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Prediction of Folding Stability and Degradability of the *De Novo* Designed Protein MB-1 in Cow Rumen

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ABSTRACT

The authors have recently reported on the design of a protein (MB-1) enriched in methionine, threonine, lysine, and leucine. The protein is intended to be produced by rumen bacteria, in a way that would provide high producing lactating cows with limiting amino acids. In this report, MB-1 stability in the rumen is assessed, i.e., where the protein might be found after cell lysis or after being secreted by rumen bacteria. Current in vitro methods used to predict proteolytic degradability in the rumen were used for MB-1, as well as other natural proteins for comparison. MB-1 was found to be more susceptible to degradation than cytochrome c and ribonuclease A. Data indicate that MB-1 will be rapidly degraded if exposed to the rumen environment without protection. The contribution of folding stability to proteolytic stability was also examined. Rumen liquor components were selected to formulate a solution compatible with constraints of thermal denaturation studies. Denaturation curves show that the natural proteins were folded at rumen temperature. The MB-1 denaturation curves indicated that MB-1 does not unfold in a cooperative transition when heated from 20 to 70°C. This suggests that MB-1 structure may be progressively modified as temperature increases, and that a continuum of conformations are available to MB-1. At 39°C, a significant (50%) portion of MB-1 molecules had their tertiary structure unfolded, contributing to proteolytic degradability. Despite the unusual constraints

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used in MB-1 design (i.e., a maximized content in selected essential amino acids), results show that MB-1 has structural properties similar to previously reported *de novo* designed proteins.

Index Entries: Synthetic protein; *de novo* design; essential amino acids; ruminant nutrition; rumen biotechnology.

INTRODUCTION

In dairy cows, most of the protein absorbed by the animal comes not from the feed, but from micro-organisms in its rumen *(1)*. Ruminally synthesized microbial protein does not possess an ideal essential amino acids (EAA) balance and is insufficient to meet the demands of lactation in high producing cows *(2)*. The result is a fairly large deficit in the amino acids methionine (M) and lysine (K), and a lesser deficit in other amino acids including threonine (T) and leucine (L) *(2–5)*. Feeding proteins that are resistant to microbial breakdown in the rumen (or feeding proteins/amino acids postruminally) significantly increased production of milk and milk protein *(3,6–11)*. Consequently, several approaches have been used to protect proteins from ruminal degradation: roasting, chemical treatment, and encapsulation *(12–14)*. Such techniques incur a cost and can modify the protein rendering it undigestible in the intestine, and can selectively decrease availability of limiting amino acids like lysine *(12,14)*. To date, no diet (be it protected or not), provides the required EAA profile to meet the demands of high-producing cows *(3)*. Thus, in order to maximize feeding efficiency, an ideal protein (or protein source) enriched in selected EAA has to be found.

Protein and/or genetic engineering has been used to develop and produce new proteins enriched in limiting EAA that would increase availability of selected limiting amino acids *(15–17)*. The use of genetically engineered rumen bacteria for production of such proteins would minimize costs and provide protection from the rumen environment. The authors recently reported on the design of MB-1, a *de novo* designed protein containing 60% of K, M, T, and L (total amino acid content) *(15)*. A major emphasis has been placed on designing a protein that would fold into a compact structure, a departure from earlier attempts in this area *(16,18–21)*. This protein has been designed to adopt the α-helical bundle fold and is engineered in a way to maximize its lifetime in bacteria. This approach has been used because MB-1 is intended for intracellular expression. Thus, the protein would be encapsulated by rumen microbes, protected from rumen degradation, and become available for digestion as the microbes reach the small intestine. This approach offers several advantages: it does not involve industrial processing of feedstuff, it is not likely to provide overprotection in the intestine because it does not involve a chemically resistant matrix, and it simply provides selected amino acids known to be limiting animal performances.

There are several indications that suggest that MB-1 is folded and stable in *Escherichia coli* (*15*, Hefford and Beauregard, unpublished), leading to the belief that it will be stable in selected rumen microbes. Nevertheless, there are two reasons why the stability of MB-1 in the rumen environment (i.e., outside bacteria) also deserves attention:

1. Intracellular production of MB-1 has be to be controlled and limited in a way to minimize the metabolic load on the transformed microbe. In order to alleviate this limitation, one could tailor the MB-1 gene to secrete the protein into the rumen liquor. This would allow for increased production of protein and avoid detrimental effects due to intracellular accumulation of heterologous proteins.
2. MB-1 could be exposed to the rumen when cells lyse before reaching the abomasum.

These two possible scenarios motivated our investigation into the fate of unprotected MB-1 in the rumen.

Of prime importance is the question of degradability of MB-1. Only a few techniques for assessment of degradability in the rumen of soluble proteins that are highly purified (>95%) and available in minute quantity (mg) are available (no in vivo techniques known to us are compatible with such limitations). Among compatible in vitro methods, the most widely used involve the use of commercially available enzymes that predict degradability in the rumen with a reasonable level of accuracy. Published procedures have been adapted based on the bacterial protease type XIV from *Streptomyces griseus* and the neutral protease from *Bacillus subtilus* because of their well known performance for predicting rumen degradation of proteins in feedstuff *(22–25)*.

In addition to degradability, the authors wanted to assess MB-1 structural stability in the rumen environment. The impact of folding stability on protein susceptibility to proteases is well-documented *(26–30)*. Thus, the authors wanted to assess the impact of the rumen environment on folding stability and its consequent contribution to proteolytic degradability. They have, therefore, done thermal denaturation studies on MB-1 in a solution with pH, salt, and volatile fatty acid content similar to rumen levels.

METHODS

Proteins

Ribonuclease A (RNAse A) was purchased from Sigma Chemical Co., (St. Louis, MO cat. no. R-5500). Cytochrome c (cyt c) from horse heart was obtained from ICN Biomedicals (Costa Mesa, CA cat. no. 101467). MB-1 was expressed and purified as previously reported *(15)*. Bacterial protease type XIV from *Streptomyces griseus* (hereafter referred to as Pronase E) was

obtained from Sigma (cat. no. P-5147). Neutral protease from *Bacillus subtilis* (hereafter referred to as Neutrase) was obtained as a gift from Novo Nordisk Bioindustrials Inc., Danbury, CT (ID# 108376).

Proteolytic Studies

Assessment of degradability was done by using previously published methods *(22,24)*. Minor modifications were made in order to assess degradability of small amounts of purified proteins: When using Pronase E *(24)*, 1 mg each of MB-1, cyt c, and RNase A were dissolved in 2 mL of borate-phosphate buffer (pH 6.8) and dialyzed at 4°C against 200 vol of the same buffer 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 this time at 6.6 U per g of protein. Samples were taken at time zero and at subsequent intervals. Reactions were stopped by the addition of 2% sodium dodecyl sulfate (SDS) buffer, and then heated for 3 min at 100°C and stored at –20°C.

In the Neutrase studies *(22)*, 1 mg of MB-1, cyt c, and RNAse A were dissolved in 2 mL citrate buffer (pH 6.5) and dialyzed at 4°C against 200 vol of the same buffer overnight. Incubations with Neutrase were done using the protocol followed in the Pronase E studies, except that a 1% Neutrase solution was used instead of Pronase E.

Electrophoresis and Estimation of Degradation

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted using a Bio-Rad Miniprotean apparatus. For MB-1, electrophoresis was conducted in a Tris-tricine buffer for 1 h at 100 V. Tricine was used to insure optimal band resolution of smaller molecular weight proteins *(31)*. Silver staining was performed using a Bio-Rad staining kit. For RNAse A and cyt c, electrophoresis was conducted in a Tris-glycine buffer for 1 h at 100 V and stained with Coomassie Blue. 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). SDS-PAGE experiments were conducted prior to proteolytic studies and thermal stability measurements to confirm protein purity (95% or better for all proteins used, not shown).

Thermal Stability Measurements

It was attempted to use fresh rumen fluid from a cannulated bovine animal without success: it was impossible to monitor conformational changes of any of the three proteins selected. A number of different molecules other than proteins may interfere in such experiments. Autoclaving, ultrafiltration, solvent extraction, and protein precipitation were used, but found to be ineffective in removing interfering substances. Rather than fur-

ther modifying the rumen liquor properties to a point where it would become unrecognizable it was decided to formulate a solution that would have selected properties of rumen fluid. The components selected were pH, salt content, and volatile fatty acid (VFA) content, i.e., the properties that were believed to have the most important impact on protein thermal stability in the rumen. This choice is supported by earlier work on solvent composition and protein stability *(32–35)*. A buffered VFA mixture was made up 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, *(1,36)*. Phenylmethylsufonyl fluoride (PMSF) at a concentration of 0.5 m*M* was added to prevent proteolytic degradation and 1 m*M* sodium azide (NaN_3) was added to prevent the growth of air-borne micro-organisms.

The amount of 0.5 milligrams each of RNAse A, cyt c, and MB-1 were dissolved in 1 mL of the buffered VFA mixture. The samples were dialyzed overnight at 4°C against 100 vol of the respective buffer. The following day, samples were equilibrated for 1 h at room temperature. All solutions were filtered (0.2 μm) and experiments were carried out using a PTI Rf-M2204 fluorometer. For cyt c, tryptophyls were excited at 290 nm and the spectra were obtained between 300 nm and 400 nm, using a bandpass of 2.5 nm. For RNAse A and MB-1, 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 all three proteins (in triplicates) 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.

Thermal stability was calculated assuming a unimolecular process as previously described *(37)*. Assuming a two-state mechanism (folded → unfolded), T_m was calculated (T_m i.e., the temperature at which the concentrations of unfolded and folded protein are equal). For MB-1 and RNase A, I_{303} (fluorescence intensity at 303 nm) was used as the property (y) indicative of the extent of unfolding of tertiary structure. Whereas for cyt c, the fluorescence intensity was measured at 320 nm (I_{320}). 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, one 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 (T_m) are obtained at $K_u = 1$ *(37)*.

RESULTS AND DISCUSSION

Two natural proteins of a similar size to MB-1 were used in order to compare MB-1 to highly resistant, native, natural proteins. Degradation (or

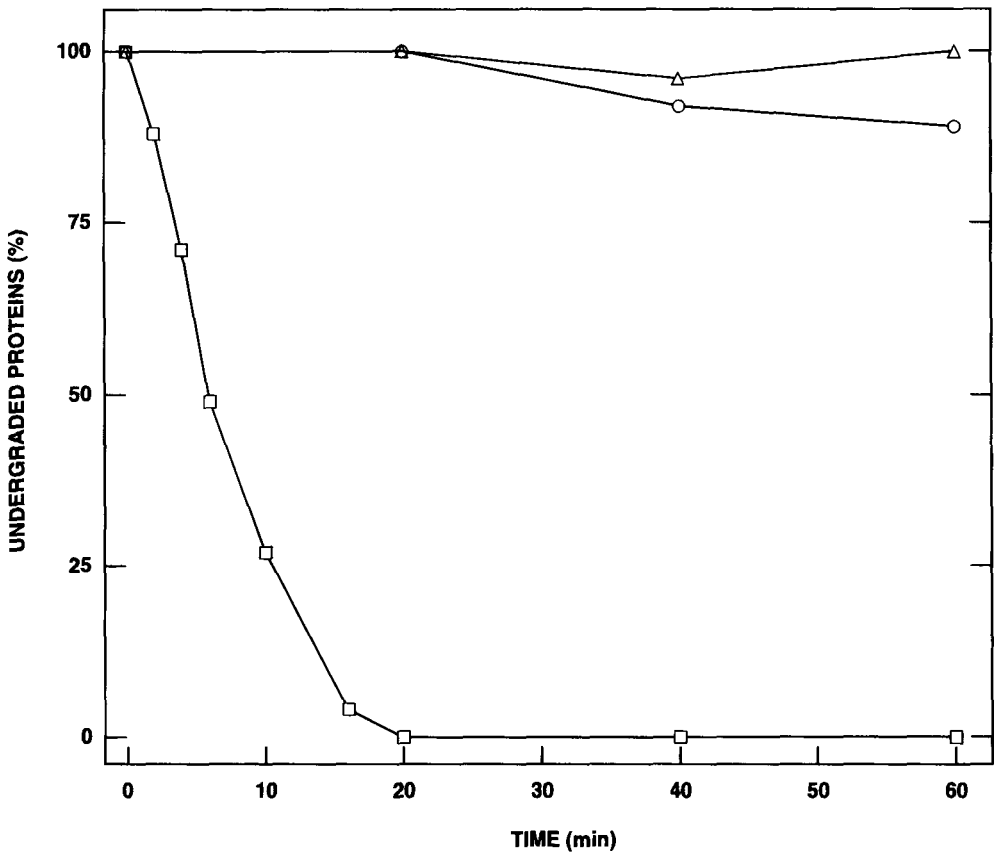

Fig. 1. Degradation of MB-1 (squares), cyt c (circles), and RNAse A (triangles) by Pronase E. The results of a typical experiment are shown. Error was calculated to be less than 5% for all results obtained from triplicate experiments.

absence of degradation) of these natural proteins (cyt c and RNAse A) by Pronase E is shown in Fig. 1. Both proteins resisted degradation better as compared with MB-1, which was completely degraded after 20 min. A similar trend was observed in Neutrase digestion experiments (Fig. 2).

Modification of the tertiary structure was reflected by changes in the fluorescence properties of the aromatic residues. The fraction of unfolded protein found at different temperature is shown in Fig. 3. Melting temperatures of 39.1 ± 1.7; 60.2 ± 1.1; and 75.3 ± 1.3°C (triplicate experiments) were obtained for MB-1, cyt c, and RNAse A respectively. Thus, the natural proteins are folded at the rumen temperature (39°C), minimizing exposure of targets for proteases. The values obtained for the natural proteins are close to previously reported denaturation temperature measured in different buffers, albeit lower by 5–10°C *(38,39)*. Because of the differences between these experiments and the authors', it is difficult to identify the VFA buffer component(s) responsible for a lower folding stability.

MB-1 denaturation was achieved at lower temperature. The pretransition region was very narrow, i.e., MB-1 "looked" native between 15 and

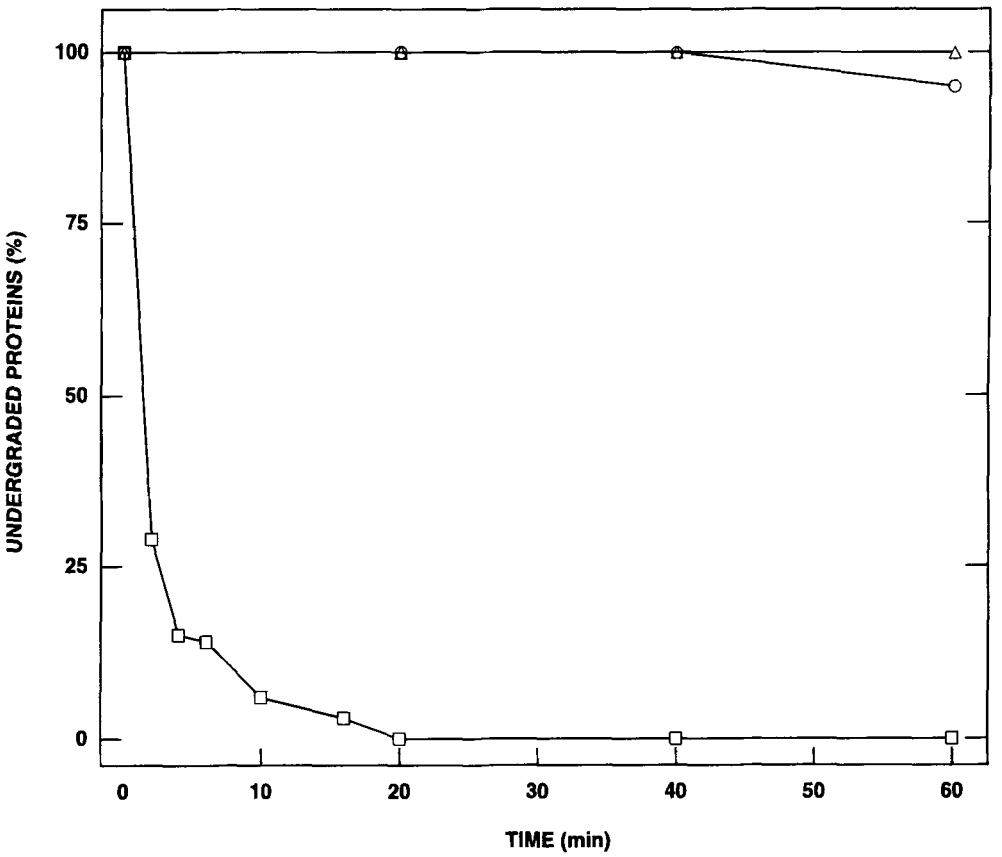

Fig. 2. Degradation by Neutrase. *See* Fig. 1 for symbols. Error was calculated to be less than 5% for all results obtained from triplicate experiments.

20°C only. It appears that 50% of MB-1 population has an unfolded tertiary structure at 39°C, which coincides with the temperature of the rumen. The denaturation (transition region) of MB-1 occurs over a broad range of temperature as compared to RNAse A and cyt c. This suggests less cooperativity in thermal unfolding for MB-1, a feature of other *de novo* designed proteins for which temperature *(40,41)* or chemical denaturant *(42)* were used for denaturation studies.

The degradability study predicts that MB-1 will be rapidly and totally degraded if exposed to the rumen environment. This property will be a disadvantage if MB-1 leaks or is deliberately secreted into the rumen. However, when delivered to the abomasum by the rumen microbes, MB-1 degradability will become an advantage. Physical and chemical treatments for protein protection can lead to overprotection, whereas some non-protected feeds are not fully digestible *(2,14)*. The very nature of MB-1 and the results shown here, suggest that MB-1 will also be fully degraded in the intestine.

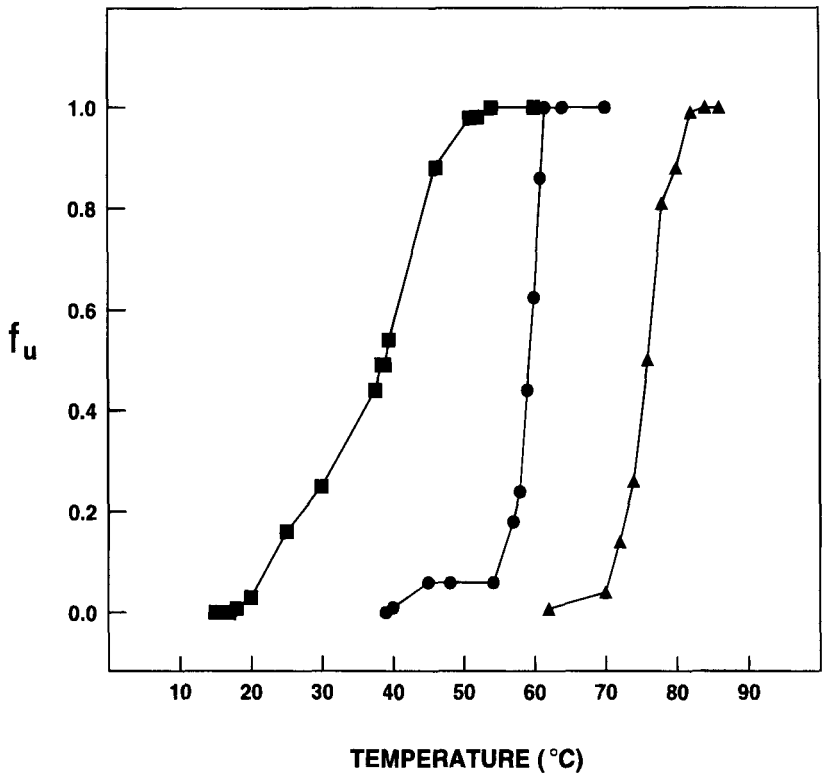

Fig. 3. Thermal unfolding of proteins in buffer VFA. Results of a typical experiment are shown. The fraction of unfolded protein is scaled from 0 to 1 on the y-axis (corresponding to 0 to 100% unfolded). Symbols are same as in Fig. 1.

The natural proteins used for comparison are well-known for their extreme cooperative unfolding and their resistance to proteolytic degradation *(38,43)*. In accordance, they were not as degraded as MB-1. Cyt c and RNAse A both contain disulfide bridges whereas MB-1 has none. It has been recognized that these structural features provide protection against degradation in the rumen *(44,45)*. The authors have identified another factor that will contribute to MB-1 degradability: the low thermal stability of its tertiary structure. Several studies have demonstrated that unfolding promotes proteolytic degradation as it leads to exposure of targets *(27,30,46–48)*. The results show that, in rumen-like fluid, the tertiary structure of MB-1 will be significantly unfolded at 39°C (50% of the total protein population). Such instability will contribute to higher degradability as predicted by the experiments done with Pronase E and Neutrase.

A number of studies on protein degradability in rumen fluids have focused on analysis of isolated proteins. Among the proteins studied, caseins and other milk proteins have been shown to be degraded in few minutes *(45,49)*, i.e., similar to MB-1 predicted lifetime in the rumen. Similar results were obtained for pea proteins like convicilin, vicilin, as

well as for other proteins of rapeseed and lupin *(45)*. Thus, MB-1 degradability in rumen would compare to that of number of proteins found in ruminant diets. Other proteins were shown to be more resistant to rumen degradation than MB-1: bovine serum albumin, RNAse (in agreement with this study), and plant albumins have been shown to resist degradation for about 8 h *(44,45,49)*. It was inferred that the difference in susceptibility among different proteins could be explained, at least in part, by the presence of disulfide bridges in the resistant proteins *(44,45)*.

It would have been interesting to compare this data to that of others related to different synthetic proteins designed for nutritional purposes. As far as it is known the stability (structural or proteolytic) of such polypeptides (in solution, in microbes, or in the rumen) has not yet been investigated or reported. It appears that MB-1 is the best characterized synthetic protein developed for nutrition purposes (this study and ref. *15*).

MB-1 can, however, be compared to *de novo* designed proteins that were created for other purposes. The few *de novo* proteins that were assayed for thermal denaturation have similar features to those of MB-1: a low T_m and a low cooperativity in unfolding *(40–42,50–52)*. For other *de novo* proteins that were not investigated for thermal denaturation, modification of tertiary structure at relatively low temperature (i.e., 30–40°C) has been observed *(53,54)*. Because of these observations, it has been suggested that most *de novo* proteins designed were in a "molten globule state" (MGS), i.e., a state that corresponds to a folding intermediate of high fluidity, with stable secondary structures, but with a tertiary structure that can adopt a number of similarly stable conformations *(55,56)*. Our results (this study, and ref. 15) suggest that MB-1 could be in a MGS state, and that MB-1 is quite comparable to most other *de novo* designed proteins reported. The achievement of a *de novo* designed protein that compares to those that were designed previously was not taken for granted when the project was initiated. Whereas most new proteins designed from scratch were created using the best amino acids that would promote a given target fold, MB-1 was submitted to more stringent constraints. These constraints were as follows:

1. Its content in selected EAA had to be maximized;
2. Its sequence should be as natural as possible;
3. It had to be encoded by a stable, nonrepetitive gene; and
4. It had to be produced and stable in *Escherichia coli*.

Thus, to meet requirements no. 2 and 4, a fold had to be achieved.

For years it has been claimed that rational *de novo* design would allow for the creation of new proteins for applications in various fields of economical importance. This study proves that the EAA content of protein can be maximized using adequate design criteria. The design approach used for MB-1 (the consensus residue approach), was similar to the approach used for the design of octarellin *(57)*, but improved in that emphasis was placed on property profile, rather than identity consensus *(15)*. In addition,

a number of global factors relevant to the α-helical bundle were considered. Comparison of MB-1 with octarellin and other *de novo* designed proteins seems to indicate that MB-1 project success is comparable to previous ones. This is an achievement, though, in view of the constraints imposed on MB-1 design.

NOTE

The name of the protein MB-1 was mistakenly changed to MB1 in ref. *15*. Both studies (this study and ref. *15*) refer to the same *de novo* protein.

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