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Development of an Optimized Feeding Technology for Dairy Cows

Improvement in Resistance to Ruminal Proteases in the De Novo*–Designed Protein MB-1*

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Abstract

We have previously reported on MB-1, a designer protein with potential application in animal nutrition. Having a high content of selected essential amino acids, MB-1 should provide limiting nutrients for animals and promote growth and production. However, the protein was found to have marginal conformational and proteolytic stability, and, thus, strategies for stabilizing MB-1 were elaborated. We discuss the synthesis of MB-1-Cys dimer, a protein with an intermolecular disulfide bridge. This mutant was exposed to Pronase E protease preparation as well as to proteases extracted from ruminal microbes. It was found that in both cases, MB-1-Cys dimer had a better resistance to proteolytic degradation than MB-1. Denaturation and hydrophobic dye binding studies revealed that this enhanced stability was not owing to conformational stabilization, but rather to changes in surface exposure as a consequence of dimerization. In particular, it was found that binding of ANSA to MB-1-Cys dimer was comparable to that observed

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for native, compact, natural proteins. We discuss the implications of these results for the design of transgenic protein production systems.

Index Entries: Agro-biotechnology; protein design; essential amino acids; proteolytic degradation; rumen protease.

Introduction

Feeding dairy cattle essential amino acids (EAA)-enriched proteins resistant to microbial breakdown in the rumen, or feeding proteins/amino acids postruminally, has been shown to significantly increase the production of milk and milk protein *(1–5)*. Several approaches have been used to protect proteins from ruminal degradation, including roasting, chemical treatment, and encapsulation (e.g., SmartAmine™ by RhonePoulenc) *(6–8)*. Such techniques increase the inherent cost of feeding. At the same time, they can modify the protein, rendering it indigestible in the intestine and selectively decrease availability of limiting amino acids such as lysine *(6,8)*.

The expense of feed modification and the difficulties associated with postruminal feeding have led to a new approach in feeding technology. Currently, many researchers are focusing their investigations on the production of high-quality proteins either directly in the rumen or in the feed. By genetically altering ruminal bacteria or creating transgenic crops, more efficient and less costly sources of EAA may be found *(9–11)*. An ideal protein would contain a high level of methionine and lysine and other selected EAA in various amounts, depending on the application. Mainly three approaches for the improvement of protein quality in a given organism are being explored *(12)*. The first approach involves the transfer of a gene coding for a high-quality protein from one organism to another that is more suitable for farming practices (heterologous expression). In this approach, the amino acid composition of a natural protein is predetermined and may not conform to the desired ratio of EAA. The second approach implies the modification of genes within the organism in such a way as to insert more selected EAA into the corresponding proteins. Successful engineering for increased methionine content has been reported, but the percentage of residues modified was rather small *(13)*. Attempts to change the amino acid content often destabilize the protein, if not prevent it from folding or being recovered at all. This limits the usefulness of the proteins created *(9,12,14,15)*. The third approach (chosen here) involves the creation of new proteins, with a biased composition of selected EAA *(16,17)*. This strategy theoretically allows for full control of the amino acid composition of the protein, an advantage over other options. Previous efforts to express synthetic proteins with high EAA content in bacteria did not yield high quantities of protein, largely owing to lack of stability of the final product. This is possibly attributable to lack of structural stability in particular, among other reasons *(17–19)*.

Rational *de novo* protein design has proven to be a useful way to both test and expand our understanding of the factors that contribute to protein

folding and stability *(20)*. New sequences encoding conformational information have been created from first principles and synthesized or expressed *(20–23)*. Experimental results indicated that *de novo* design is indeed possible, because these designed proteins have at least some of the desired structural qualities.

We have previously reported on the design of MB-1, a protein with a high content in selected EAA *(16)*. This tailored protein was designed *de novo* using a rational approach by which the sequence was made to specify a target fold: the square α-helical bundle. Approximately 60% of its amino acid content is made up of the EAA methionine, threonine, lysine, and leucine *(16)*. Initial results indicated that the design resulted in a protein that is stable in *Escherichia coli*, largely helical, and characterized by a folded core. Its native behavior and expression levels in vivo suggest that MB-1 is superior to earlier designs *(17–19,24)*.

The efficiency of MB-1 as a source of EAA for lactating cows will depend on its resistance to degradation in the producing organism, in the ruminal environment, and finally in the abomasum. Recently, we reported on the proteolytic degradation of MB-1 by Pronase E. The model experiment predicted that MB-1 would be degraded rapidly (in less than 1 h) in the ruminal environment *(24)*. In an attempt to identify sources of instabilities in MB-1, we have used a range of techniques and found that its conformational stability was low. In fact, the melting temperature for MB-1 matched the ruminal temperature of 39°C. This means that for a significant population of MB-1, most targets, including those that should be buried in the core, would be exposed to proteases in the rumen.

In an effort to improve MB-1's resistance to degradation, strategies involving the insertion of disulfide bonds in its structure were considered. An intramolecular disulfide bridge would bring stability to the protein by reducing the entropy of the unfolded state. At the same time, it would bring stiffness to the region where it is formed. This protein feature has been correlated with resistance to degradation in the rumen *(25–27)*. An intermolecular bridge would provide protection at the interface between the proteins, where the surface area accessible to proteases would be reduced. Interactions between closely situated proteins may also confer an increase in conformational stability, which in turn could enhance resistance to proteases. Finally, dimerization of MB-1 through disulfide linkage would give insight into the impact of genetic dimerization (i.e., linking of two MB-1-coding regions in one single gene to express a protein of twice the molecular weight) on protein stability.

In this article, we present the characterization of MB-1-Cys, a mutant of MB-1 capable of forming an intermolecular disulfide bond. The new dimeric molecule is compared to MB-1 with respect to its resistance to degradation to Pronase E protease extract, its resistance to intracellular proteases extracted from ruminal microorganisms, its conformational stability, and its binding to the hydrophobic dye 8-anilino-1-naphthalene sulfonic acid (ANSA).

Materials and Methods

Preparation of MB-1 Mutant

Restriction enzymes *Pst*I and *Hind*III each cleave the pCMG20 4-x plasmid at a single site. The region between these two sites corresponds to the last eight amino acids of helix IV, the last helix of MB-1. Synthetic oligonucleotides were designed such that, when annealed, they would form a cassette housing the cysteine mutation at amino acid position 93, MB-1 flanking sequences, and *Pst*I and *Hind*III sticky ends for directional subcloning. A gift from R. Teather (Agriculture Canada, Ottawa), the oligonucleotides (strands 1 and 2) were purified using denaturing polyacrylamide gel electrophoresis (PAGE), heated together at 100°C for 2 min, and then cooled slowly for 1 h until annealed. Then the corresponding MB-1 sequence was replaced by the mutant cassette through directional subcloning.

1. Strand 1: 5'-GTGCTTGATGCAAAAAGGCGTAGCTTA-3'.
2. Strand 2: 3'-ACGTCACGAACTACGTTTTTCCGCATCGAATTCGA-5'.
   *Pst*I *Hind*III

E. coli B834 cells (genotype: F^- *ompT gal met* r_Bm_B) were transformed with the ligated plasmid and selected for ampicillin resistance. The mutant construct was confirmed by sequencing (*see* Fig. 1) using the *Bst*I sequencing kit (Bio-Rad).

Protein Production

Bacteria carrying the mutant vectors were grown in 1 L of LB Miller medium (Difco) to an optical density of 0.6. Transcription was induced using isopropyl β-D-thiogalactopyranoside at 1 m*M* for 3 h. Then the cells were harvested by centrifugation at 3000*g*. The purification procedure was essentially as described in Beauregard et al. *(16)* with a few modifications. Precipitated cells were resuspended in ice-cold column buffer (10 m*M* Tris, 200 m*M* NaCl, 10 m*M* EDTA, 1 m*M* NaN_3, pH 7.4). Phenylmethylsulfonyl fluoride, EGTA, benzamidine-hydrochloride, and benzamide were added to a final concentration of 0.1, 10, 2, and 2 m*M*, respectively. Cells were then lysed by ten 30-s sonication pulses using a Branson Sonifier 250 at 60% output control. The sonicate was centrifuged at 11,500*g* for 30 min at 4°C.

The supernatant was loaded onto a 15-mL amylose column. MBP-MB-1 was eluted by washing the column with column buffer containing 10 m*M* maltose (elution buffer). Pooled peak fractions were placed in dialysis tubing (Spectra/Por; molecular weight cutoff of 3500 Daltons) with 50 µL of factor Xa/10 mL of fusion protein. The dialysis bag was placed in 20 m*M* Tris, 100 m*M* NaCl, and 3 m*M* $CaCl_2$ (cleavage buffer) overnight at 4°C. The following morning, the bag was transferred to Tris-EDTA (TE) buffer, pH 8.0. After dialysis, the sample was applied to DEAE-Sepharose equilibrated in TE buffer, pH 8.0 (Fast Flow; Pharmacia) and washed with the same buffer. MB-1 was collected as the flowthrough.

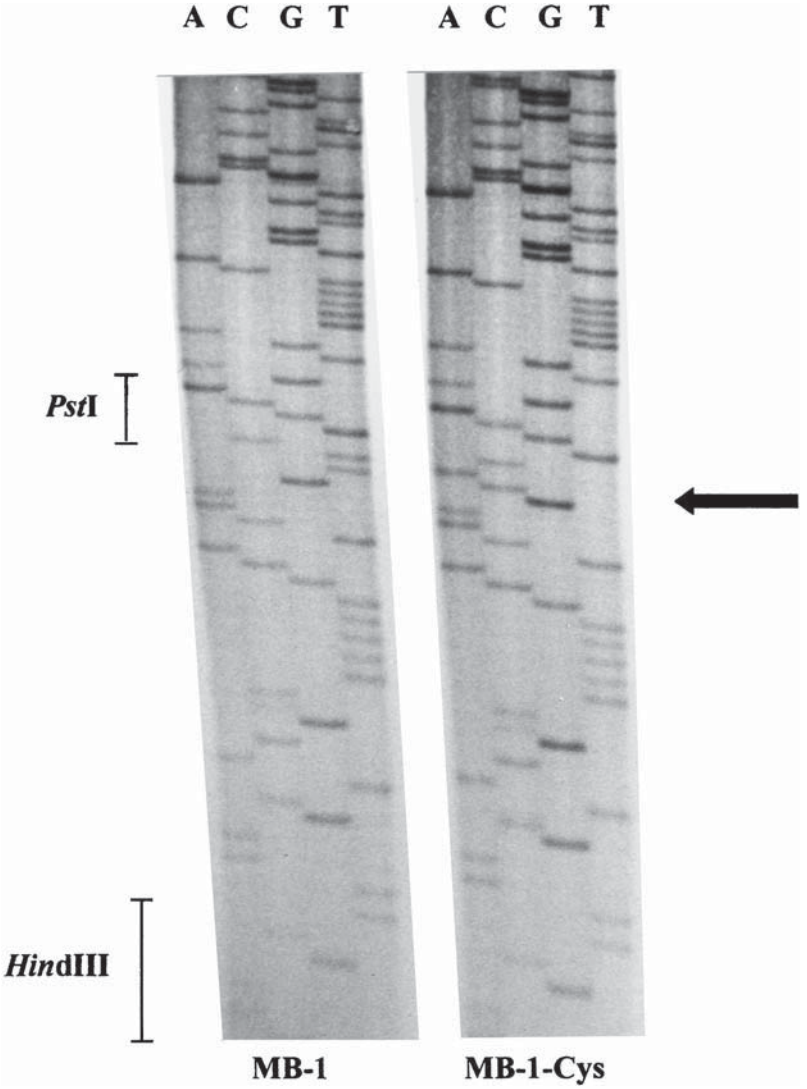


Fig. 1. Sequencing gel autoradiograms for MB-1 and MB-1-Cys plasmids. Noncoding strands were sequenced 5' to 3' in the C-terminal region. The mutation is indicated by an arrow as the sequence is read from the bottom to the top of the gel. The GTT coding for N93 is changed to a GCA, which codes for a cysteine. *Pst*I and *Hind*III sites are shown for reference points.

MB-1-Cys was purified using the same procedure; however, 2 m*M* dithiothreitol (DTT) (Sigma, St. Louis, MO) was used in all solutions except for cleavage buffer.

Protein Quantification and Electrophoresis

The concentration of protein was determined by the bicinchoninic acid (BCA) assay (Sigma), using bovine serum albumin as the standard. Protein was visualized by sodium dodecyl sulfate (SDS) PAGE using

12% polyacrylamide-tricine gels *(28)*, followed by staining with silver nitrate. SDS-PAGE experiments were conducted prior to measurements to confirm protein purity.

Dimerization of MB-1-Cys

Removal of the DTT by dialysis into TE buffer (pH 8.0) showed rapid and complete conversion of MB-1-Cys to the MB-1-Cys dimer (monitored by tricine SDS-PAGE under nonreducing conditions).

Extraction of Ruminal Proteases

Ruminal liquor was extracted from a 7-yr-old fistulated cow. The fluid was subsequently purified to a powdered form containing intracellular and membrane-bound proteases as described by Mahadevan et al. *(29)*, with the following changes: 1 L of ruminal fluid was used, centrifuging was done for 1 h at 5000*g*, 200 mL of water and butanol were used, and the filter cake was resuspended in 300 mL of acetone. The powder was not purified further after being collected from the desiccator.

Characterization of Powder Proteolytic Activity

To solubilize proteases from ruminal microbes, 0.2 g of rumen-extracted proteases (REPs) were added to 10 mL of water and stirred for 1 h. The solution was then centrifuged at 10,000*g* for 30 min to pellet down insoluble particles. The pellet was discarded, and the protein content in the supernatant was measured by BCA.

To determine the proteolytic activity of the REPs, hydrolysis of casein (purified powder from bovine milk; Sigma) was carried out at 39°C as described in Mahadevan et al. *(29)*. The presence of amino acids in the REPs and the production of amino acids by autodegradation of REPs during incubation were subtracted from total amino acid production. It followed that the rate of casein hydrolysis for our extract was 100 mg of casein/h, which is lower than that reported by Mahadevan et al. *(29)* (500 mg of casein/h). This difference in proteolytic activity may be attributed to the difficulty in reproducing a precisely defined ruminal flora in different cows. However, removal of insoluble particles by the additional centrifugation step that was performed may also have contributed to the discrepancy.

Assessment of Proteolytic Degradation of MB-1 and Mutant

REP was prepared at a concentration of 3 μg/mL, a concentration that would produce a protein background faint enough on gel so as not to interfere with the MB-1 and dimer bands. Solutions for degradation assays were made of 3 μg/mL of REP, 40 μg/mL of protein in 300 μL of buffer (borate phosphate, pH 6.8). Under these conditions, REP activity was 0.22 U/g of MB-1. REP was added after the sample had been equilibrated at 39°C for 15 min. Aliquots were taken at time zero (immediately after REP addition), and every 10 min thereafter. Reactions were stopped by the addition of

2% SDS buffer and heating for 3 min at 100°C. Samples were analyzed by SDS-PAGE followed by silver staining. The amount of protein remaining after incubation with proteases was determined by measuring the density of each band on the gels using a densitometer (LKB 2222-020 Ultroscan XL).

In addition to REPs, we used the bacterial protease type XIV from *Streptomyces griseus*, better known as Pronase E (P-5147; Sigma). This protease extract is used for prediction of foodstuff degradability in the rumen *(30,31)*. For these experiments, 1 mg of MB-1 was dissolved in 2 mL of borate-phosphate buffer (pH 6.8) and dialyzed at 4°C against 200 vol of the same buffer with two bath changes overnight. A 1-mL aliquot was withdrawn from the dialysis bag and incubated at 39°C for 15 min in a water bath. Pronase E was added at 6.6 U/g of protein. Samples were processed as described for REP digestion experiments.

Measurement of Thermal Stability

For thermal denaturation studies, a solution of 50 m*M* sodium acetate, 20 m*M* sodium propionate, 20 m*M* sodium butyrate, and 17 m*M* potassium phosphate adjusted to pH 6.5 (volatile fatty acids [VFA] buffer) was used to mimic the VFA content and pH of ruminal liquor *(32,33)*. MB-1 (0.5 mg) or MB-1-Cys dimer was dissolved in 1 mL of VFA buffer and dialyzed overnight at 4°C. All solutions were filtered (0.2 μm) prior to fluorescence measurements. Tyrosine residues were excited at 284 nm, and the spectra were obtained from 295 to 350 nm, using a band pass of 3 nm. Denaturation studies for both proteins (in triplicate) were conducted from 15 to 80°C with an equilibration period of 15 min between each temperature. The sample temperature was controlled with a circulating bath (Lauda R6S). The cell temperature was calibrated against the bath temperature with a Parr 1671 precision thermometer. All spectra were corrected for buffer and water emission and for loss of efficiency in the detector and gratings of the PTI Rf-M2204 fluorometer.

Thermal stability was calculated assuming a unimolecular, two-state process as previously described *(34)*. For MB-1 and MB-1-Cys dimer, I_{303} (tyrosine fluorescence emission at 303 nm) was used as the property (y) indicative of the extent of unfolding of tertiary structure. In the folded state, the parameter $y = y_f$ and the fraction of folded protein, f_f, is equal to 1. When the protein is unfolded, the parameter $y = y_u$ and the fraction of unfolded protein, f_u, is equal to 1. For intermediate states, y is given by $y_f f_f + y_u f_u$. Thus, by measuring y, we can calculate the fraction of protein unfolded:

$$f_u = (y_f - y)/(y_f - y_u)$$

The equilibrium constant for the unfolding process is $K_u = f_u/(1 - f_u)$, and melting temperatures (Tm) are obtained at $K_u = 1$ *(34)*.

Determination of Affinity for Hydrophobic Dye ANSA

The impact of MB-1 and MB-1-Cys dimer on ANSA fluorescence was investigated. Solutions prepared with 0.1 mg/mL of protein were added to

10 μ*M* ANSA in a 10 m*M* acetate buffer (pH 5.0). After a 5-min incubation, ANSA was excited at 350 nm and its fluorescence emission was detected from 400 to 500 nm. The same experiments were performed in the absence of proteins, and the ratio of I_{480} (ANSA + protein) over I_{480} (ANSA alone) was calculated for each. This ratio is known as the enhancement factor.

Calculation of ANSA binding parameters was done according to Scatchard *(35)*, with modifications by Cardamone and Puri *(36)*. In the first study, the protein concentration was varied from 0 to 0.8 mg/mL and the amount of ANSA was held constant (2 μ*M*). In the second experiment, the protein concentration was kept constant at 0.1 mg/mL, and the concentration of ANSA was increased from 0 to 500 μ*M*. A 10 m*M* acetate buffer (pH 5.0) was used for the analysis.

For both studies, a value referred to as Δ*F* was calculated. The Δ*F* was determined using the fluorescence intensity at the wavelength of maximal fluorescence for each individual spectrum, as suggested by Cardamone and Puri *(36)*. The equation defining Δ*F* is as follows:

$$\Delta F = \{[I_{480}\ (\text{Protein + ANSA}) - I_{480}\ (\text{ANSA})]/I_{480}\ (\text{Buffer})\}$$

Using the data collected in the first experiment, a plot of $1/\Delta F$ vs 1/[protein] was prepared. This curve was extrapolated to 1/[protein] = 0 to determine the change in fluorescence that would be observed for 2 μ*M* bound ANSA. Using this value, the increase in fluorescence intensity that would be observed for 1 μ*M* bound ANSA, otherwise known as the arbitrary unit, was obtained.

Employing the arbitrary unit calculated, the amount of bound and unbound ANSA was calculated for each of the samples in the second experiment (0.1 mg/mL of protein, ANSA varies). The Δ*F* was determined for each spectrum, and the amount of bound ANSA in each solution was calculated by dividing the Δ*F* by the arbitrary unit. Once the concentration of bound ANSA was calculated, the concentration of free ANSA could be determined from the known concentration of ANSA added. These data were then plotted according to Scatchard's equation:

$$b/f = [(\text{n} - \text{b})/K_D]$$

in which *b* is the concentration of bound ANSA; *f* is the concentration of free ANSA; *n* is the number of ANSA binding sites per protein; and K_D is the dissociation constant.

Results

The design strategy adopted for MB-1-Cys is best illustrated by the sketch in Fig. 2. An exposed asparagine was replaced by a cysteinyl residue so that it would be available for reaction with another exposed cysteine. Once oxidized, the proteins are linked and their exposed surface area is reduced. DNA sequencing of the mutated plasmid is shown in Fig. 1. The noncoding strand was sequenced from 5' to 3', and the GTT to GCA change

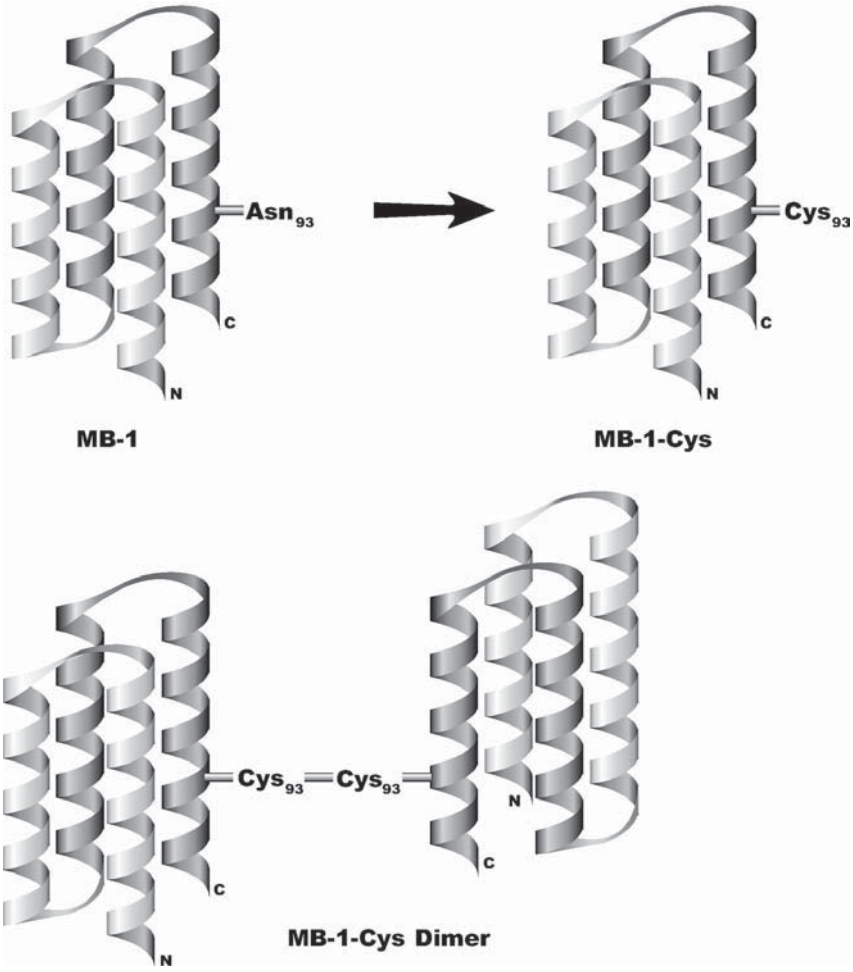


Fig. 2. Design of MB-1-Cys dimer. The pictogram shows the mutation N93C, allowing for formation of a disulfide bridge between two proteins.

was found as expected. This corresponds to an AAC → TGC change on the coding strand that is translated to the N93 → C mutation in the protein. SDS-PAGE analysis of the protein after production and purification revealed that on removal of the reducing agent DTT, the apparent mass of MB-1 increased from 11.3 to 22.8 kDa (not shown).

The impact of the mutation and dimerization on resistance to proteases was verified using Pronase E digestion experiments. This protease extract was used under conditions reported previously, conditions that allowed a fairly reliable prediction of foodstuff degradability in the ruminal environment *(30,31)*. Figure 3 shows degradation curves, and Fig. 3B clearly demonstrates the improvement of resistance to Pronase E brought by dimerization. After 50 min, all the MB-1 had vanished, whereas the initial population of MB-1-Cys dimer had decreased by only 50%.

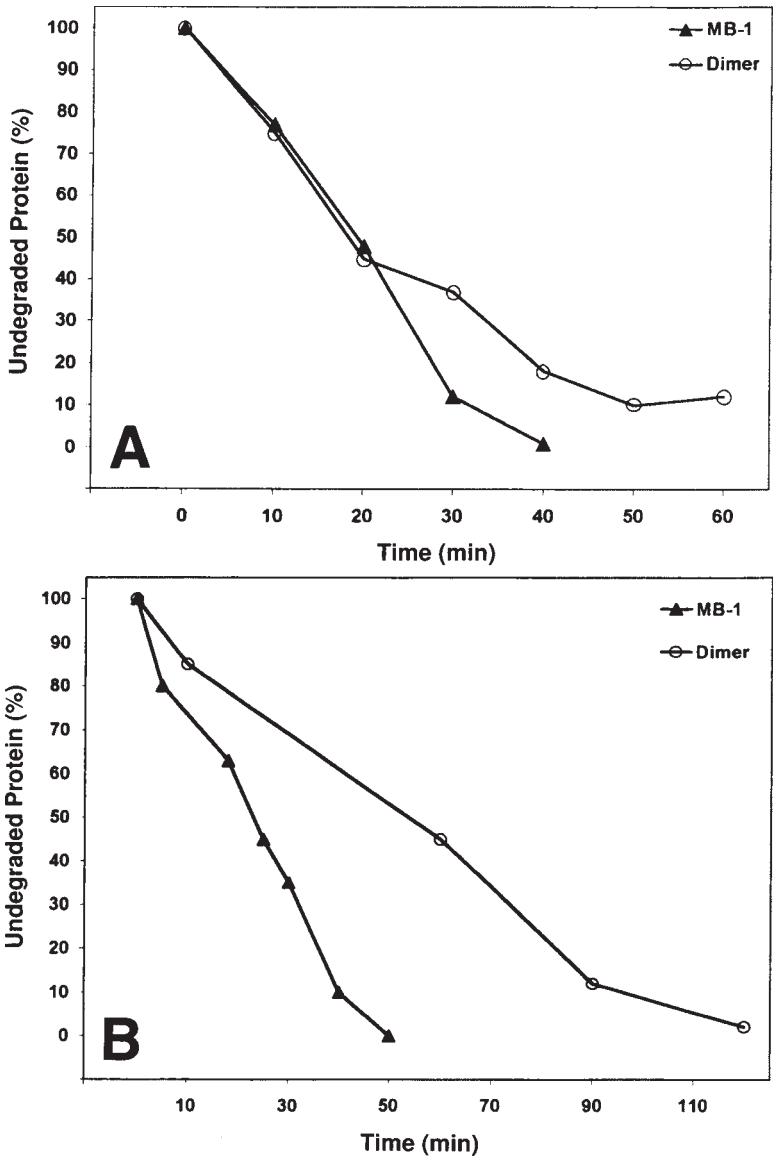


Fig. 3. Comparison of degradation by Pronase E and rumen proteases. **(A)** Degradation of MB-1 (▲) and the dimer (○) by REP; **(B)** degradation by Pronase E using the same symbols.

The Pronase E technique has been shown to mimic degradation in the rumen, where extracellular proteases represent the bulk of proteases present in solution *(30,31)*. In terms of MB-1 production, this assay may provide information on MB-1 performance when supplied as a feed additive, being produced in a crop. However, in terms of MB-1 production in ruminal microbes where degradation by intracellular proteases dominates, this model experiment may not be adequate. To circumvent this, we have

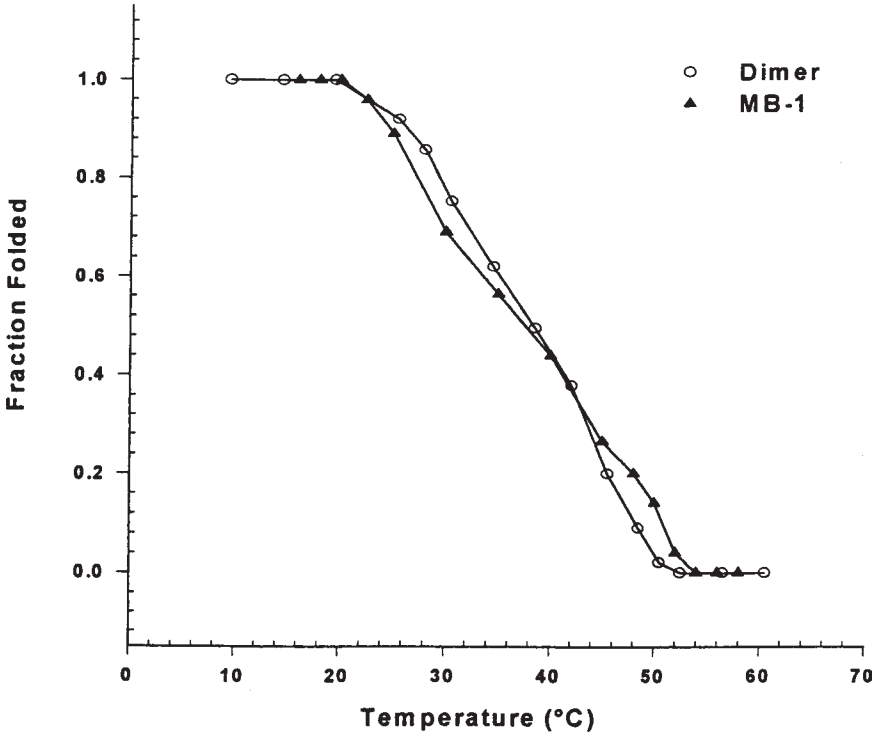


Fig. 4. Thermal denaturation curves for MB-1 and dimer. The melting temperature (T_m) is the temperature at which half of the proteins are folded (fraction folded = 0.5).

adapted an extraction procedure first published by Mahadevan et al. *(29)* that focused on the purification of intracellular proteases from ruminal microflora. Using the same approach as for Pronase E, MB-1 and MB-1-Cys dimer were exposed to ruminal proteases and analyzed as described in Materials and Methods. Under these conditions, MB-1-Cys dimer appears to degrade in two phases: a rapid one that parallels degradation of MB-1, followed by a slower phase that allows MB-1-Cys dimer to outlive MB-1 (Fig. 3A). It is not clear at present why such behavior was not observed with Pronase E.

The difference in proteolytic stability observed for MB-1-Cys dimer was predicted to originate mainly from the reduction in surface area available to proteases. Yet, other factors may influence resistance to proteases, especially conformational stability and exposure of hydrophobic clusters, or lack of compactness. To determine whether these factors were contributing to the enhanced proteolytic stability of MB-1-Cys dimer, further studies were performed. Thermal denaturation studies were carried out on MB-1-Cys dimer and MB-1 to assess any conformational change in the protein. The unfolding curves obtained are shown in Fig. 4, where it appears that the mutation and dimerization had little impact on conformational stability. At 39°C, both protein populations were 50% unfolded, with the

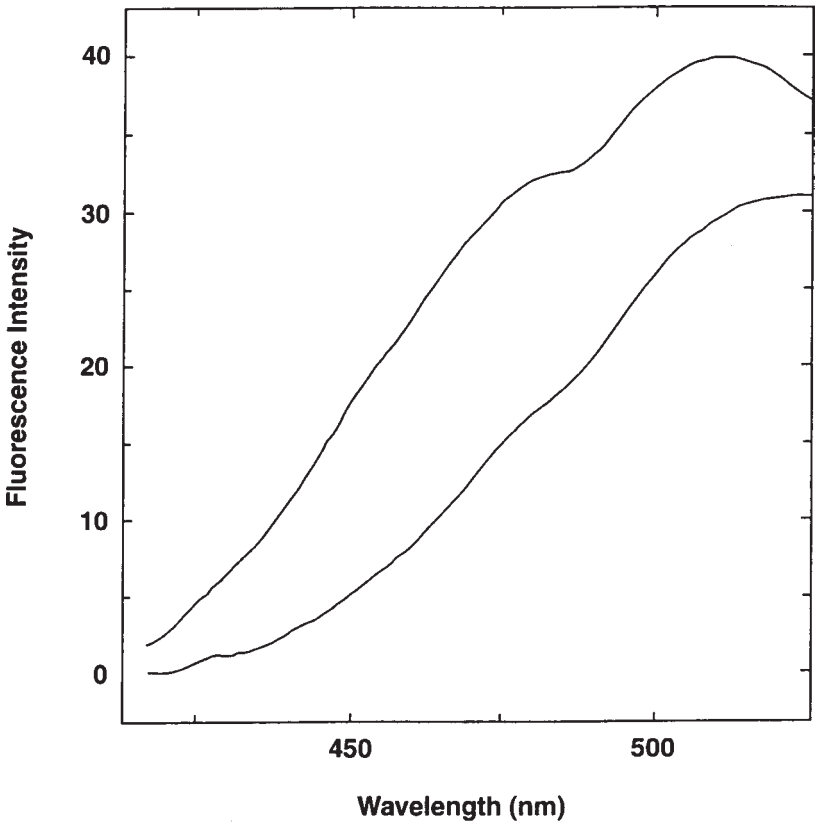


Fig. 5. Enhancement of ANSA fluorescence by MB-1. The intensities at 480 nm (where emission from bound ANSA is maximal) were used to calculate the enhancement factor. Bottom curve, ANSA alone; top curve, ANSA + MB-1. An enhancement factor of 2 is calculated for MB-1.

detrimental effect being a high exposure of protease targets and a low resistance to degradation in the rumen.

Lack of compactness and exposure of hydrophobic targets in the folded state of a protein are also known to increase susceptibility to proteases *(37,38)*. Because the fluorescent dye ANSA is a diagnostic probe of loosely folded cores and exposed hydrophobic clusters *(39,40)*, it can be used to detect any changes in these properties when a mutation is introduced into a protein. The impact of MB-1 and mutant on ANSA fluorescence (enhancement factor) was therefore measured as shown in Figs. 5 and 6. An enhancement factor of approx 2 was determined for MB-1, and a value of approx 1.5 was observed for the dimer. These values indicate a low exposure of hydrophobic clusters to ANSA in both proteins. They suggest that the proteins are compact and that the small difference between the enhancement factors for MB-1 and MB-1-Cys dimer is insufficient to explain the large difference in proteolytic stability observed with Pronase E.

A Scatchard analysis of ANSA binding was then performed to determine the affinity of ANSA for the proteins (K_D). Figure 7 shows the fluores-

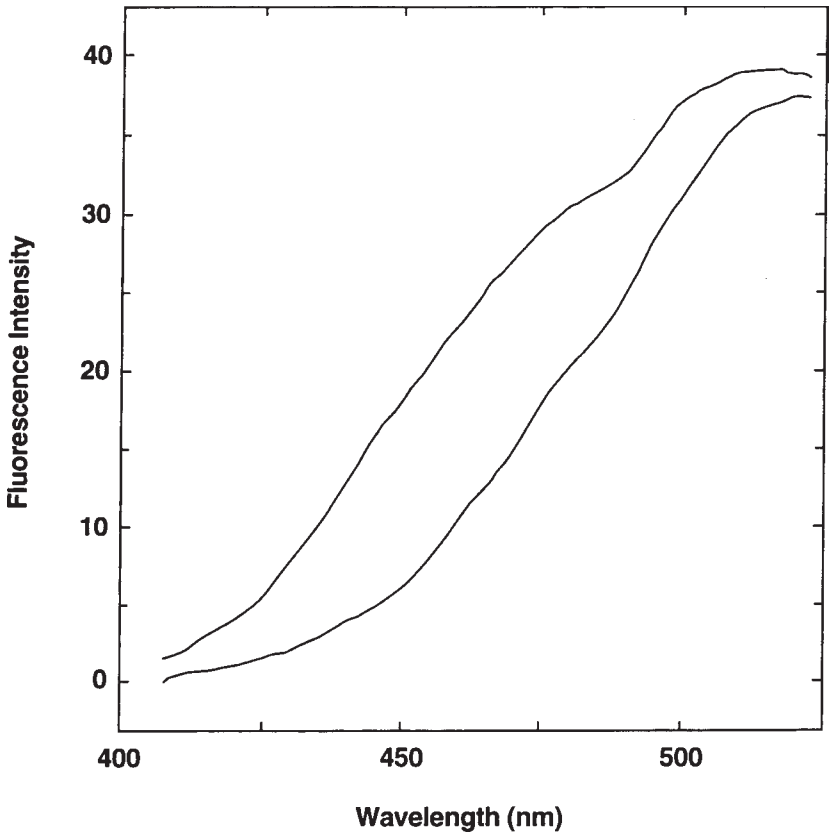


Fig. 6. Enhancement of ANSA fluorescence by MB-1-Cys dimer. The intensities at 480 nm (where emission from bound ANSA is maximal) were used to calculate the enhancement factor. Bottom curve, ANSA alone; top curve, ANSA + MB-1-Cys dimer. The enhancement factor calculated from these data is 1.5.

cence intensities recorded for various concentrations of ANSA in the presence of a fixed amount of protein. Figure 7 demonstrates that MB-1-Cys dimer offered a limited number of binding sites and that ANSA fluorescence tended to saturate as the concentration increased. Meanwhile, in the presence of MB-1, ANSA fluorescence increased linearly, showing no sign of saturation within the 0–500 μ*M* concentration range. To determine the dissociation constant, K_D, the maximal change in ANSA fluorescence on binding to various concentrations of the proteins was found. Extrapolation of the plot (Fig. 8) to infinite dimer concentration reveals that the binding of 1 μ*M* ANSA to MB-1-Cys dimer resulted in a maximal fluorescence increase of 75 arbitrary units. This analysis did not work for MB-1, since no saturation was observed (as seen in Fig. 7). A Scatchard analysis of ANSA binding to MB-1-Cys dimer was performed as described in Fig. 9. K_D was calculated from the slope of this Scatchard plot to give a value of 230 μ*M* (*see* Materials and Methods).

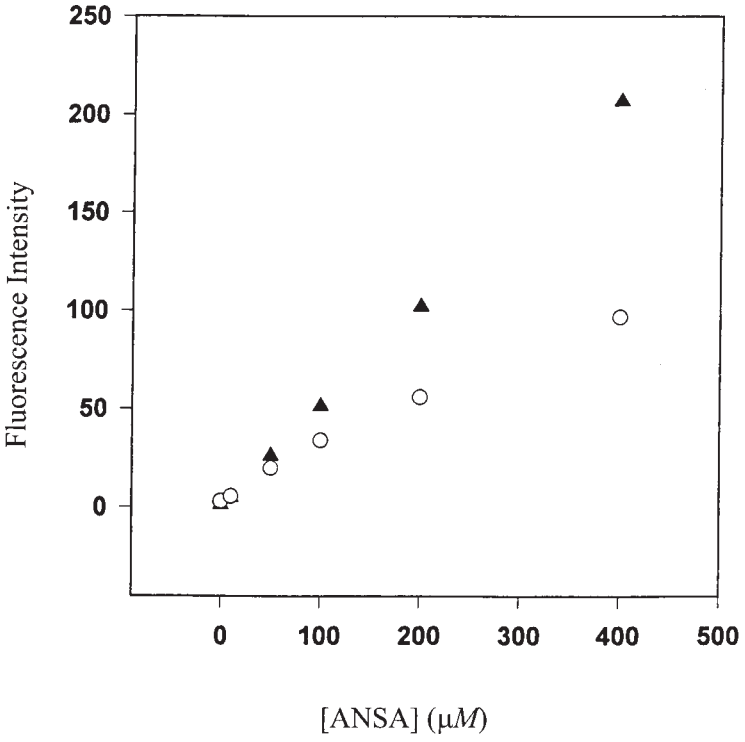
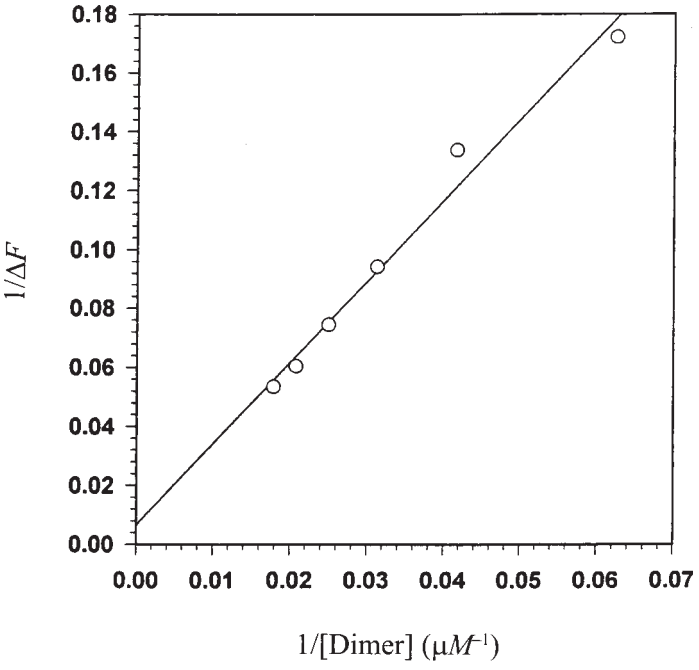

Fluorescence Intensity
250
200
150
100
50
0
0
100
200
300
400
500
[ANSA] (μM)

Fig. 7


1/ΔF
0.18
0.16
0.14
0.12
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0.08
0.06
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0.06
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1/[Dimer] (μM⁻¹)

Fig. 8

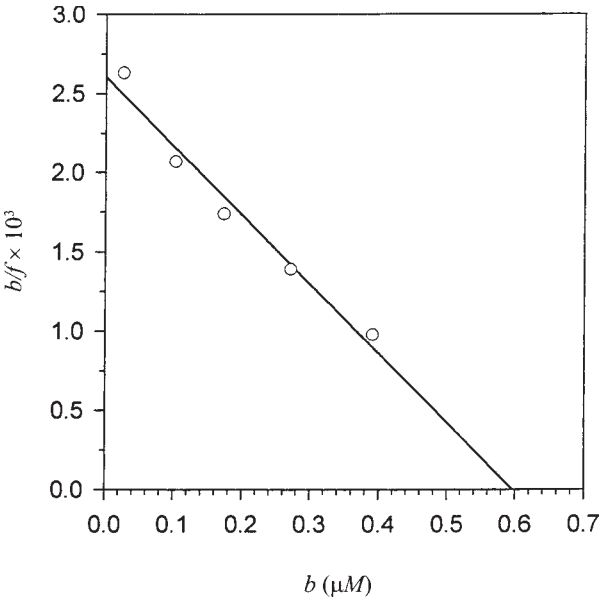

Fig. 9. Scatchard analysis of ANSA binding to MB-1-Cys dimer. The dissociation constant is given by the slope, which corresponds to $-1/K_D$. In this case, K_D is 230 μ*M*.

Discussion

MB-1 was designed to help meet the nutritional requirements of high-producing dairy cows, and of all the comparable proteins reported so far, it appears to be the best-characterized synthetic protein designed for nutrition. It is expressed and stable in vivo, folded as per design, and has several features of native proteins *(16,41)*. Its resistance to proteases compares to that of some natural plant proteins but is still rather weak *(24)*. Our strategy for solving this limitation involved the insertion of disulfide bridges. MB-1-Cys was created by replacing N(93) with a cysteine residue. Oxidation of the cysteine sulfhydryl groups on two closely situated MB-1 units resulted in the formation of a disulfide product with twice the molecular weight. This linkage was expected to place the units appropriately, so that each would protect a portion of the other from the surroundings and reduce the number of accessible protease targets. Proteolytic studies with both

Fig. 7. *(previous page)* Titration of ANSA fluorescence at 480 nm. Protein concentration was maintained at 0.1 mg/mL, and ANSA concentration was increased from 0 to 400 μ*M*. ANSA binding to MB-1-Cys dimer (○) saturates at higher ANSA concentrations, whereas binding to MB-1 (▲) does not.

Fig. 8. *(previous page)* Double reciprocal plot of the change in fluorescence (Δ*F*) induced by addition of dimer to ANSA. The concentration of ANSA used was fixed at 2 μ*M*. Extrapolation to infinite dimer concentration revealed an increase in fluorescence intensity of 75 for 1 μ*M* ANSA. This arbitrary unit was used for the determination of the dissociation constant (*see* Materials and Methods).

Pronase E and REP indicated that dimerization had rendered MB-1 more resistant to proteolytic degradation. Whereas Pronase E is believed to mimic degradation by ruminal liquor proteases *(30,31)*, REP was formulated to contain intracellular and membrane-bound proteases from ruminal microflora. Because MB-1-Cys dimer was found to be relatively more stable than MB-1 in both tests, it is quite reasonable to assume that this will be the case in vivo.

We then tried to explain the gain in resistance to proteolytic degradation observed for MB-1-Cys dimer. It was postulated that the enhanced proteolytic stability could be owing to the following:

1. A decrease in the exposed surface area surrounding the disulfide bond.
2. The change in amino acid sequence having an impact on conformational stability, and in turn on proteolytic stability *(42,43)*.
3. Stabilizing interactions between surface residues of the disulfide-linked subunits.
4. A change in the degree of exposure of hydrophobic regions *(37,44)*.

The first experiment involved thermal denaturation and comparison of the melting temperatures of MB-1 and MB-1-Cys dimer. The results obtained suggest that the increased proteolytic stability of the dimer could not be attributed to an increased conformational stability. Because options 2 and 3 in the previous list would have resulted in an increase in conformational stability, they may be discarded from the list of possible explanations.

Differences between proteins in their folded states may also have an impact on degradability. In particular, exposure of hydrophobic regions might enhance proteolytic degradation. To verify whether or not there was a difference between exposure of hydrophobic regions in MB-1 and MB-1-Cys dimer, the hydrophobic dye ANSA was employed at a temperature of 18°C at which the two proteins would be in their folded states. Such dye is believed to have little affinity for proteins in their folded state, and a higher affinity when proteins either are destabilized enough to permit ANSA penetration, or when they expose hydrophobic clusters *(39,40,45)*. Because the presence of an exposed hydrophobic surface, or a highly fluid core, is expected to contribute to proteolytic susceptibility *(37,44)*, it was postulated that a change in exposure may have occurred in the mutant, rendering it less susceptible to degradation. The interest of ANSA binding measurements was therefore twofold. On the one hand, the results would establish whether or not option 4 was a factor in the enhanced proteolytic stability of the dimer. On the other hand, the binding properties would determine whether or not MB-1 and MB-1-Cys dimer compare to native proteins in terms of compactness. Achievement of a compact core with low affinity to ANSA is a current challenge in designer protein projects *(44)*.

The change in ANSA fluorescence (i.e., the enhancement factor) on binding to MB-1 and MB-1-Cys dimer was determined under conditions that minimize destabilization of protein conformation *(40)*. The enhancement factors measured were similar to the values of 1.5–10 reported for

native proteins, and clearly out of the range for nonnative, molten, globule-like proteins (enhancement factors of 10 and above) *(45–47)*. This means that the slight change in core compactness observed for the dimer, as indicated by its smaller enhancement factor, cannot explain the large enhancement in proteolytic stability. Although no significant change in enhancement factors was observed, ANSA binding to MB-1-Cys dimer was found to differ from that for MB-1. At high concentrations, ANSA appeared to continuously create more binding sites in MB-1, while binding saturated in the dimer. Such behavior has been observed in the past when ANSA at a high concentration was placed in contact with proteins for long periods of time *(40)*. Under such conditions, ANSA appears to continuously create new binding sites in the protein, which in turn leads to destabilization of the core. Clearly, MB-1 did not resist destabilization by ANSA, and MB-1-Cys dimer was unaffected by the amphiphilic ANSA molecules. Taken together, these results rule out option 4 in the list of possible explanations and lend support to the concept of a reduction in exposed surface after dimerization as the cause of enhanced resistance to proteases.

Our investigation allows for some progress regarding MB-1's (and its derivatives) potential application as a high-quality protein for lactating cows. The gain in stability was more profound for the Pronase E experiment, suggesting that MB-1-Cys dimer would be more effective when fed via a transgenic crop as compared to a ruminal microbe expression system. When comparing protein digestibility in the rumen, the dimer would be comparable to sunflower albumin 8, more resistant to degradation than pea vicilin and convicilin, but less resistant than ovalbumin and the large subunit of RubisCo *(26)*. A protein having a tailored EAA content and such stability is an achievement in protein biotechnology.

The question of how long a protein should resist degradation in the rumen depends, to a large extent, on the way it is going to be supplied. When one considers a transgenic organism such as a crop or ruminal bacteria, then the compartment where expression of a high-quality protein will occur is of prime importance. For instance, in the case of a disulfide-linked oligomer, the redox potential of such a compartment becomes critical. Experiments involving different transgenic organisms that would shed light on the question of protection in the rumen are under way.

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