この研究プログラムは，散発的クロイツフェルト・ヤコブ病(CJD)が神経外科手術で伝播するという所見によって進められた。新規変異株は野生型サブチリシンのアルカリプロテアーゼ中，3 つのアミノ酸が置換されており，より高い熱安定性ならびにより速い触媒速度を示す。著者らは本稿において，プリオンに感染したマウスの脳ホモジネートを MC3 とともに pH 8，10 および 12 で in vitro でインキュベートしたところ，すべての免疫反応性 PrP^{Sc}（感染性プリオンタンパク質）が完全に分解されたことをウェスタンブロット法で確認した。同条件下においては部分的にしか分解しないプロテイナーゼ K と比較すると，MC3 はより高い有効性を示した。これらの結果は in vivo の実験によって裏付けられており，この実験では MC3 で分解した感染性マウス脳ホモジネート（iMBH）でスパイクしたマウスの 66.6%が 18 ヵ月を超えて生存し（平均インキュベート期間 447±154 日間），一方 10^{-8} に希釈した iMBH をスパイクしたマウスの生存率は 30.4%（326±162 日間）であった。この有意差は 7 log 超のプリオンクリアランスに相当する。これに対して，プロテイナーゼ K で分解した iMBH をスパイクしたマウスの全生存率は 22.7%（327±151 日）と低かった。著者らは，アルカリ条件下における MC3 による分解は，簡単かつ安全な汚染除去法であり，医療器具に対し非破壊的であることから，医療上の管理および手術器具の汚染除去において広範に適用可能であると結論付けている。

報告企業の意見	今後の対応
この新規手法は，手術器具のプリオン汚染除去に関する興味深いものであり，また，適用された場合は，集団における外科的伝播のリスクならびに比較的高い有病率（英国など）を低減させるのに寄与すると考えられる。しかしながら，このような技術が血漿分画製剤の製造工程においてプリオンに対する病原体除去・不活化能力をさらに向上させることができるか否かは未だ不明である。	現時点で新たな安全対策上の措置を講じる必要はないと考える。 今後，本手法の血漿分画製剤の製造工程への適用に関する情報収集に努める。

使用上の注意記載状況・その他参考事項等

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Decontamination of prion protein (BSE301V) using a genetically engineered protease

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KEYWORDS
Bacillus lentus subtilisin; Bovine spongiform encephalopathy; Inactivation processes; Variant Creutzfeldt–Jakob disease

Summary A previous study has demonstrated the potential of alkaline proteases to inactivate bovine spongiform encephalopathy (BSE301V). Here we explored the use of MC3, a genetically engineered variant of *Bacillus lentus* subtilisin. MC3 was used to digest BSE301V infectious mouse brain homogenate (iMBH). MC3 eliminated all detectable 6H4-immunoreactive material at pH 10 and 12; however, Proteinase K was only partially effective at pH 12. When bioassayed in VM mice, MC3- and Proteinase K-digested iMBH gave respectively 66.6% and 22.7% survival rates. Using a titration series for disease incubation, this equates to a >7 log reduction in infectivity for MC3 and >6 log reduction for Proteinase K. This study demonstrates the potential for thermostable proteases to be developed as effective inactivation processes for prion agents in healthcare management.

Introduction

The emergence of bovine spongiform encephalopathy (BSE) in cattle, believed to have given rise to variant Creutzfeldt–Jakob disease (vCJD) in humans, has generated a number of public health issues. Although any residual risk of eating BSE-contaminated food has been minimised, there continues to be a threat of onward transmission by iatrogenic routes.

Defining the levels of risk of transmission of human prion disorders via surgery, transplant or transfusion has proved difficult due to the unique nature of these agents, uncertainties about the underlying prevalence of the disease and the absence of ante-mortem diagnostic methods. The number of clinical vCJD cases remains low: 167

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(dead and alive) definite or probable cases of vCJD in the UK with a further 43 cases worldwide to June 2008 [University of Edinburgh's National Creutzfeldt–Jakob Disease Surveillance Unit (NCJDSU) website]. All clinical cases have been restricted to a single genotype, being homozygous for methionine at codon 129 of the prion protein gene (*PRNP*). Further cases of potential infection, with no known clinical disease, have been identified in the two remaining genotypes: two valine homozygotes from a retrospective study looking at appendix samples and a heterozygote case of blood transfusion related transmission.[1–3] Studies suggest that clinical manifestation in these genotypes may require extended incubations and possibly present with different clinical symptoms.[4,5]

The estimation of transmission risk via vCJD is difficult as certain aspects of its presentation differ significantly from the historical forms of CJD. PrP^{Sc}, a biomarker for prion infectivity, has been found at much higher levels in vCJD-infected lymphoid and peripheral nervous tissue than sporadic CJD (sCJD) infected tissue.[6,7] This suggests that the iatrogenic transmission of vCJD is more likely than sCJD, which is supported by the identification of four cases of transfusion-related vCJD.[3,8,9] However, there are well-documented cases of transmission of sCJD via neurosurgery, transplant of dura mater and human growth hormone.[10–14] Some studies have also suggested that an increased rate of sCJD is associated with an increase in the number of surgical events an individual may have undergone.[15,16] In addition, PrP^{Sc} signal has been shown in sCJD-infected skeletal muscle and adrenal gland, suggesting that infectivity may be more widespread than originally thought.[17,18]

Given the high levels of uncertainty, validated methods for the decontamination of surgical instruments are urgently required. It is widely accepted that autoclaving only partially inactivates TSE agents.[19,20] The World Health Organization (WHO) recommends extended treatment with high concentrations of sodium hypochlorite or sodium hydroxide but these are not suitable for many applications, including routine decontamination of surgical instruments.[21] We report here an extension of our previous work using genetically engineered alkaline proteases in an inactivation model relevant to treatment of surgical instruments.

Methods

Reagents and models

Proteinase K was supplied by Finnzymes (Espoo, Finland) and MC3 ('Prionzyme™') by Danisco US Inc., Genencor Division (Rochester, NY, USA). Preparation of BSE301V mouse brain homogenate, analysis by sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis, western blotting and bioassays were carried out as previously described.[22] Animal studies were conducted in compliance with current UK Home Office regulations and licences.

Assessment of the subtilisin variants

The kinetic parameters k_{cat}, K_M, and k_{cat}/K_M were measured by hydrolysis of succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide (AAPFpna).[23] To assess thermal stability, purified enzyme (15 μg/mL in 0.1 mol/L glycine, 0.01% Tween-80, pH 10.0) ± 50 mmol/L $CaCl_2$ was incubated at 10 °C for 5 min, 10 °C to 60 °C over 1 min and 60 °C for 20 min, then placed on ice for 10 min and enzyme activity measured by hydrolysis as above.

Results

Protease digestion of infectious MBH (iMBH)

Digestion of BSE301V iMBH by MC3 was assessed at different pH values. At all pH values tested, MC3 was able to reduce the levels of 6H4-reactive material as detected by western blot (Figure 1). The western blots showed the presence of the characteristic triple bands corresponding to the unglycosylated, monoglycosylated and diglycosylated forms of PrP^{Sc}. Bands were fully digested at pH 8, 10 and 12: this is in line with previous results showing that alkaline pH is critical to effective digestion of PrP^{Sc} by subtilisin-type proteases.[22] At pH 4, PrP^{Sc} is only partially digested, whereas greater digestion is observed at pH 2 and 6, suggesting that the conformation at pH 4 may be especially resistant to proteolysis. Proteinase K was relatively poor at eliminating PrP^{Sc} with a smear of material evident even at pH 12, partial digestion of PrP^{Sc} at pH 8 and 10, resolution to PrP^{Sc} at pH 6 and little or no effect at pH 2 and 4 (Figure 1). Digestions of iMBH with MC3 at 50, 60, 70 and 80 °C were analysed by western blot; digestion at 60 °C appeared to be most effective (data not shown).

Assessment of MC3-digested iMBH by bioassay gave a highly significant reduction in the overall levels of infectivity compared with our previous study (Figure 2). Of the mice challenged with MC3-digested iMBH, 66.6% survived to the end of the study with a mean incubation of 447 ± 154 days (assuming that all animals had been culled at 18

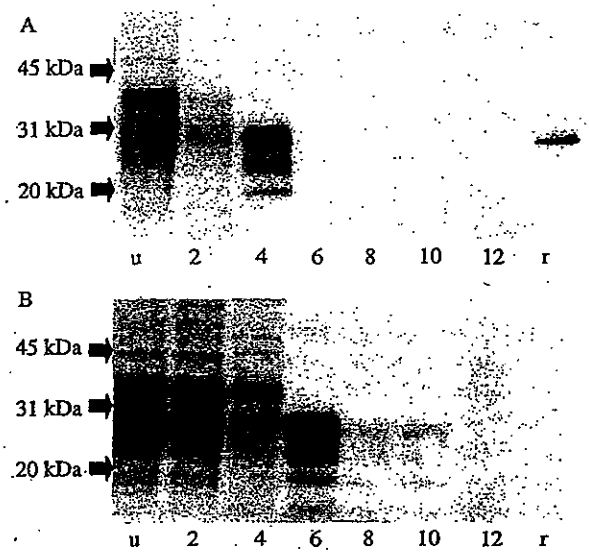

Figure 1 Degradation of 6H4-immunoreactive material by proteases under alkaline conditions. Infectious mouse brain homogenate (iMBH) dialysed against pH buffer indicated, digested for 30 min at 60 °C with 2 mg/mL MC3 (A) and Proteinase K (B). Digestion was assessed by western blot with 6H4. Lane r: recombinant PrP (Prionics); lane u: undigested iMBH in PBS.

months post challenge). This compares with 29.2% (315 ± 173 days) and 30.4% (326 ± 162 days) survival for 10^{-7} and 10^{-8} dilutions of iMBH respectively.[22] The incubation period (447 ± 154 days) is significantly different from the 10^{-8} dilution (326 ± 162 days), equating to a >7 log clearance, as assessed by Kaplan–Meier survival analysis ($P = 0.0195$). Data from our previous study show incubation of iMBH with pH 12 buffer resulted in a reduction in infectivity of ~1 log from 135.8 ± 5.59 to 142.8 ± 9.62 for an equivalent dilution of iMBH. Extended incubation periods of 327 ± 151 days were also obtained for Proteinase K digestion although there was a lower overall survival rate of 22.7%. These values were not significantly different from the 10^{-8} dilution ($P = 0.7971$). Histology was carried out to confirm disease pathology and showed complete correlation between clinical endpoint and signs of disease in the brain (results not shown).

Relevance of the genetic modifications to MC3 and its ability to digest prion material

MC3, a proprietary alkaline protease, represents a genetically engineered variant of the *Bacillus lentus* subtilisin with amino acid changes N76D/

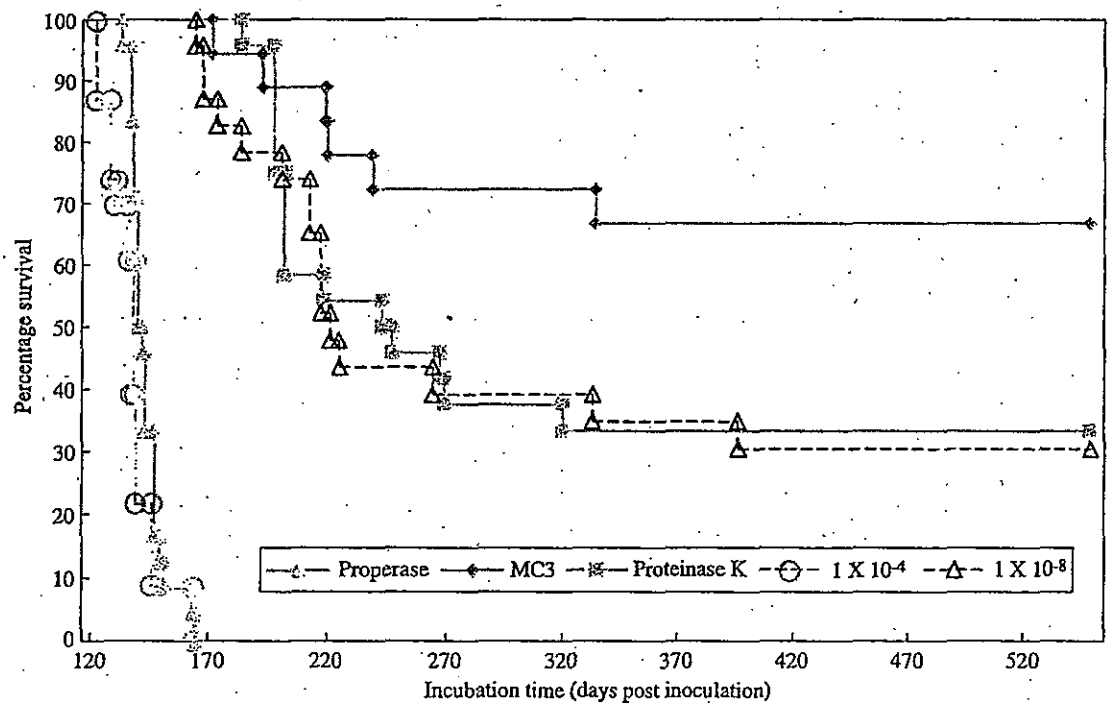

Figure 2 Bioassay survival curves of protease-treated BSE-301V infectious mouse brain homogenate (iMBH) in VM mice. Percentage survival of mice was assessed following protease digestion of 10% BSE301V iMBH. The values were compared to a titration of iMBH in naive MBH (nMBH) as published previously.[22]

Table I Thermal stability and enzymatic characteristics of engineered proteases[a]

	Thermal stability ($T_{1/2}$ min)[b] (% of Purafect)	Enzyme kinetic analysis		
		k_{cat} (1/s)	K_M (M)	k_{cat}/K_M
Purafect	100	170	0.78	2.20E+05
Properase	100	435	1.89	2.30E+05
MC3	460	830	1.6	5.20E+05

[a] Measured by hydrolysis of succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide at pH 8.6, 25 °C.
[b] pH 10 buffer.

S103A/V104I.[24] The effects of the three amino acid modifications in MC3 were compared with both the parent enzyme (wild type *Bacillus lentus* subtilisin; Purafect) and Properase (Table I). At pH 10, MC3 shows significantly improved thermal stability with the half-life of the enzyme extended by >4.5-fold compared with Purafect and Properase. Enzyme kinetics were measured by hydrolysis of the substrate AAPFpna. MC3 showed significantly improved catalytic properties with a higher catalytic rate (k_{cat}/K_M) of 5.2×10^5 compared with 2.3×10^5 for Properase and 2.2×10^5 for Purafect.

Discussion

This study demonstrates the ability of genetically engineered proteases, selected for improved stability and catalytic properties at alkaline pH, to digest prion material. We have reduced BSE301V infectivity from ~3 log with Properase in our previous study to >7 log with MC3. This compares favourably with established decontamination/disinfection processes and is beyond the 5 log reduction recommended by the Spongiform Encephalopathy Advisory Committee (SEAC) on its website. MC3 can be easily introduced into existing healthcare practice and avoids the harsh conditions for instruments, operators and the environment that are associated with the WHO guideline conditions of 20 000 ppm active chlorine or 1 mol/L sodium hydroxide. The alkaline pH required for inactivation is comparable with other alkaline prion inactivating cleaners on the market (neodisher® Septo-Clean, Serchem Delta) and is compatible with stainless steel instruments. The 30 min contact time of the enzyme with iMBH was selected on the basis of compatibility with a pre-soak process for the final product.

Since the publication of our earlier study, several other groups have described approaches to the inactivation of prions including further protease-based, detergent-based and gas-phase inactivants.[20,25–35] Comparing data between these models is extremely difficult as a variety of TSE agents, animal models and challenge regimes have been used to assess decontamination. The relevance of different scrapie strains to the inactivation of human prion agents (and BSE) has been questioned, given their lower stability to thermal, chemical and enzymatic denaturation.[20,36,37] Given this uncertainty it would seem most appropriate to use a high titre TSE agent that is directly relevant to human disease in an animal model with no transmission barrier. At the moment such models are limited to the type of strain used here, a murine passaged BSE strain as a model for BSE and vCJD, or vCJD/sCJD in a human transgenic murine model.

Many inactivation studies have used the wire implant model as pioneered by Weissman and colleagues.[38] Although these studies are clearly directly relevant for assessing the effectiveness of prion removal from steel surfaces, they may be limited in their ability to show large reduction values due to the limited volume of material on the wire. Titration curves for 263K scrapie on wires consistently show a very rapid decrease in infectivity at ~4–6 log dilutions compared to the 8 log dilutions typical of this agent in solution.[30,31] Limited data using wire implants in overexpressing transgenic mouse models offer no improvement in sensitivity.[25,33] SEAC has highlighted on its website the need for a standardised model which can be used to compare the efficacy of decontamination technologies. The guidance suggests that a suitable model should mimic the clinical situation, for which the decontamination technology is to be used, as closely as possible. SEAC recognises that infectivity bound to a metal surface, particularly material that is dried on, may represent a tougher challenge than infectivity in solution. However, the guidance also suggests that any decontamination process should demonstrate a reduction in infectivity of ≥5 log, to offer an appreciable improvement over existing practices. In reality this may mean that two models need to be employed: a wire model to demonstrate clinical efficacy and an 'in-solution' model to ensure sufficient dynamic range.