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IN VITRO STABILITY OF NATURAL AND SYNTHETIC PROTEINS FOR POTENTIAL APPLICATION IN ANIMAL FEEDING

A Thesis

Submitted to the Graduate faculty

in Partial Fulfilment of the Requirements

for the Degree of

Master of Science

in the Department of Health Management

Faculty of Veterinary Medicine

University of Prince Edward Island

Semir Omar

Charlottetown, P.E.I.

August, 1995

ISBN 0-612-07901-5

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ABSTRACT

Milk Bundle-1 is a *de novo* designed protein with 100 amino acids, having a molecular weight of 11.4 kilodaltons. MB-1 is enriched with 57% of selected essential amino acids (methionine, threonine, lysine and leucine) and is intended as a potential feed additive for dairy cows. Before MB-1 can be used as a feed additive, it has to be assessed for its stability and degradation. In order to predict the behaviour of MB-1 in rumen environment, *in vitro* experiments were conducted to investigate its structural as well as its proteolytic stability.

Optimization of fluorescence technique was conducted by studying the structural stability of jack bean urease. For thermal denaturation, the fluorescence peak position shifted from 330 nm to 335 nm upon heating urease from room temperature to 75°C, with a T_m corresponding to 72.4°C. For chemical denaturation, the shift in maximum wavelength was from 330 nm to 346 nm with a $[GuHCl]_{1/2}$ of 2 M. The results indicated the hexameric urease structure dissociates into folded dimers which then aggregate or dissociate into unfolded monomers.

Fluorescence spectroscopy was used to study the thermal stability of MB-1. Denaturation studies of MB-1 were conducted in the presence of physiological levels of volatile fatty acids buffer with ionic strength and pH similar to rumen fluid. Under these conditions melting temperature was obtained to be around 50°C. This suggested that will have a folded structure at the rumen temperature of 39°C. This feature is important in that it indicates that MB-1's structure and folding will contribute to a resistance to *in vivo* degradation.

Proteolytic stability investigated using two proteases of microbial origin. Pronase E (from *Streptomyces griseus*) and Neutrase (from *Bacillus subtilis*) were used according to a procedure currently used as a standard methodology in feed evaluation laboratories. The results of these studies revealed that MB-1 is more degradable than cytochrome c, ribonuclease A and urease.

Comparison to other similar projects showed that MB-1 is the first synthetic protein intended for nutrition that has shown to behave like natural proteins in terms of its thermal and proteolytic stability. However, if MB-1 were to be used as a feed additive (as is), then some structural modification will be required in order to make it less degradable.

ACKNOWLEDGEMENTS

A sincere thanks and heartfelt gratitude goes to my supervisor, Dr. M. Beauregard for his guidance, support, patience and assistance throughout my Master of Science program. I also extend my appreciation to members of my supervisory committee, Drs. F. Kibenge, M. McNiven, T. Ogilvie and J. Pringle for their advice and critical reading of this manuscript.

I am grateful to the faculty and staff of Departments of Chemistry and Health Management, UPEI. Special thanks to Dr. Mary Hefford and Mrs. Sharon Allard for their assistance during my research work at Agriculture and Agri-Food Canada, Ottawa. I would also like to thank Dr. F. Markham for allowing me to use his chromatography instrument. The technical help of Mrs. Dawna Lund, Ms. Charlene Peters (Chemistry) and Ms. Judy Furlong (Anatomy and Physiology) is greatly appreciated. Last but not least I thank my extended family for their love, patience and moral support.

This research project was supported by the National Research Council (Industrial Research Assistance Program), Diagnostic Chemicals Ltd., the Canadian Space agency and SCRUG grants from UPEI.

DEDICATED TO

MY MOTHER, BIRHAN AND MY LATE FATHER, OMAR HASSANO

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COMMONLY USED ABBREVIATIONS

TERM	ABBREVIATION
Standard units of measurements	
degree Celsius	°C
centrifugal force	g
millilitre	mL
millimolar	mM
molecular weight	MW
microlitre	μL
microgram	μg
nanometre	nm
maximum wavelength	λ_{max}
hydrogen ion activity	pH
percent	%
hours	hr
photons/sec	p/s
Chemicals	
ethylenediaminetetracacetate	EDTA
sodium hydroxide	NaOH
guanadinium hydrochloride	GuHCl
phenylmethylsulfonlyfluoride	PMSF
ribonuclease	RNAse
cytochrome c	Cyt. c
milk bundle-1	MB-1
maltose binding protein	MBP
sodium dodecyl sulphate	SDS
polyacrylamide gel electrophoresis	PAGE
diethylaminoethyl	DEAE
volatile fatty acids	VFA

Chapter 1: Introduction

1.1 Proteins

Proteins occupy a very prominent role in life. Their functions are unequalled among the compounds that are synthesized by living things. Proteins have diverse structures and functions; as enzymes, they mediate the biosynthesis and degradation of biochemical compounds; as food they supply energy and provide amino acids used for building up tissues and muscles in humans and animals (Cheftel *et al.*, 1985).

Proteins are made of amino acids which are the building-blocks for all living tissues. There are 20 naturally occurring amino acids and they all contain one or more amino groups, one or more carboxylic acid groups and a carbon chain. Only methionine and cystine contain sulphur (Cheftel *et al.*, 1985). Amino acids are classified as either essential or nonessential. Histidine (H), Isoleucine (I), Lysine (K), Leucine (L), Methionine (M), Threonine (T), Phenylalanine (F), Tryptophan (W) and Valine (V) are considered essential amino acids (Purser, 1970) while the rest are classified as non essential. Non essential amino acids can be synthesized by the body of all animals, whereas essential amino acids are not synthesized (Miller, 1979).

1.2 Proteins in dairy cows

1.2.1 Protein source

The major sources of protein in cows are from plants and rumen microorganisms. Plant source is usually from forages, concentrates and protein supplements such as the oilseed meals (Miller, 1979). Among the high-protein supplementary feeds, the one used in greatest amount is soybean meal, and generally, contains 45-50% of crude protein (Cullision, 1975). Cows also get their protein from rumen microbes. Protein is synthesized by rumen microbes from the amino acids and ammonia released by degradation of the crude protein, non protein nitrogen and sulphur present in the rumen (Chalupa, 1975).

1.2.2 Digestion and absorption

Protein hydrolysis in the rumen is a multi-step process. In the first step, the protein is solubilized and secondly, the peptide bond is cleaved enzymatically by a variety of proteases to form peptides, amino acids and ammonia. A portion of the protein remains undegraded, and this fraction as well as the rumen microbes bypass the ruminal digestion and go to the abomasum. There the main enzyme pepsin, which is active at the abomasal pH 2, cleaves the peptide bonds (Asplund, 1994). Further digestion in the small intestine is then achieved by secretions of the liver, pancreas

and small intestinal mucosa (Church, 1988). The end products of this hydrolysis process are small peptides and amino acids. These products are then absorbed by the small intestine and circulated into the blood where they are used for many functions including milk protein synthesis (Church, 1988).

1.2.3 Milk synthesis

Dairy cows consume protein in order to supply nitrogen (N) for microbial growth in the rumen and to provide amino acids required for body maintenance and milk production (Miller, 1979). Dietary protein was originally thought to have little influence on milk protein; however, more recent research suggested that both the amount and type of dietary protein can influence the protein content in milk (DePeters and Cant, 1992). Thus, milk synthesis can be increased when dairy cows (deficient in essential amino acids) receive proteins which bypass rumen fermentation (Owens and Zinn, 1988).

1.2.4 Amino acid availability

The essential amino acids that are absorbed from the small intestine come from four sources: (1) digestion of rumen microorganisms; (2) digestion of feed protein escaping degradation in the rumen; (3) amino acids bypassing the rumen; and (4) reabsorption of proteins endogenously secreted into the intestinal tract (Chalupa, 1975). Many

studies have confirmed that the amino acid balance obtained from digestion of the rumen microbes and dietary proteins is not sufficient to meet the essential amino acid needs of lactating cows (Burroughs *et al.*, 1975; Chalupa, 1975). Indirect methods utilizing amino acid concentrations in plasma have been used to identify the limiting amino acids for milk synthesis in lactating dairy cows (Schwab *et al.*, 1975). Chandler and Polan (1972) calculated the transfer efficiency of amino acids from blood to milk protein and suggested that methionine, threonine, tyrosine, phenylalanine and lysine were limiting amino acids for milk synthesis. In a more recent study, King *et al.*, (1990) confirmed that methionine, lysine and threonine are the most limiting amino acids. The non essential amino acids were not found to be limiting for milk synthesis (Schwab *et al.*, 1975).

1.3 Protection of proteins

Several studies have proven that proteins of low degradability increase the milk production in dairy cows (Johnson *et al.*, 1987; Kung and Huber, 1983). In view of this, several groups have been trying to create "by-pass" protein sources that would resist degradation in the rumen (Beauregard *et al.*, 1995; McNiven *et al.*, 1994; Robinson *et al.*, unpublished).

There are two commonly used methods available for protecting proteins from degradation by rumen microorganisms; chemical and physical approaches. In the

chemical approach, certain chemical agents such as formaldehyde or tannin form cross linkages with amino and amide groups which then decrease solubility of proteins at the pH of the rumen. Chemically-treated proteins subsequently are made available to the animal by destruction of these linkages in the acidic abomasum (Chalupa, 1975). In the physical approach, heat treatment is frequently used to treat grains and forages so that the resulting product has decreased solubility and degradation in the rumen (Stern *et al.*, 1985).

Protection of protein by some chemical and physical treatments of proteins has shown to increase the milk production in dairy cows (Robinson, unpublished; McNiven *et al.*, 1994). However, in some cases, overprotection of proteins occurs and this causes lower digestibility of the protein in the small intestine (Chalupa, 1975). Therefore, other methods are under considerations in order to solve this problem.

The alternative approach would be to create a new protein that would be resistant to ruminal degradation. Previous attempts were based on the design of simple genes made of repeating segments coding for essential amino acid (Doel *et al.*, 1980; Jaynes *et al.*, 1985; Beauregard *et al.*, 1994). This approach presented several problems. Firstly, highly repetitive DNA segments may be unstable; and secondly, polypeptides with repeating sequences deviate from the usual, natural protein format and may not be useful for nutrition (Boeble and Baker, 1982).

The approach used in our group was to design a *de novo* protein that has a high content of selected amino acids but also resembles natural proteins in terms of its structural stability. A synthetic protein, Milk Bundle-1 (MB-1) enriched in four essential amino acids (methionine, threonine, lysine and leucine) was designed, expressed and produced (Beauregard *et al.*, 1995).

1.4 Factors affecting protein degradation

The rate at which feed protein is digested in the rumen is complicated by numerous factors which involve the nature of the protein (Roe *et al.*, 1991). Structural stability, solubility and solvent effects are some of the important physical factors that affect protein degradation.

1.4.1 Structural stability

Structural stability of a protein is known to have a major impact on proteolytic stability (Golberg *et al.*, 1978). Folding stability in natural proteins is important for resistance to proteases as unfolding exposes regions targeted by proteases (Schein, 1989; Parsell and Sauer, 1989; Goldberg *et al.*, 1978). The impact of structure on the degradation of proteins in rumen fluids has been investigated by many workers. Mahadevan *et al.*, (1980) observed the stabilization of structure by disulphide linkages and confirmed that these linkages were involved in providing resistance to degradation

by rumen enzymes.

1.4.2 Solubility

Solubility of proteins is also an important factor in ruminal degradation of protein (Shirley, 1986). *In vitro* studies with rumen fluid of comparable ionic strength and pH has confirmed that the degradation of proteins by rumen microorganisms is directly related to solubility of the protein (Crooker *et al.*, 1978; Wohlt *et al.*, 1973). Thus, soluble proteins are more susceptible to proteolytic attacks.

1.4.3 Effects of environment

Among the physical factors that are known to affect the structural stability of proteins are temperature, pH, ionic strength and amphiphilic molecules in their environment (Privalov, 1979). Amphiphilics are compounds having both polar and nonpolar segments that act as detergents and destabilize proteins (Creighton, 1989).

1.5 Protein stability studies by fluorescence spectroscopy

Protein folding is mainly directed by the relatively weak intermolecular forces between noncovalently bonded atoms such as electrostatic, van der Waals and hydrophobic forces (Creighton, 1989). Some amino acids have apolar side chains (hydrophobic

residues) and these residues tend not to interact with water. The effect of these hydrophobic forces is believed to be the main force leading to the conformational stability of a protein (Privalov, 1979).

In protein chemistry, fluorescence spectroscopy is a sensitive technique used to study the structures, dynamics, conformational changes and interactions of proteins in solution (Permyakov, 1992). Fluorescence is a phenomenon that usually occurs with organic compounds containing aromatic rings or highly conjugated alkene systems (Permyakov, 1992). Fluorescence occurs when an excited electron relaxes from the first excited state (S_1) to the ground state (S_0). First the electron is excited by absorption of light to reach S_1 state, then the electron loses energy before stabilizing at S_1. Finally the electron relaxes to S_0 and emits light at a lower energy than was absorbed. Thus the emission spectrum is shifted to a longer wavelength than the absorption spectrum (Lakowicz, 1983).

The fluorescence of proteins originates from three amino acids; phenylalanine, tyrosine and tryptophan. The position of the maximum of the fluorescence spectrum of tryptophan residues in proteins varies within the limits of 307 to 353 nm; for tyrosine from 303 to 306 nm and for phenylalanine 280 to 282 nm. The fluorescence of these amino acids are widely used as an indicator of the folding stability of the proteins (Lakowicz, 1983).

Protein stability and unfolding can be studied by denaturing the proteins using either heat or chemical treatment with guanidinium hydrochloride (GuHCl) or urea (Privalov, 1979). Thermal or chemical denaturation of proteins containing tryptophan results in a shift of tryptophan fluorescence spectrum to a longer wavelength. Tryptophan, a nonpolar amino acid, is often found inside the core of a protein where there is little exposure to solvent (Creighton, 1989). As the protein denatures, the hydrophobic core becomes exposed to solvent and the tryptophan will interact with the aqueous phase (Lakowicz, 1983). In the case of proteins that do not contain tryptophan, changes in tyrosine emission are predominantly changes in fluorescence intensity. The intensity of tyrosine fluorescence is highly dependent on its environment and the fluorescence maximum remains around 303 nm, irrespective of the molecular environment of the fluorophores (Permyakov, 1992). Thus, fluorescence can be used to monitor unfolding of proteins containing either tryptophan or tyrosine. The purposes of thermodynamic investigation of protein denaturation is to evaluate the folding free energy of protein, i.e. the energy required to convert the protein from its native three-dimensional structure to the completely denatured state (Ahmad and Bigelow, 1982). It is an accurate measure of structural stability, and as mentioned above, it can reveal information on protein susceptibility to proteolytic enzymes.

1.6 Research objectives

The long term objectives of our research are to explore the potential of MB-1 as a

potential feed additive for dairy cows. This study focused on the behaviour of MB-1 *in vitro*, in conditions that resemble the rumen environment. These conditions were selected because they allow one to monitor MB-1's structural stability and to predict its proteolytic degradability.

The immediate goal of this work was to investigate the *in vitro* structural stability of MB-1 since the structural stability of protein has an impact on proteolytic stability and half-life. Thus, thermal denaturation studies were conducted to find out if MB-1 will stay folded in a rumen-like environment. *In vitro* proteolytic studies were performed to predict MB-1's degradation in the rumen. The information obtained from these studies will then be used in the future to identify ways of improving the stability of MB-1.

Chapter 2: OPTIMIZATION OF FLUORESCENCE TECHNIQUE: Structural stability studies of jack bean urease by fluorescence emission spectroscopy.

2.1 INTRODUCTION

The current small scale method used to produce and purify MB-1 yields low amounts of product. To establish optimized techniques requires abundant sample of proteins. Thus, urease was selected primarily because it is an industrially useful, inexpensive protein, and more importantly, some aspects of its structural stability have not been previously studied.

Urease is an enzyme with the systematic name of urea amidohydrolase that acts on non-peptide C-N bonds in linear amides. It was first described in 1876 by Musculus after filtering bacteria on filter paper (Varner, 1960). Urease has since been reported to occur in 200 species of bacteria, yeast, fungi as well as higher plants. Ruminal bacteria secrete urease to hydrolyse urea to form ammonia which is used for microbial synthesis of protein (Miller, 1979). The richest plant source of urease is jack beans, *Canavalia ensiformis* and *Canavalia obstusifolia* which contain up to 0.15% urease on dry weight basis (Sumner, 1953).

More than a dozen types of urease are found to exist, though the traditional molecular form, α-urease is the dominant form encountered in jack bean meal

(Fishbein *et al.*, 1971). There are important applications of jack bean urease in clinical chemistry (Zerner, 1991). Accordingly, several investigators have studied its thermal inactivation and have proposed ways to stabilize its activity (Margolin *et al.*, 1985; Wang and Johnston, 1993; Kallury *et al.*, 1993). Further advances in this field will depend increasingly on our knowledge of urease structure and stability.

Jack bean urease is a hexameric protein (MW 545 kDa), made of 6 identical subunits, each with a MW of 91 kDa (Hirai *et al.*, 1993; Takashima *et al.*, 1988). This enzyme can undergo dissociation from hexamer to an active dissociation product (previously believed to be a trimer) when subjected to either low pH, or high pH and high concentration of polyalcohols (Fishbein *et al.*, 1969; Gorin *et al.*, 1968; Blattler *et al.*, 1967; Reithel and Robbins, 1967; Sehgal and Naylor, 1966). Further dissociation to inactive monomers can be achieved by treatment with high concentration of sodium dodecyl sulphate (SDS) or guanidinium hydrochloride (GuHCl) (Blatter and Gorin, 1969). Active monomers have been obtained using low concentration of SDS (Hirai *et al.*, 1993). At present, the structure of urease and its dissociation products remains unknown (Hirai *et al.*, 1993; Fishbein *et al.*, 1973). Previous methods used to monitor dissociation of urease involved time-consuming techniques and, to our knowledge, no thermodynamic studies of urease structural stability have been published. Further information on the structure and stability of urease would provide insight into the mechanism of α-urease inactivation and how to prevent it. The presence of four tryptophan residues per subunit makes urease a

suitable candidate for analysis using fluorescence spectroscopy (Lakowicz, 1983). In this study, a spectrofluorometric study of urease unfolding is presented. This investigation is the first thermodynamic study of urease structural stability and provides new insight regarding urease structure.

2.2 Material and methods

Urease was purchased from different sources: Sigma U-0251 (Sigma Chemical Co., St.Louis, MO), ICN 103211 (ICN Biochemicals, St.Laurent, PQ) and MV30J (Diagnostic Chemical Ltd., Charlottetown, PEI). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to check for homogeneity and bands were scanned for relative amounts of protein using gel scanning densitometer (Ultroscan XL Laser Densitometer, Pharmacia LKB Biotechnology, Montreal, PQ). The sample that was found to be the purest was to be used for further work. Buffer A containing 2 mM phosphate buffer, 1 mM sodium EDTA, 5 mM dithiotreitol, 0.5 mM phenylmethylsufonylfluoride (PMSF), 0.5 mM sodium azide and 20% ethylene glycol was prepared (Diagnostic Chemicals Ltd., 1983).

2.2.1 Electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% polyacrylamide gels was performed according to the method of Hames and Rickwood

(1981). Staining was done with Coomassie Blue (0.25% Coomassie Blue, 50% methanol and 7% acetic acid) solution.

2.2.2 Chemical denaturation

For chemical denaturation studies, different molarities (0 M, 0.3 M, 0.5 M, 0.8 M, 1.0 M , 1.3 M, 1.8 M, 1.9 M, 2 M, 2.1 M, 2.2 M, 2.3 M, 2.4 M, 2.5 M 2.6 M, 2.7 M, 2.8 M, 2.9 M, and 7 M) of GuHCl were added to Buffer A and the pH was adjusted to 6.0 using 0.1 M potassium hydroxide. In each case, 1 mg of urease was dissolved in 1 mL of the GuHCl solution and dialysed against respective concentration of GuHCl solution at 4°C overnight. Following this, the protein solutions were removed from the dialysis bags, kept for 4 hr at room temperature and fluorescence measurements were conducted at 25°C. These experiments were conducted in triplicate.

2.2.3 Thermal denaturation

For thermal denaturation studies, urease (1 mg) was dissolved in 1 mL Buffer A and dialysed overnight at 4°C. This solution was then put in a quartz cuvette. The experiments were then conducted between 25°C and 80°C (25°C, 30°C, 35°C, 40°C, 45°C, 52°C, 55°C, 58°C, 60°C, 62°C, 65°C, 68°C, 70°C, 71°C, 72°C, 73°C, 74°C, 75°C, 76°C, 77°C, 78°C, 79°C, and 80°C), allowing 30 minutes for

equilibration at each temperature. The sample temperature was controlled with a Lauda R6S circulating bath (Lauda Instruments, Koln, Germany). The cell temperature was calibrated against the bath temperature with a Parr 1671 precision thermometer (Fisher Scientific, Montreal, PQ). All solutions were filtered using a 0.2 μm filter (Nalgene Co., Rochester, NY) prior to measurements. These experiments were done in triplicates.

2.2.4 Fluorescence studies

Fluorescence measurements were carried out using Rf-M2204 fluorometer (Photon Technology Instruments, South Brunswick, NJ). Urease fluorescence was excited with light of 290 nm and the spectra were scanned between 300 nm and 400 nm, using a bandpass of 2.5 nm. All spectra were corrected for buffer and water emission and for loss of efficiency in the detector and gratings. No changes in pH were detected in the solution after denaturation.

2.2.5 Thermodynamic calculations

Unfolding free energy was calculated as described in Pace *et al.*, (1989) assuming a two state mechanism (folded --> unfolded). Due to its sensitivity to tertiary fold modification, I_{320} (fluorescence intensity at 320 nm) was chosen as the property (y) indicative of the extent of unfolding (Eftink, 1994). For experiments with 0.33 μM

urease, the unfolded state in 2.5 M GuHCl was characterized by I_{320} of 1.3 x 10^5 photons/s, (y_u), while the folded state has I_{320} of 5 x 10^5 photons/s (y_f). Thus at 346 nm the fraction of unfolded protein (f_u) is equal to 1, while the fraction of folded protein (f_f or 1-f_u) is 0. Anywhere between these two states, I_{320} (y) is equal to $y_f f_f$ + $y_u f_u$. Thus, by measuring y, we can calculate the fraction of protein unfolded: f_u = (y_f - y) / (y_f - y_u). For a unimolecular process, the equilibrium constant for the unfolding process is $K_u = f_u / (1-f_u)$, where f_u is concentration independent. For a process where a folded dimer dissociates into two monomers, f_u becomes sensitive to concentration and $K_u = 2 f_u^2 [M_t] / (1 - f_u)$, where $[M_t]$ is the total monomer concentration in molar units. A process involving a trimer-to-monomer transition would also be concentration sensitive with $K_u = 3 f_u^3 [M_t]^2 / (1 - f_u)$. The free energy of unfolding, ΔG_u, is obtained with $\Delta G_u = - RT \ln K_u$. The linear model was used for extrapolation of ΔG_u at 0 M GuHCl: $\Delta G_u = \Delta G_u(H_2O)$ - m [GuHCl], where m is a measure of the dependence of ΔG_u on denaturant concentration (Schellman, 1978; Pace *et al.*, 1989).

2.2.6 Reversibility studies

For reversibility studies, urease which was denatured using 2.6 M GuHCl was dialysed gradually down to 0 M GuHCl using several baths of Buffer A. The samples were then analyzed for enzymatic activity as well as fluorescence. Thermally denatured urease was left at room temperature for several days and then measured for

its fluorescence. Urease activity was measured using a colorimetric discontinuous assay method based on a refined version of the Berthelot colour reaction (Diagnostic Chemicals Limited, 1983; Kaplan, 1965).

2.3 RESULTS

Table 1 shows the comparison of urease purity from different samples done using SDS-PAGE. The samples obtained from ICN contained three equal bands and the one from DCL contained two major bands with each sband having equal relative composition as assessed by a gel scanning densitometer. Urease from Sigma was found to be 95% homogenous and was used for further experiments.

Fluorescence emission spectra of urease measured in different conditions are shown in Figure 1. Native urease (no GuHCl, 25 °C) had its fluorescence maximum at 330 nm, indicating that tryptophan residues are rather protected from the aqueous solvent (Lakowicz, 1983). After heating to 75 °C, the λ_{max} shifted to 335 nm, due to an increase in tryptophan exposure. This effect was even greater in 5 M GuHCl, where the tryptophan were mostly exposed to water (maximum at 346 nm). As commonly found, GuHCl is a stronger denaturant than heat (Jaenicke and Rudolphi, 1989). Both denatured samples had a lower fluorescence intensity compared to native urease. For thermally denatured urease, this was due in part to some aggregation followed by precipitation.

Table 1. Purity check of urease from different sources by SDS-PAGE

Sample	Molecular weight (Da)	Relative composition
Sigma urease	90,000	95%
	45,000	5%
ICN urease	90,000	33%
	60,000	33%
	45,000	33%
DCL urease	90,000	50%
	58,000	50%

2.3.1 Thermal denaturation

The λ_{max} was monitored at several temperatures and a denaturation curve was constructed (Figure 2). The conformation of urease was stable below 40 °C, corresponding to the native state. Then a modest change in tryptophan environment with increasing temperature is reflected by a slow increase in λ_{max} from 330 nm to 332 nm, where it stabilizes. In order to identify the new conformation obtained, a a dissociating buffer (acetate pH 3.5 or 90% ethylene glycol pH 9.2) was prepared. Under such conditions, urease had been earlier shown to dissociate into halves (Contaxis and Reithel, 1971; Gorin *et al.*, 1968). It was also observed this small change of λ_{max} and this may be due to the dissociation of the hexamer into folded dissociation products. A more important transition can be seen at about 72 °C, indicative of a change in protein structure leading to further exposure of tryptophan to water. The main transition in the denaturation curve happens around 72 °C, close to the reported inactivation temperature for urease (Margolin *et al.*, 1985; Wang and Johnston, 1993). The new state was unaffected by further heating and it was impossible to promote refolding of urease by cooling the solution. Formation of aggregates was detected visually upon heating to 60 °C and above.

2.3.2 Chemical denaturation

As indicated in Figure 3, a similar denaturation curve was obtained by treatment of

urease with increasing concentration of GuHCl. The main difference between denaturation curves in Figure 2 and 3 was the magnitude of change of maximum wavelength suggesting a greater extent of unfolding in the latter case. The native state and the intermediate state with maximum wavelength, λ_{max} of 332 nm were still visible, and a large change in conformation was obtained after addition of 2 M GuHCl. In this case, the tryptophan residues are at their greatest exposure to the environment, and no changes were found upon increasing denaturant concentration up to 7 M. It has been previously reported that in 6 M GuHCl, urease was dissociated into inactive monomer (Blatter and Gorin, 1969). It was shown here that under such conditions, these monomers were unfolded.

Using GuHCl as the denaturant we found that the denaturation was mostly reversible: the λ_{max} of 330 nm could be recovered, as well as some catalytic activity with the removal of GuHCl. Thus, a thermodynamic analysis was done on these data. Different unfolding reactions may be considered. However, on the basis of previous investigations, the transition should involve the dissociation of a folded intermediate (obtained after the dissociation of the hexamer) into unfolded monomers (Blatter and Gorin, 1969; Hirai *et al.*, 1993). Unimolecular reactions would have equilibrium populations of folded and unfolded protein that are independent of protein concentration.

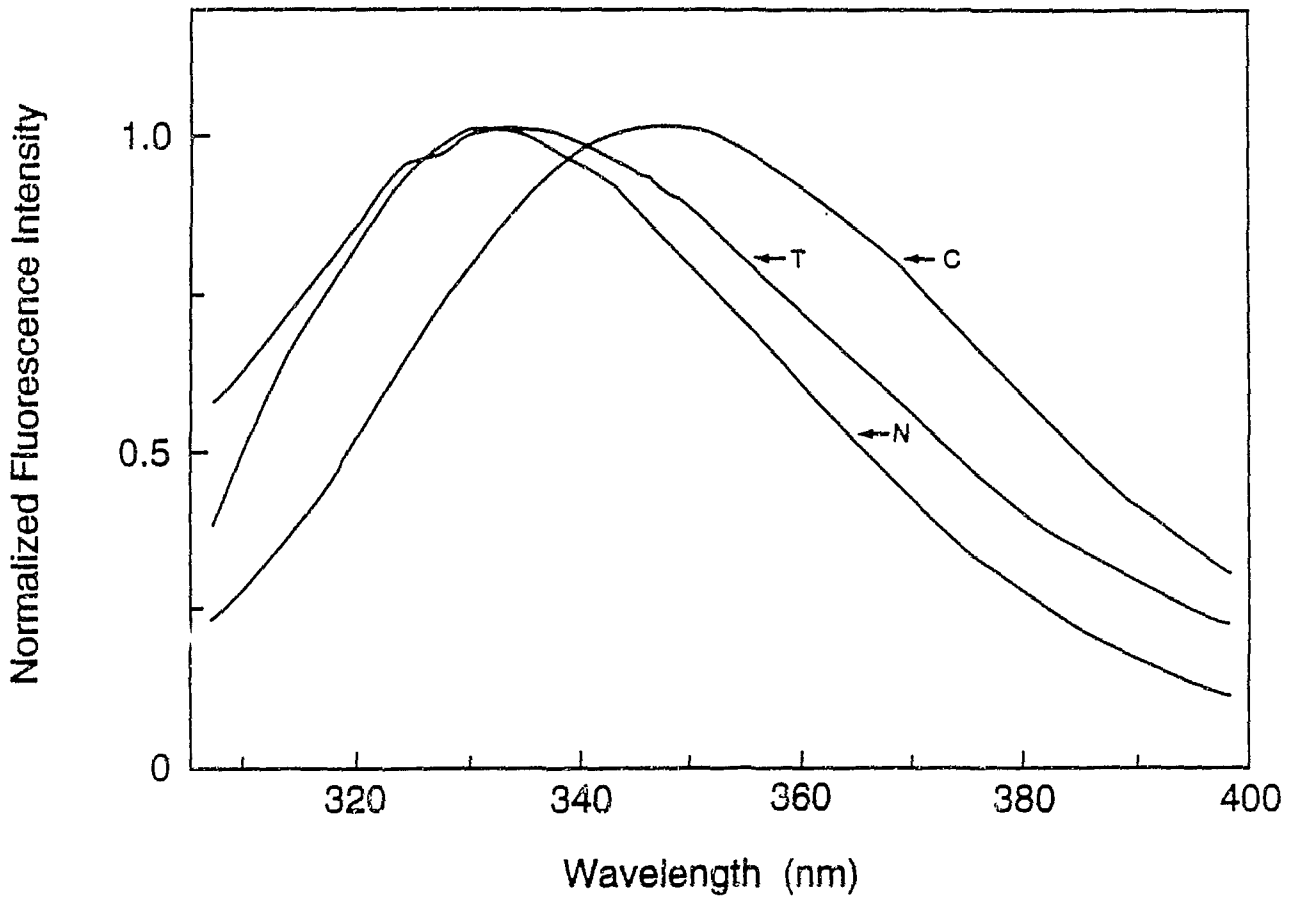

Figure 1. Fluorescence spectra of native (N), thermally (T) and chemically (C) denatured urease. Fluorescence intensity at peak have been normalized to 1 abitrary unit for comparison.

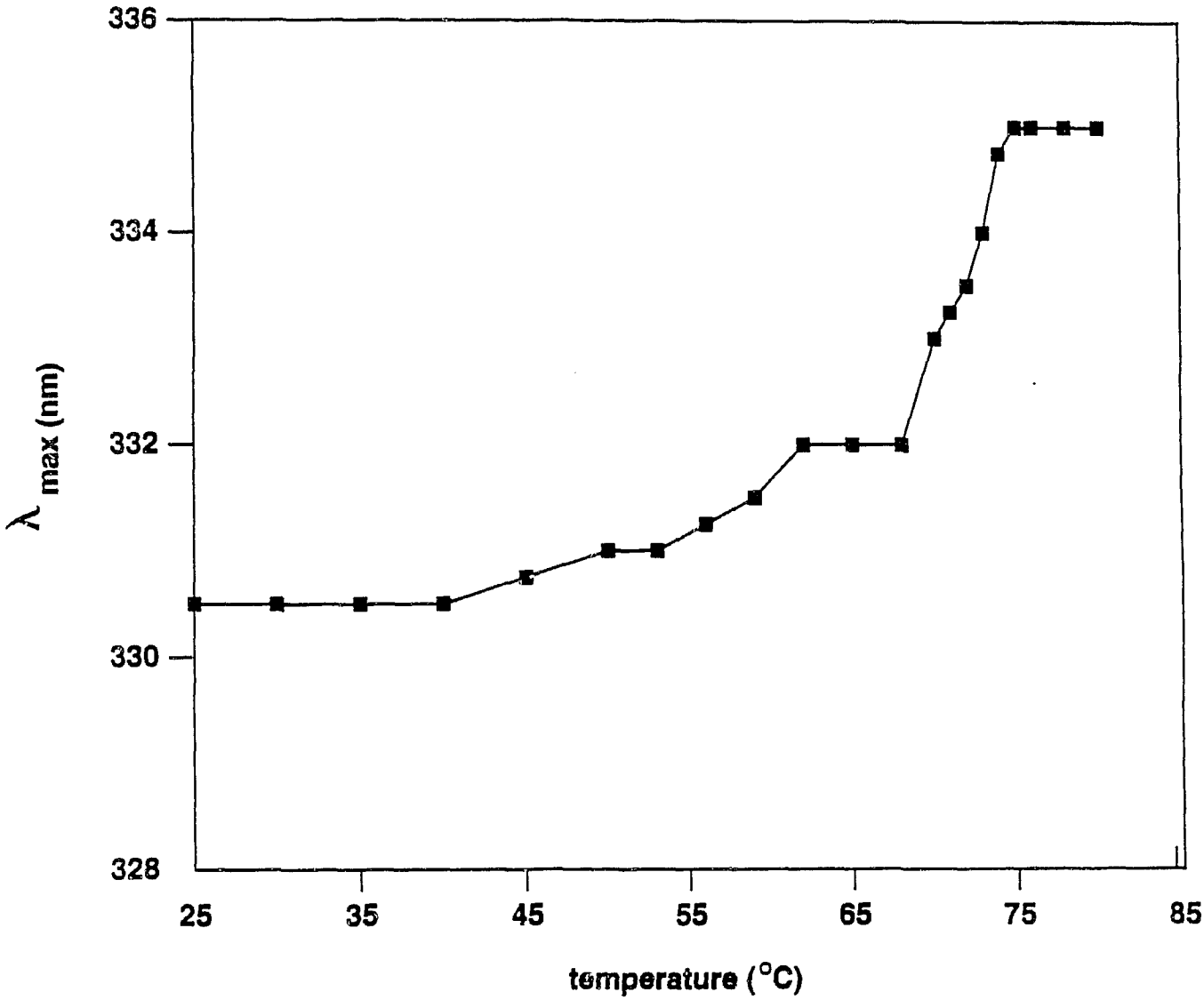

Figure 2. Thermal denaturation curve. Fluorescence emission maximum dependence on heat.

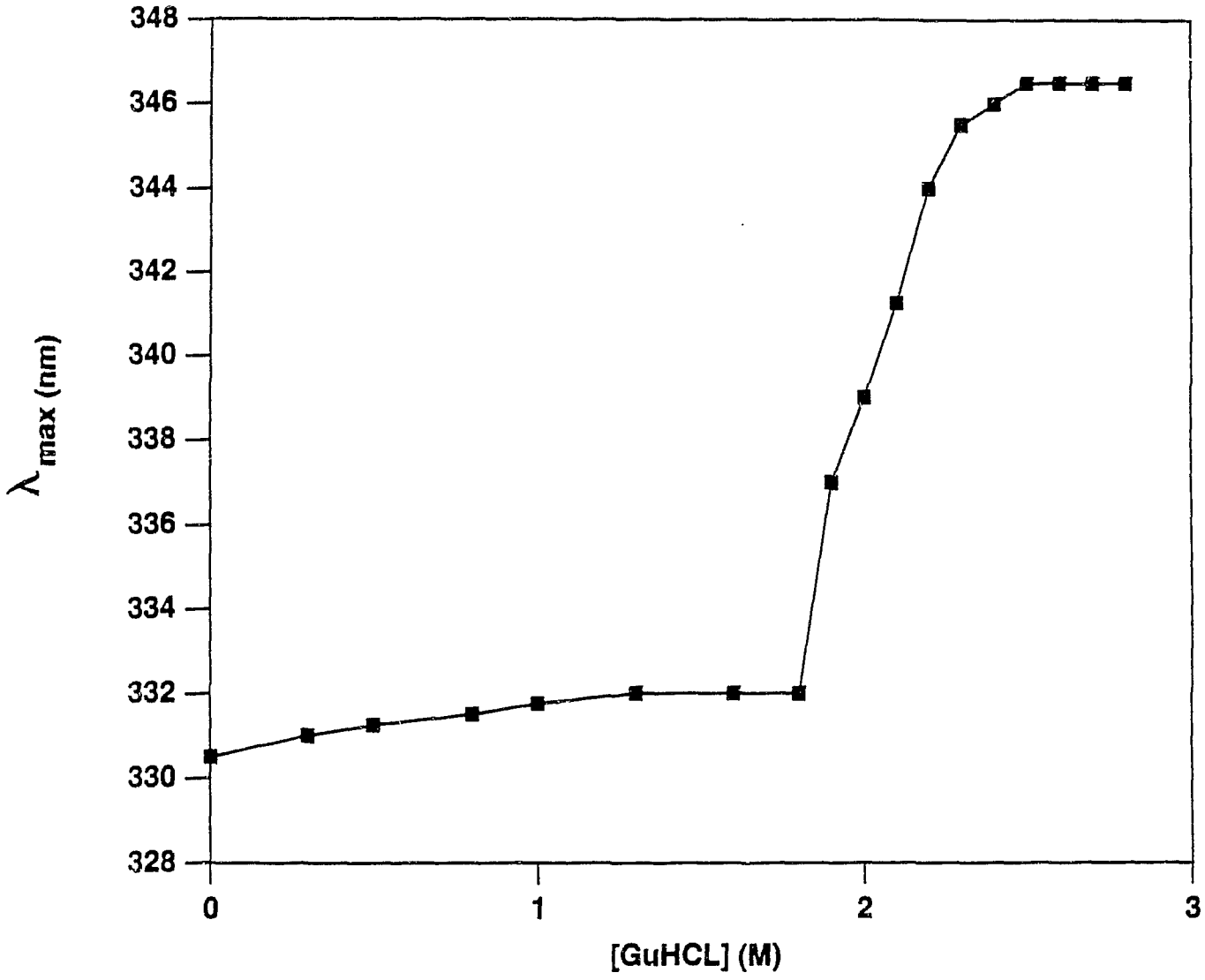

Figure 3. GuHCl denaturation curve. Fluorescence emission maximum dependence on GuHCl.

The equilibrium population of folded versus unfolded urease was estimated using I_{320} values in the transition region. While λ_{max} yields useful information on tryptophan exposure, it may lead to erroneous estimates due to differences in fluorescence yield between the native and the denatured state (Eftink, 1994). As shown in Figure 4, for a given concentration of denaturant in the transition zone, the populations of folded versus unfolded urease did change in response to a difference in urease concentration. The unimolecular reaction therefore, must be rejected since it should not have been affected by a change in concentration. A reaction where folded trimer would dissociate into three unfolded monomers would be described as:

$$F_3 <=> 3U \quad (1)$$

where F_3 is the folded trimer, and U is the unfolded monomer. The unfolding equilibrium constant K_u for this reaction is equal to $[U]^3/[F_3]$. K_u can be related to f_u (the fraction of unfolded urease) by equation 2. These equations were derived from the equations given by Bowie and Sauer, (1989) where;

$$K_u = 3f_u^3[M_t]^2 / (1-f_u) \quad (2)$$

M_t is the total concentration in monomer expressed in molar units, and $(1-f_u)$ is the fraction of folded protein.

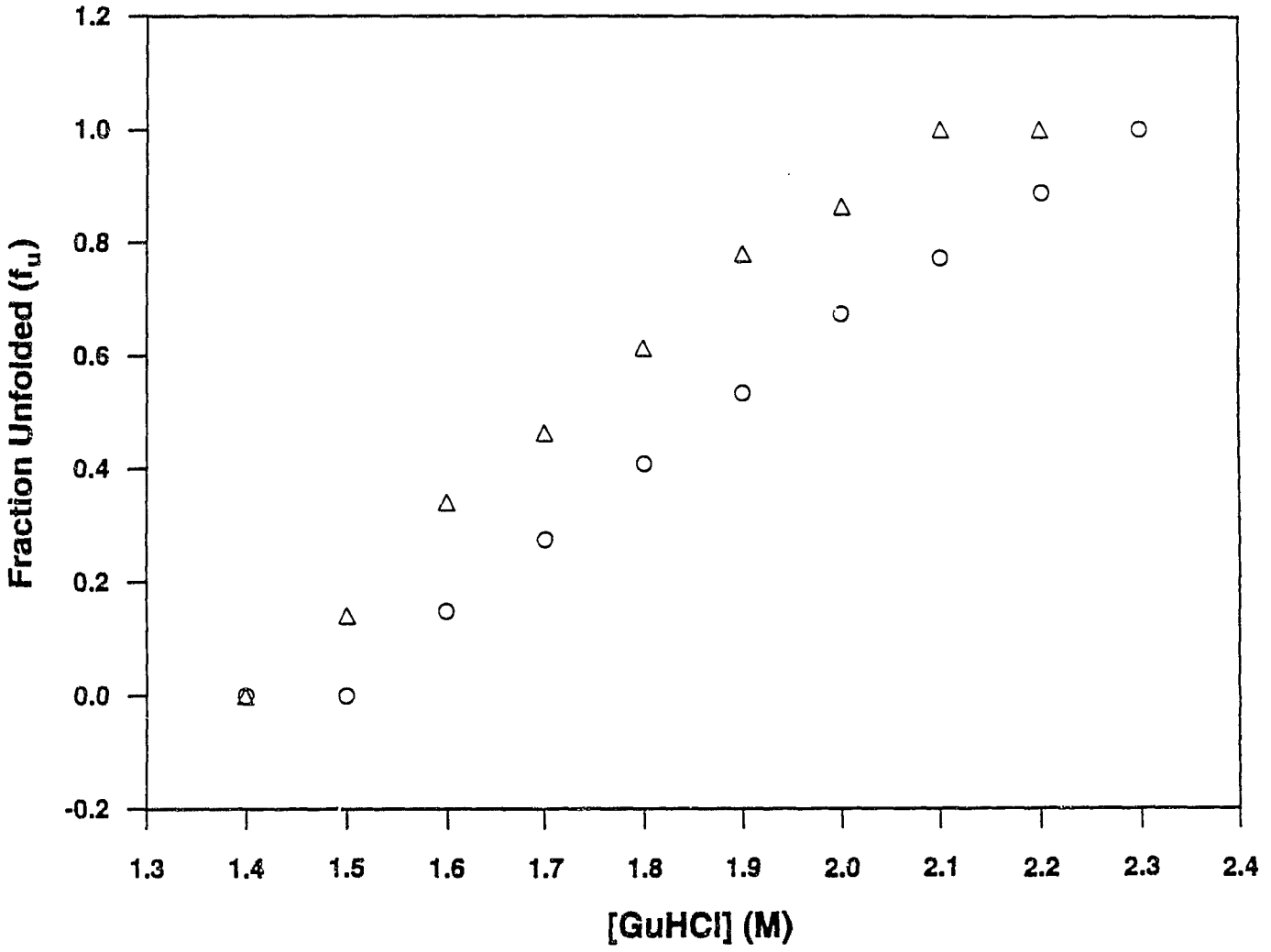

Figure 4. Effect of urease concentration on its denaturation by GuHCl. Denaturation is expressed as the fraction unfolded (f_u). Triangles: 33 x 10^{-7} M urease; circles; 3.3 x 10^{-7} M.

If this model provides a reasonable thermodynamic description of the denaturation reaction, then one could calculate the same value of K_u or ΔG_u (calculated as -RT ln K_u) from experiments performed at different concentrations of urease. As shown in Figure 5, ΔG_u was then obtained by extrapolating at 0 M GuHCl. This figure demonstrated that the values of ΔG_u are not the same when calculated with denaturation curves done at different concentrations of urease.

A process involving the denaturation of a dimer into unfolded monomers was described by Bowie and Sauer (1989),

$$F_2 <=> 2U \quad (3)$$

Where F_2 is the folded dimer and U is the unfolded monomer.

In this case, K_u can be related to f_u (the fraction of unfolded urease) by equation 4:

$$K_u = 2f_u^2[M_t] / (1-f_u) \quad (4)$$

The consistent extrapolation of $\Delta G_u = 19.0 \pm 0.3$ kcal mol^{-1} for different urease concentrations confirms that the large change in structure involves a dissociation of dimers into unfolded monomers (Figure 5). The irreversibility of this experiment precludes the use of equilibrium thermodynamic calculations.

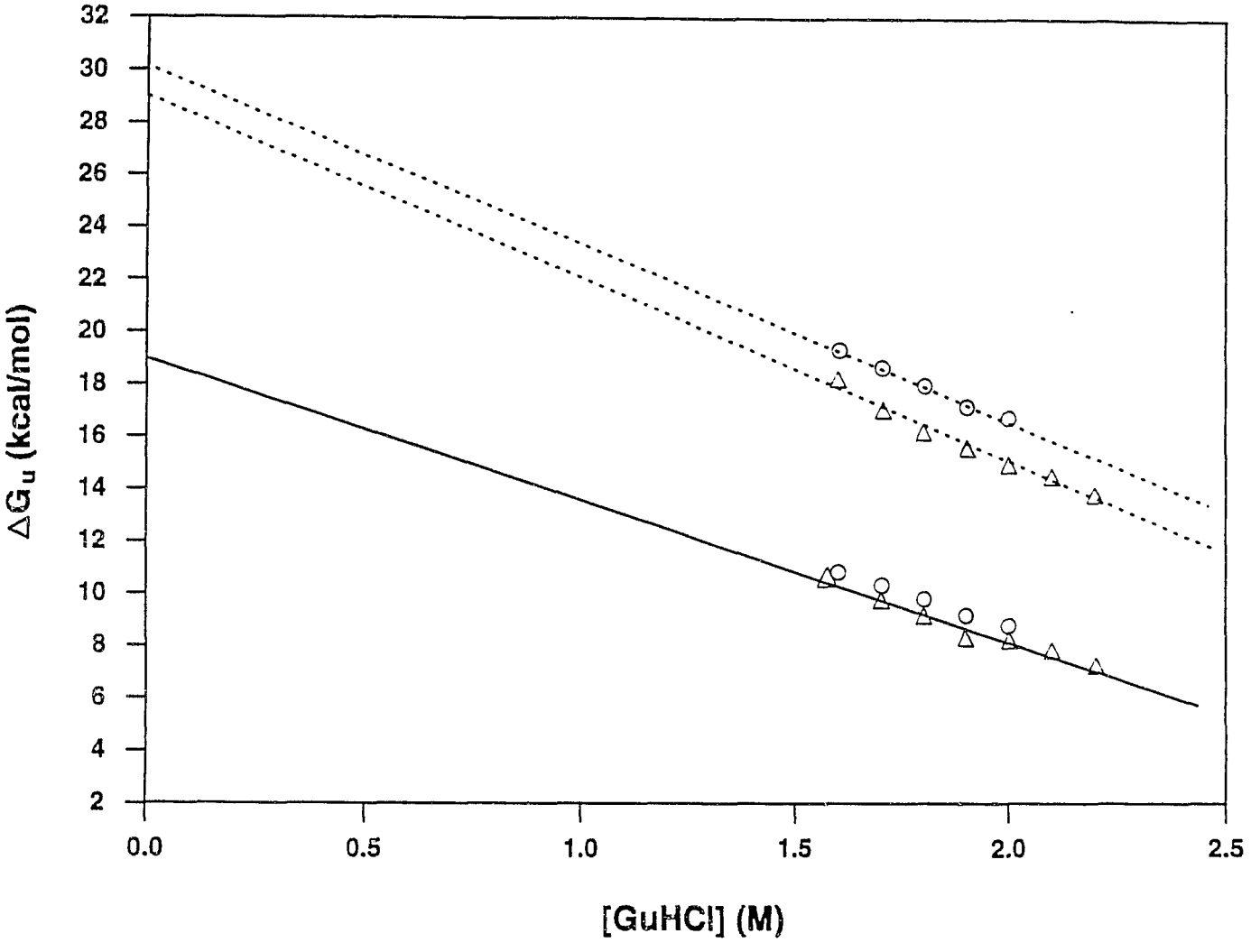

Figure 5. Extrapolation of ΔG_u at 0 M GuHCl obtained with a trimer to monomer (dashed lines) and dimer to monomer model (solid line).

2.4 DISCUSSION

Tryptophan is known for its sensitivity to the polarity of its environment: its emission maximum shifts from around 325 nm in a non-polar environment to around 350 nm in a polar environment (Lakowicz, 1983). We found that in native urease, the tryptophan molecules were mostly protected from water. The tryptophan environment changed drastically when urease was treated with a concentration of 2.0 M GuHCl or higher, going from non-polar to polar. This indicates that tryptophan molecules become exposed to water under such conditions. The thermodynamic study of GuHCl-induced unfolding of urease indicated that this drastic change in structure was due to a dissociation of dimers into monomers. Tryptophan residues in dimers appear to be protected as their fluorescence maximum is at 332 nm prior to the transition, suggesting that the dimers are folded. The λ_{max} = 346 nm obtained after the transition (from 2.5 M to 7 M GuHCl) reveals that the monomers were unfolded. Support for these interpretation also comes from an earlier study showing that in 6 M GuHCl, urease monomers were obtained (Blatter and Gorin, 1969).

The thermally denatured state obtained at 75 °C was different from the one obtained in 2.5 M GuHCl. These denatured structures were different on two counts: 1) the process was irreversible, leading to protein aggregation; and 2) the tryptophan was not exposed to solvent to a comparable extent. The lower exposure of tryptophan observed in the thermally denatured samples could be ascribed to the formation of

aggregates. These study on urease structure stability is compatible with several earlier studies described below.

GuHCl studies: It was found that urease could retain its activity in solutions containing up to 2 M GuHCl. Under such conditions, urease formed a precipitate upon prolonged exposure (Dixon *et al.*, 1980). By increasing [GuHCl] to 2.5 M, the same investigators found that urease was inactivated, and that the process was partially reversible. This study revealed that in 2 M GuHCl, some dimers were folded and reversibly dissociated into unfolded monomers at higher concentrations of GuHCl. Other experiments have shown that urease was dissociated into monomers in 6 M GuHCl (Blatter and Gorin, 1969). The thermodynamic analyses corroborates the earlier findings and demonstrates that such monomers are unfolded.

Thermal inactivation studies: Thermal inactivation studies have shown that urease remains active up to 70 °C (Wang and Johnston, 1993; Kallury *et al.*, 1993), but that the inactivation at high temperature was irreversible (Wang and Johnston, 1993). The proposed unfolding mechanism is consistent with these studies.

SDS studies: The observation by Hirai *et al.*, (1993) that active monomers can be obtained in low SDS concentration (0.23 g SDS per g urease) is not in contradiction with the proposed model. At low concentration, SDS would stabilize a hydrophobic surface in aqueous solution by forming an amphiphilic interface. When SDS

concentration was increased (1.4 g SDS per g urease), the monomer unfolded as expected (Blatter and Gorin, 1969).

Low pH/high pH and polyalcohols studies: Early studies on urease dissociation have shown that an active dissociation intermediate could be obtained with various treatments of urease (Contaxis and Reithel, 1971; Gorin *et al.*, 1968; Blatter *et al.*, 1967). The size of such an intermediate was found to be in the range of 160-190 kDa (Gorin *et al.*, 1968; Reithel and Robbins, 1967), indicating that it consisted of a dimer (2 subunits of 91 kDa each). The secondary structures of such dimers were not different from the hexamer (Contaxis and Reithel, 1971). It is shown here that the dimers could also be obtained by addition of GuHCl, and that their tertiary structure was left unchanged when compared to the hexamer.

A schematic model for α-urease dissociation is shown in Figure 6. This pathway summarizes these findings and integrates data available from the literature (Hirai *et al.*, 1993; Dixon *et al.*, 1980; Contaxis and Reithel, 1971; Gorin *et al.*, 1968). These data suggest that native urease can be dissociated into active, folded dimers and the active dimer and monomer are prone to aggregation. Then, depending on the treatment, the dimers can be dissociated into SDS-stabilized active monomers, or inactive monomers that for aggregates.

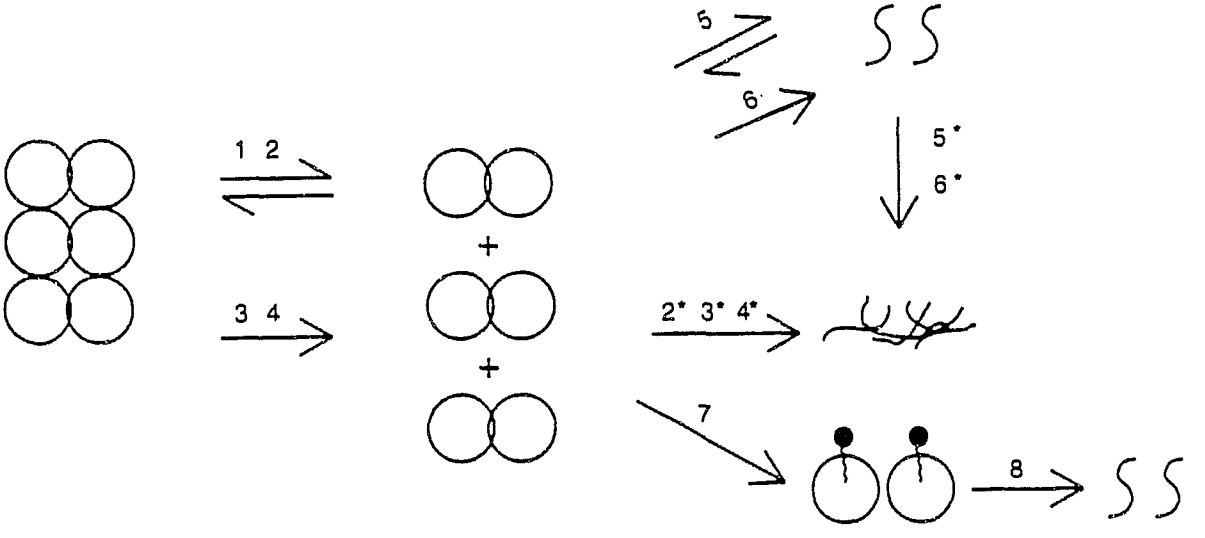

Figure 6. Dissociation/denaturation pathways for α-urease.
Symbols related to urease structure: Open circle, folded and active subunit; S-shape line, unfolded and inactive subunit; isolated open circle with detergent molecule inserted, SDS-stabilized subunits. Symbols for treatments: 1, high pH and polyalcohols; 2, GuHCl 2 M; 3, heating 60 °C; 4, low pH; 5, GuHCl 2.5 M; 6, heating 75 °C; 7, low SDS; 8, high SDS. Asterisks indicate that treatment is prolonged. Arrows indicate reversibility (where known).

These studies (and others cited above) show that the active dimer and the monomer are prone to aggregation. The stabilization of the monomers by detergent (Hirai *et al.*, 1993) indicates that exposure of hydrophobic segments is responsible for aggregation. Thus, the use of amphiphilic molecules could prove to be a powerful way for stabilizing urease.

The dissociation mechanism for urease would provide a rationale for recent studies on stabilization of urease by immobilization (Kallury *et al.*, 1993; Margolin *et al.*, 1985). By restricting urease translational freedom, it was found that urease could remain active after treatment at 100 °C. Such an improvement in stability would be explained by the nature of urease inactivation: the dissociation of a dimeric protein.

In the immobilized system the subunits cannot migrate freely after dissociation. The result is that the dimer conformation can be reformed, and that the formation of non-active aggregates is minimized. When free in solution, urease monomers can change their position and orientation to form non-native aggregates.

Thermodynamics of unfolding have been studied for a large number of proteins (Privalov, 1979). Globular protein have unfolding free energies in the range of 5 - 15 kcal mol^{-1}, while dimeric proteins appear to be more stable with unfolding free energies of 10-20 kcal mol^{-1} (Bowie and Sauer, 1993). Urease denaturation involves a dimer dissociation mechanism and in accordance has a very high free energy of

unfolding. Its value (19.0 $\pm$ 0.3 kcal mol-1) is among the highest for proteins reported so far.

This study demonstrated that urease dissociation and denaturation can be monitored using fluorescence spectroscopy. This work also revealed that urease denaturation involves a dimer dissociation mechanism and in accordance has a very high free energy of unfolding. Such studies will be useful industrially for thermal stability of urease.

Chapter 3: STRUCTURAL STABILITY OF A SYNTHETIC PROTEIN (MB-1) EXPOSED TO VOLATILE FATTY ACIDS.

3.1 INTRODUCTION

Milk Bundle protein, MB-1, is a *de novo* protein (100 residues long) enriched with a high content of methionine (M), threonine (T), lysine (K), and leucine (L). MB-1 represents the first example of a *de novo* designed protein with a potential application in ruminant nutrition (Beauregard *et al.*, 1995). In order to assess the feasibility of MB-1 as a feed additive, it should first be evaluated for the physical aspects of its structural stability.

The thermodynamic stability of a protein determines the fraction of the protein that will be in an unfolded and proteolytically susceptible state (Privalov, 1979). Therefore, the structural stability of a protein is an important determinant of its proteolytic susceptibility (Parsell and Sauer, 1989). The physical stability of a protein is influenced by many factors in its environment; the pH, ionic strength and the presence of chemicals that stabilize it (Privalov, 1979).

Among the major components of a rumen fluid that would affect the stability of MB-1 are the amphiphilics. Amphiphilics act as detergents and destabilize the protein molecules (Privalov, 1979; Loucks, 1993). Volatile fatty acids are the major

amphiphilics found in the rumen that could have an impact on MB-1 stability. The main volatile fatty acids present in the rumen fluid are acetic, propionic and butyric acid (Hungate, 1966).

Preliminary structural stability studies of MB-1 were conducted using rumen fluid that was obtained from fistulated cows. The proteins and microorganisms were removed from the fluid by filtration, and then concentrated. In spite of these treatments, the fluid could not be used for these studies. Rumen fluid contains many chromophores such as chlorophyll, carotenoids and many other related substances (Van Soest, 1983). Spectroscopic investigation confirmed that these pigments absorb light between 300 nm and 400 nm (Katz, 1994). Hence, it was impossible to employ fluorescence techniques to conduct structural studies using this fluid since it would interfere with the wavelength of interest. The only way to remove the pigments was through solvent extraction. The use of these solvents would result in a drastic change in the organic composition in the rumen fluid. To address this problem, an artificial pigment-free buffer which contained only the major components (in terms of protein thermodynamic stability) of the rumen solution had to be prepared.

This study was conducted to understand the following points. 1. Will MB-1 remain folded at the rumen temperature; and 2. will the presence of the amphiphilic volatile fatty acids destabilize MB-1? To investigate these, thermal stability studies were conducted in the presence of physiological levels of volatile fatty acids at a pH within

rumen range. MB-1 was exposed to these conditions and its folding behaviour was studied. Three natural proteins, RNAse A, cytochrome c, and urease were also exposed to similar conditions so that comparison could be done to these stable and highly purified proteins.

3.2 Material and Methods

3.2.1 Chemicals and Natural proteins

Ribonuclease A (RNAse A), (R-5500), and urease (U-0251) were purchased from Sigma Chemical Co. (St. Louis, MO). Cytochrome c from horse heart (101467), was obtained from ICN Biomedicals (St.Laurent, PQ). To formulate the artifical buffer, sodium acetate, sodium propionate and sodium butyrate were purchased from BDH Inc. (Toronto, ON).

3.2.2 Electrophoresis (SDS-PAGE)

Throughout these experiments, sodium dodecyl sulphate polyacrylamide gel electrophoresis was conducted in a Miniprotean Bio-Rad apparatus (Beverly, MA). Acrylamide gels of 10% resolving gel and 5% stacking gel (Sigma Chemical Co., St.Louis, MO) were prepared. Electrophoresis was conducted in Tris-tricine buffer (0.1 M Tris pH 8.25, 0.1 M Tricine, 0.1% SDS) for 1 hr at 100 V and 30 mA.

Tricine was used to insure optimal band resolution of small molecular weight proteins (Schagger and Von Jagow, 1987). Silver staining was performed according to Bio-Rad's instruction booklet (Bio-Rad, Mississauga, ON).

3.2.3 Synthetic protein, MB-1

Milk bundle-1 (MB-1) was previously designed and the gene was constructed at Agriculture and Agri-Food Canada, Ottawa (Beauregard *et al.*, 1995).

3.2.3.1 Gene expression

The MB-1 gene was expressed in *E. coli* using the pMAL-c2 (New England Biolabs) expression system (Beauregard *et al.*, 1995). Overnight cultures of *E. coli* clones containing the various constructs were diluted 1:100 in 2YT (yeast, tryptone) medium and grown 2 hr at 37°C (A_{600} = 0.3 to 0.5) with agitation. IPTG was added to a final concentration of 0.3 mM and incubation continued for 3.5 hr (A_{600} = 1.2 to 1.4).

3.2.3.2 Cell harvest and protein fractionation

Cells were harvested by centrifugation and resupended in a buffer (10 mM Tris (pH 7.4), 200 mM NaCl, 1 mM EDTA and 1 mM NaN_3). Cells were lysed by sonication (10 pulses of 30 s with a pause of 30 s between each pulse), and NaCl was added to

a final concentration of 700 mM in order to remove cell debris. The resulting bacterial extracts contained a fusion of maltose binding protein with milk bundle protein (MBP-MB1). The supernatant was then stored at -20°C until further purification by amylose affinity column chromatography.

3.2.3.3 Amylose affinity column chromatography

An amylose resin (New England Biolabs, Mississauga, ON) was poured into a column and washed for 20 minutes with Buffer B (10 mM Tris, 200 mM NaCl, 1 mM NaN_3 at pH 7.4) to remove the ethanol. The column was then equilibrated with cold Buffer B for 30 minutes. The bacterial extract containing the fusion protein was thawed on ice and loaded onto the column. The fusion protein was recovered by applying an elution buffer (10 mM Tris-HCl, 200 mM NaCl, 10 mM maltose, 1 mM EDTA, 1 mM NaN_3 adjusted to pH 7.4). SDS-PAGE was employed (as per method described in Chapter 3.2.2) to determine which fractions contained the fusion protein. The fractions containing the fusion protein were then pooled for separation and further purification.

3.2.3.4 Cleavage of fusion

Cleavage of the fusion protein was achieved by adding 50 μL of 1 mg/mL Factor Xa (New England Biolabs, Mississauga, ON) to the pooled sample. Hydrolysis was

conducted in 20 mM Tris (pH 8.0), 100 mM NaCl, and 2 mM $CaCl_2$ at room temperature for 2 hr. The sample was then dialysed overnight at 4°C using 3.5 kDa cut off dialysis bags (Spectropor, Houston, TX). The bag was transferred to 4 L of buffer containing 10 mM Tris (pH 8.25) and 1 mM EDTA and dialyzed for 2 hr to remove the maltose (according to the protocol described in New England Biolabs). Separation of MB-1, MBP and Factor Xa was achieved by ion-exchange chromatography.

3.2.3.5 Ion-Exchange Chromatography

The sample was loaded onto a diethylaminoethyl sepharose (DEAE) fast flow column (New England Biolabs, Mississauga, ON) and a salt gradient (high salt: 50 mL of 10 mM Tris (pH 8.25), 1 mM EDTA, 40 mM NaCl and low salt: 50 mL of 10 mM Tris (pH 8.25), 1 mM EDTA was applied to elute MB-1. The fractions that contained MB-1 were pooled and then dialysed against 4 L of 20 mM ammonium bicarbonate buffer. Finally, the dialysed sample was partially concentrated to about 4 mL volume using a freeze-dryer (Labcon Co., Tecumseh, ON) and then transferred into four centrifuge tubes. These samples were then concentrated by evaporation in vacuum (Savant Speedvac Concentrator, Farmingdale, NY). The dried sample was weighed and then redissolved in distilled water and further lyophilized until a constant weight was obtained. This step was used to eliminate most of the volatile salts that were present in the dried sample.

3.2.3.6 Amino acid analysis

Amino acid analysis was conducted by Dr. Mak Yaguchi at the National Research Council, Ottawa. The method used involved acid hydrolysis of MB-1 in 6 N HCl at 110°C overnight. The second step was to remove the acid and dissolve the hydrolysate in a buffer at low pH. Finally the component amino acids were separated and quantified. Separation was done using a Dowex column and post-column derivatization was done with ninhydrin for detection. Ninhydrin reacts with the amino acid's amino group to give a red coloured product. This was then detected and quantified by comparing it to a mixture of known amounts of amino acid standards.

3.3 Quantification of MB-1

Quantification of MB-1 was necessary in order to determine the actual concentration because the dried MB-1 sample contained some salts. MB-1 quantification was conducted as follows: 10 μL of protein sample was added to 3.948 mL of 10 mM acetate buffer (pH 5.0), 40 μL of 1% polyoxyethlene 23 lauryl ether (Sigma Chemical Co., St.Louis, MO), and 2 μL of 1 M PMSF. An absorbance reading was conducted at 214 nm (Stoscheck, 1990). Since MB-1 does not have a known extinction coefficient, estimation of protein was conducted by comparing it to a calibration curve constructed using a known concentration of RNAse A solution.

3.4 Purity check of MB-1

SDS-PAGE of MB-1 was conducted to determine the purity of the samples from each preparation. Known amount of MB-1 (0.5 μg, 1.5 μg and 5 μg) were loaded onto SDS-PAGE. The gel was then scanned with a gel scanning densitometer (Ultroscan XL Laser Densitometer, Pharmacia LKB Biotechnology, Montreal, PQ). Interpretation of the data was done by comparison of the relative areas of the protein bands.

3.5 Preparation of volatile fatty acids (VFA) buffer

Buffer VFA was made up of 50 mM sodium acetate, 20 mM sodium propionate, 20 mM sodium butyrate, and 17 mM potassium phosphate adjusted to pH 6.5 to approximate the rumen level of total ionic strength and pH (Stewart,1975; Hungate, 1966). A control buffer with the same pH and ionic strength but without the VFA was prepared using 90 mM NaCl and 17 mM potassium phosphate. To each buffer, PMSF, at a concentration of 0.5 mM was added to prevent proteolytic degradation and 1 mM sodium azide was added to prevent the growth of airborne microorganisms.

3.6 Structural stability studies

3.6.1 Fluorescence studies

One milligram each of RNAse A, cytochrome c, urease and MB-1 was dissolved in 1 mL of Buffer VFA as well as the control buffer. The samples were dialysed overnight at 4°C against 100 volumes of the respective buffer. On the following day, samples were kept for 1 hr at room temperature to equilibrate. All solutions were filtered using 0.2 μm filter and denaturation experiments were carried out using PTI Rf-M2204 fluorometer. For cytochrome c and urease, tryptophan was excited with light of 290 nm and the spectra were scanned between 300 nm and 400 nm, using a bandpass of 2.5 nm. For RNAse A and MB-1, tyrosine was excited with light of 284 nm and the spectra were scanned from 295 to 350 nm, using a bandpass of 3 nm. Denaturation studies for all four proteins (in triplicate) were conducted from 25° to 80°C with an equilibration period of 15 minutes between each temperature. The sample temperature was controlled with a circulating bath. All spectra were corrected for buffer and water emission and for loss of efficiency in the detector and gratings. Curves of fluorescence intensity versus temperature were constructed.

3.6.2 Thermodynamic calculations

Thermal stability was calculated assuming a unimolecular process as described in Pace

et al. (1989). Assuming a two-state mechanism (folded --> unfolded), T_m was calculated. For urease and cytochrome c, I_{320} (fluorescence intensity at 320 nm) and for MB-1 and RNAse A, I_{303} (fluorescence intensity at 303 nm) were chosen as the property (y) indicative of the extent of unfolding. As previously seen (Section 2.2.5), in the folded state, the parameter $y = y_f$ when the fraction of folded protein f_f is equal to 1. When the protein is unfolded, the parameter $y = y_u$, when the fraction of unfolded protein, f_u equal to 1. Anywhere between these two states, at y is equal to $y_f f_f + y_u f_u$. Thus, by measuring y, we can calculate the fraction of protein unfolded: $f_u = (y_f - y)/(y_f - y_u)$. For a unimolecular process, the equilibrium constant for the unfolding process is $K_u = f_u/(1-f_u)$, where f_u is concentration independent. The free energy of unfolding, ΔG_u, is obtained with $\Delta G_u = - RT \ln K_u$. Melting temperature ($T_m$), i.e. the temperature at which the concentration of unfolded and folded protein are at equilibrium, was obtained by extrapolation of ΔG_u to 0 (Pace *et al.*, 1989).

3.6.3 Aggregation test

Aggregation was visually checked by observing the presence of aggregates at each temperature interval. The solution that appeared to be cloudy (or with small chunks of aggregates) was designated as "aggregated". These aggregates were seen after the melting temperature of the proteins.

3.7 RESULTS

3.7.1 Purification

The fusion protein (MBP-MB-1) which had an apparent MW of 52 kDa was selectively retained on the amylose column. Elution of this protein was achieved in the presence of maltose indicating that the maltose binding protein (MBP) portion of the fusion was correctly folded. As indicated in Figure 7 (lane 7) , the cleavage of the MBP-MB-1 fusion using Factor Xa gave two protein bands with anticipated MW of 42 and 11 kDa corresponding to MBP and MB-1 respectively. The two proteins were then separated by DEAE (ion-exchange chromatography). The MB-1 samples were pooled and freeze-dried. The average yield obtained for each batch was about 10 mg. SDS-PAGE was employed to check for purity of MB-1 samples. As shown in Figure 8, MB-1 was found to be 99% pure as determined by a gel scanner.

3.7.2 Amino acid composition

Amino acid composition (courtesy of Dr. Mak Yaguchi, NRC, Ottawa) gave anticipated results: MB-1 consisted of 22% threonine, 14% methionine, 12% lysine, 9% leucine for a total of 57% of amino acid content of MB-1.

3.7.3 Structural stability studies

The results of thermal denaturation studies of MB-1, cytochrome c, RNAse A and urease were plotted. As can be seen in Figures 9 to 12, only those temperatures that were useful for transition phase of the proteins were plotted (details given in section 2.2.5). The thermal unfolding curves can clearly be seen to have three regions: pre-transition, transition and post-transition. Initially, the protein is in its native, folded state and fluorescence intensity is decreasing with temperature in the pre-transition region. Then, as the protein unfolds, there is an increase of fluorescence intensity in the transition region.

Finally, the protein attains a new conformation and there is again a decrease in fluorescence intensity with temperature which corresponds to the post-transition region. Therefore, the steep increase in fluorescence observed represents the unfolding of the proteins. At the inflexion point, there are two populations of protein, the original, folded population and the new, unfolded population. At the equilibrium point of the unfolding process, the free energy change of the process equals zero. The temperature at this point is known as T_m, the unfolding temperature of the protein.

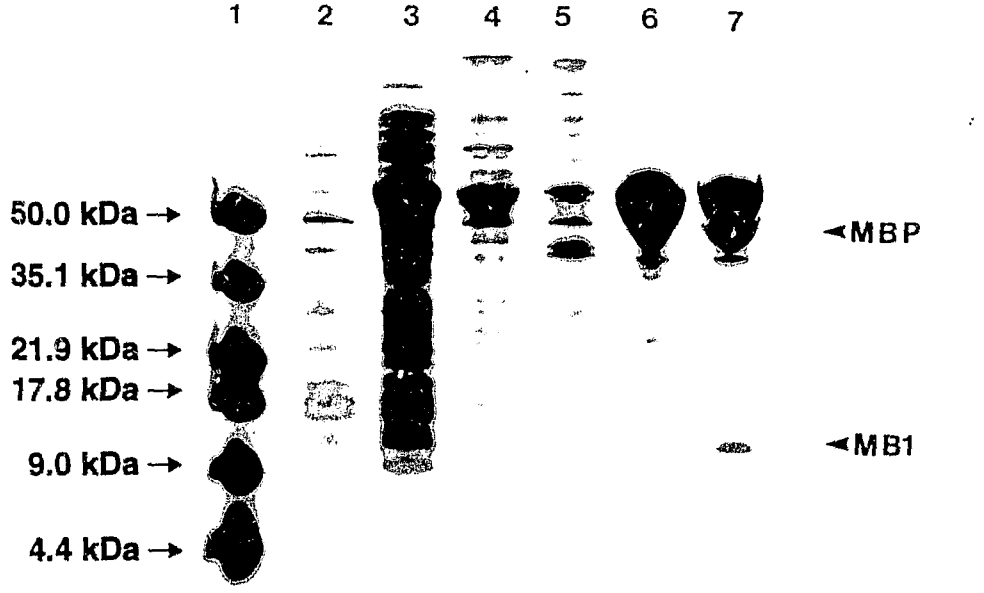

Figure 7. SDS-PAGE. Prestained molecular weight protein markers (BioRad) are in lane 1. Total protein from *E.coli* TB1 containing the MBP-MB1 gene are in lane 2 (uninduced) and 3 (induced with IPTG). Intracellular protein was released from induced cultures by sonication and the cellular debris removed by centrifugation (lane 5). The material in the soluble protein fraction (lane 4) was applied to an affinity column. Lane 6 shows the MBP-MB1 fusion protein after preliminary purification on an amylose column. Lane 7 shows the products obtained after partial cleavage of the material in lane 6 with Factor Xa.

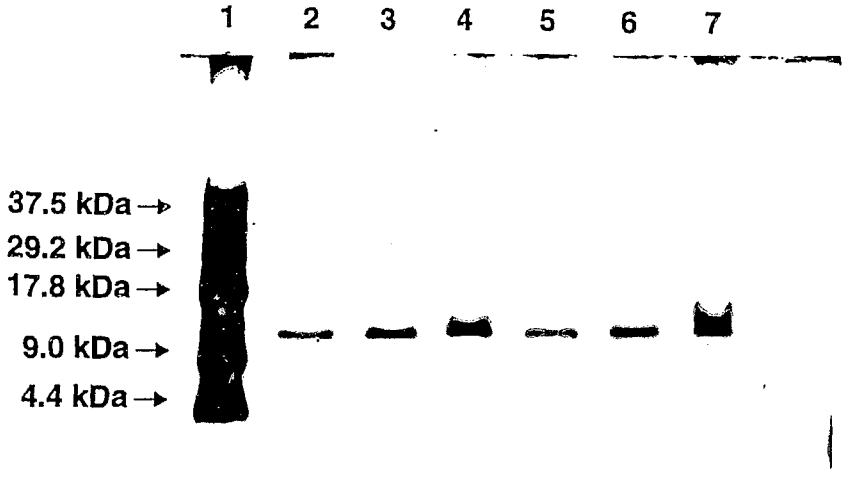

Figure 8. SDS-PAGE purity check of MB-1 for two batches. Lane 1: MW markers;lane 2: 0.5 μg of MB-1; lane 3: 1.5 μg MB-1; lane 4: 5 μg MB-1; lane 5: 0.5 μg MB-1; lane 6: 1.5 μg MB-1; lane 7: 5 μg of MB-1.

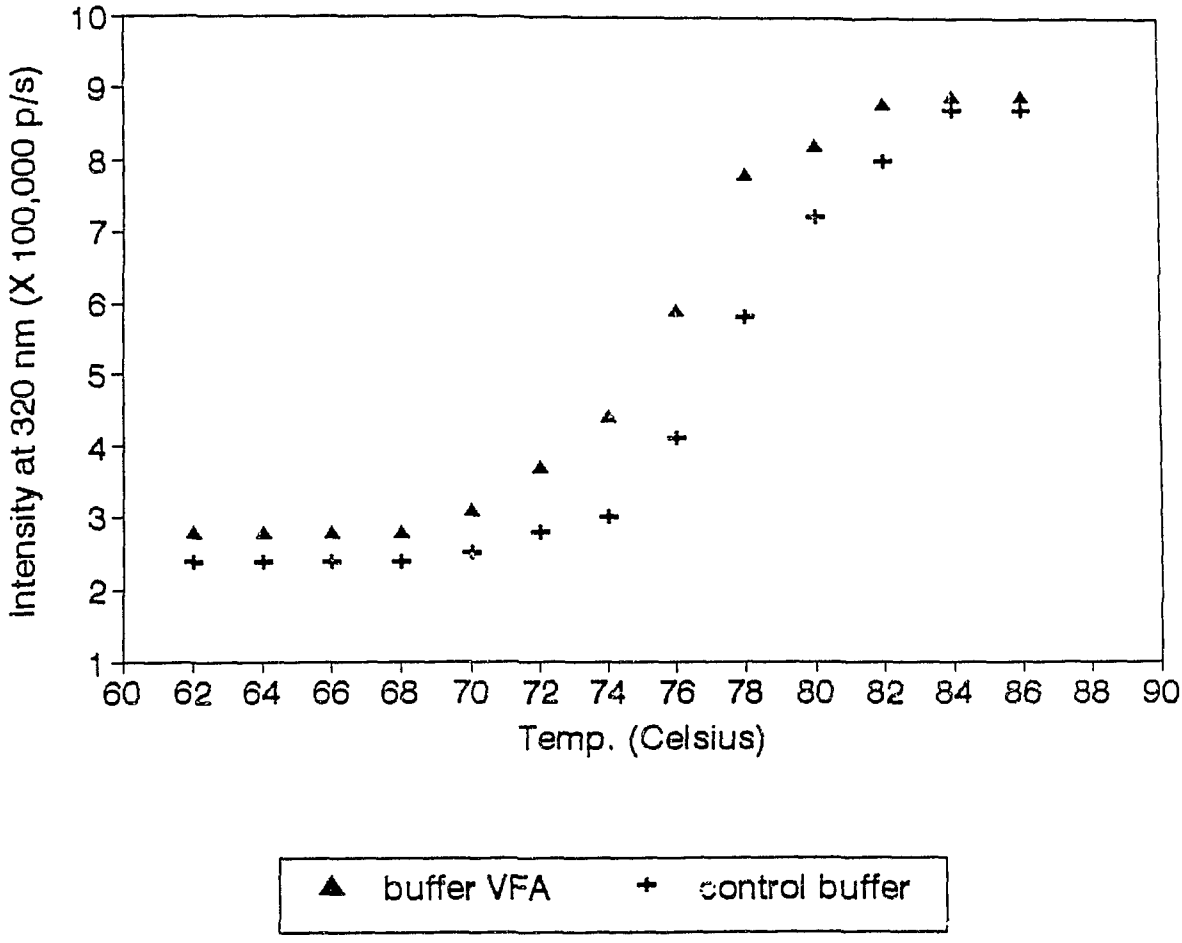

Figure 9. Thermal denaturation of cytochrome c in VFA and control buffers.

Cytochrome c is a single polypeptide protein with 104 residues having MW of 12.4 kDa, which contains tryptophan residues (Margolish *et al.*, 1961). Thermal unfolding of cytochrome c was monitored by following the behaviour of these residues. A plot of fluorescence intensity versus temperature is shown in Figure 9. The pre-transition region is seen as having no change of intensity, followed by an increase of intensity during the transition phase. This increase of intensity is the result of energy going to the nearby quenching groups around the tryptophan. Finally, there is no change of intensity at the post-transition region as the protein becomes unfolded. T_m values of 75°C was calculated in volatile fatty acids buffer and 76°C in control buffer. Investigation of thermal stability of urease in the two buffers are shown in Figure 10. The pre-transition region shows a gradual decrease of intensity followed by large decrease during the transition phase. This indicates that there are no quenchers around the tryptophan molecules. Once the protein is unfolded, the tryptophan molecules get exposed and water becomes the quencher. As the protein become unfolded, the post-transition region is shown as having no change of intensity. T_m values of 68°C in volatile fatty acids and in 70°C for control buffer were obtained. RNAse A is a single polypeptide chain protein of 124 residues, MW 13.7 kDa has tyrosine and no tryptophan residues (Anfinsen, 1961). Unfolding of RNAse A was observed by monitoring the tyrosine fluorescence. A plot of fluorescence intensity versus temperature generated is shown in Figure 11. The gradual decrease of fluorescence intensity in the pre-transition region is due to the presence of quenchers around the tyrosine molecule.

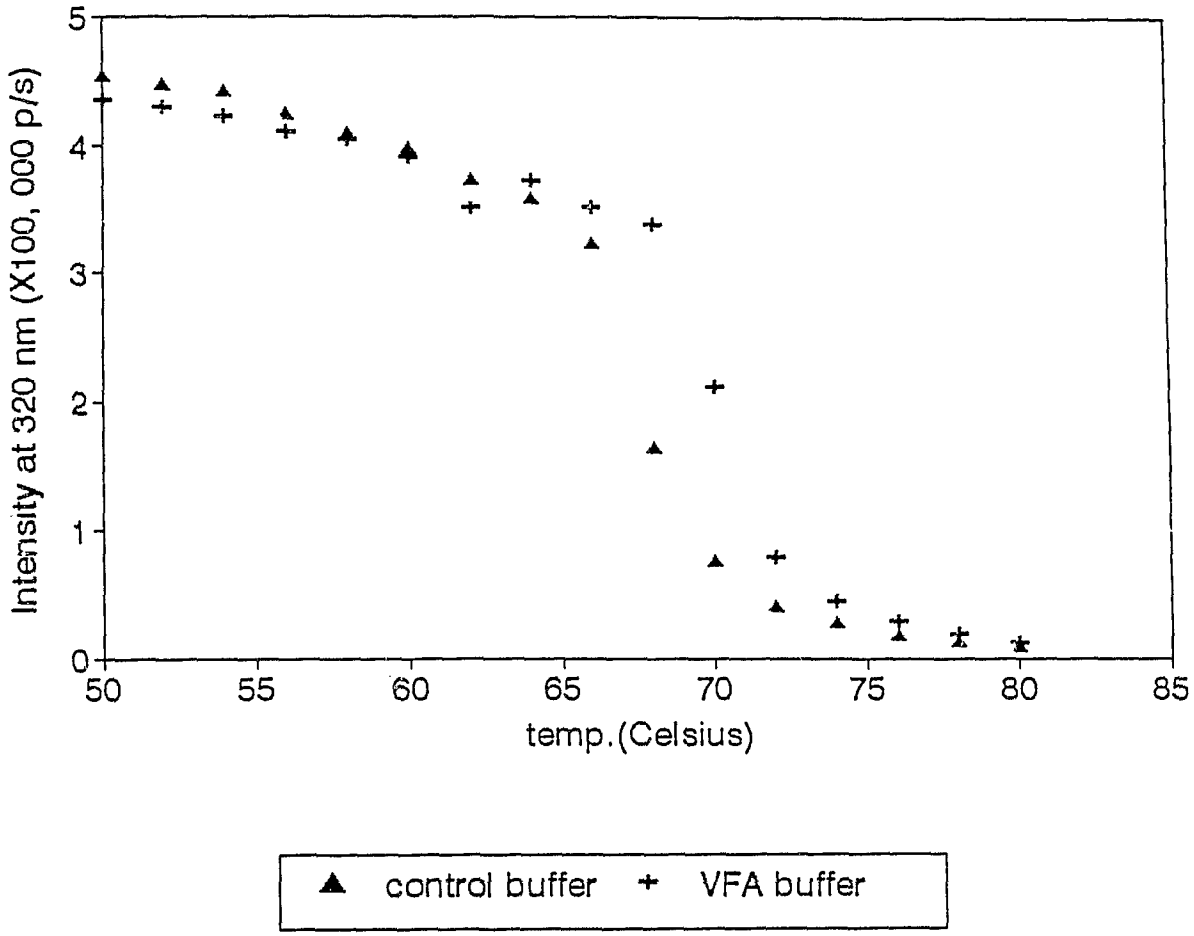

Figure 10. Thermal denaturation of urease in VFA and control buffers

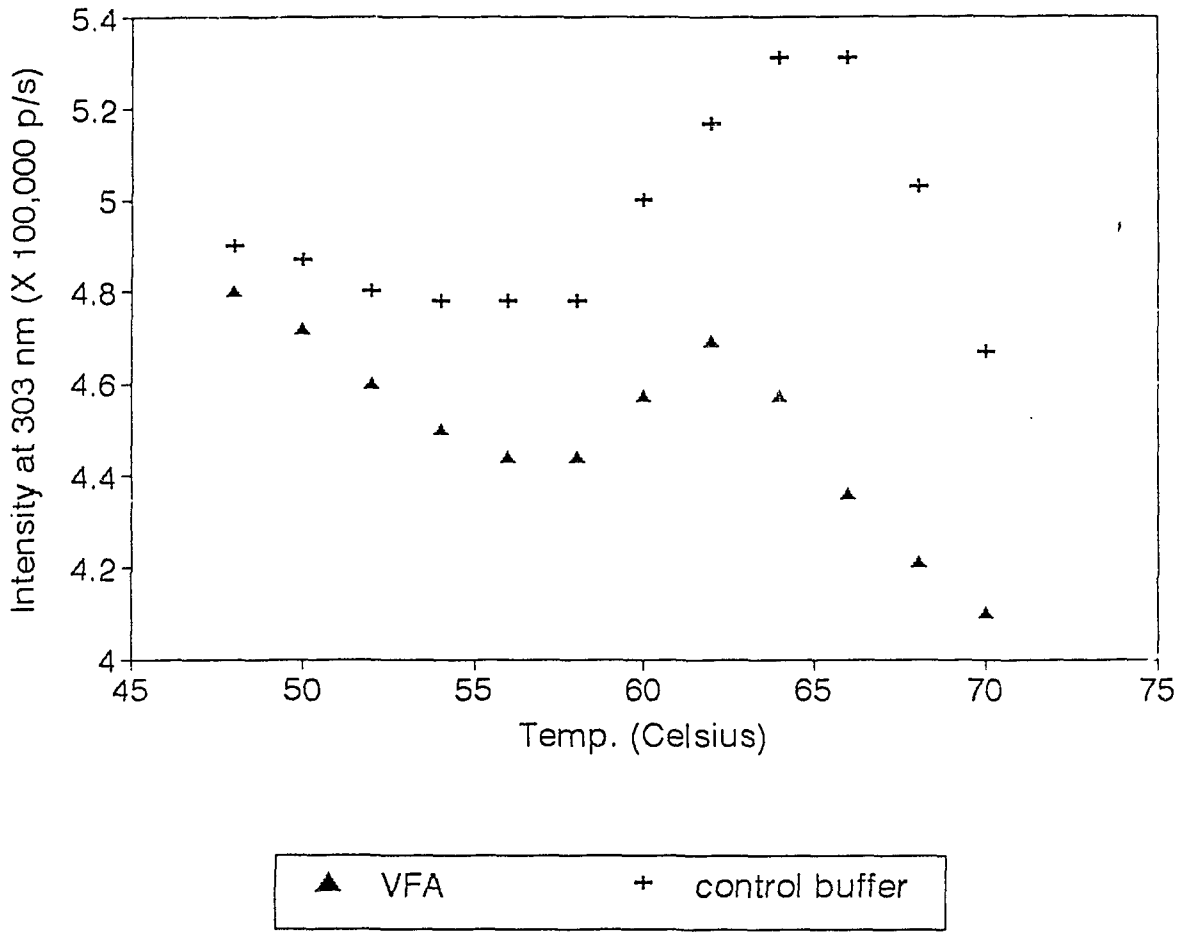

Figure 11. Thermal denaturation of RNAse A in VFA and control buffers

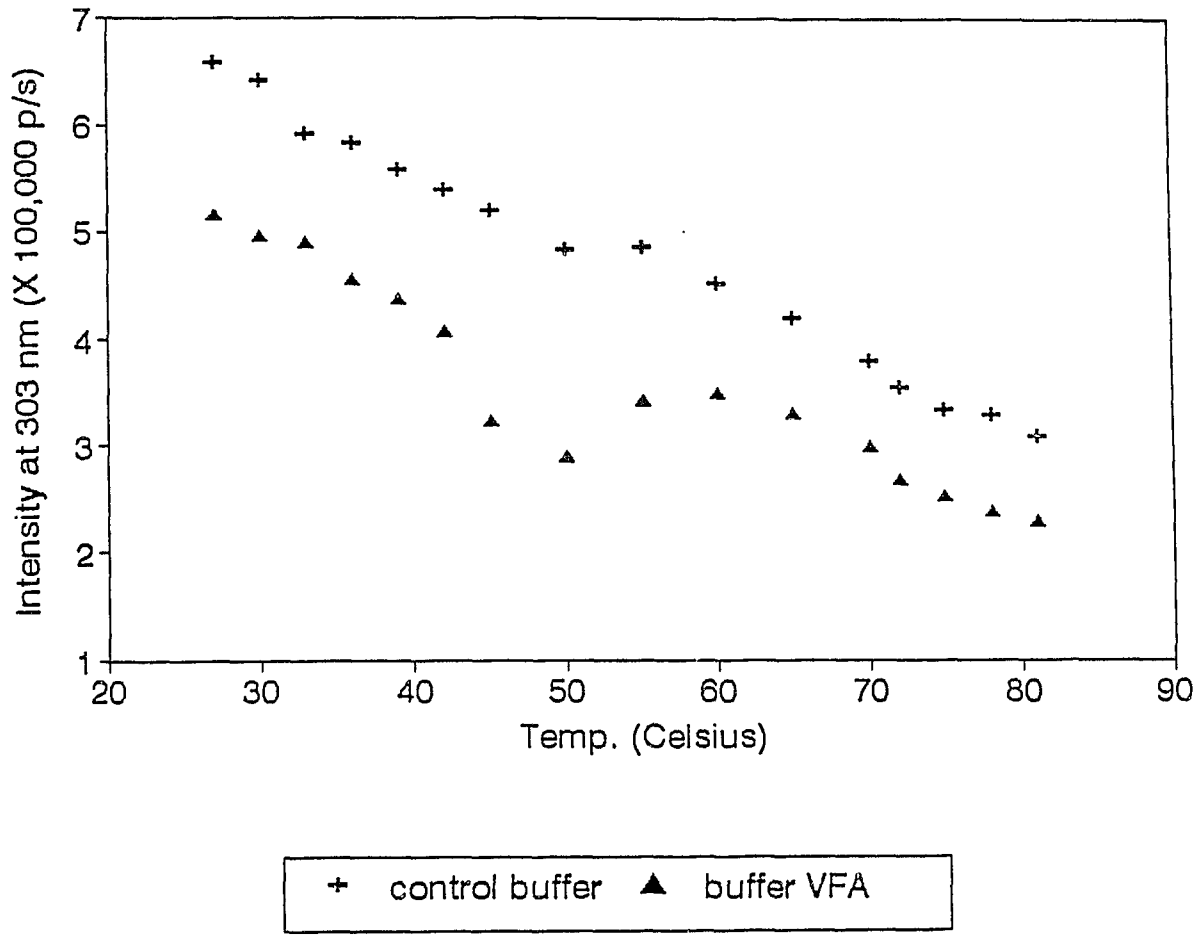

Figure 12. Thermal denaturation of MB-1 in VFA and control buffers.

Then as the protein unfolds, the quenchers move away from tyrosine molecule and an increase of fluorescence intensity is seen. T_m calculations showed that under the volatile fatty acid conditions, RNAse A denatures at T_m value of 60°C. Similar studies in a control buffer has indicated a T_m value of about 62°C.

MB-1 is a single polypeptide chain of 100 residues, MW 11.4 kDa has a tyrosine residue. Thermal denaturation of MB-1 was conducted under similar conditions. As seen in Figure 12, the decrease of fluorescence intensity was observed upon heating the protein from 25°C to 45°C. The decrease of intensity is due the presence of quenchers in the water environment. As the protein unfolds, the tyrosine molecules become exposed to this environment and the fluorescence intensity increases between 45°C and 60°C (transition region) followed by a gradual decrease in the post-transition region. Thermodynamic calculation gave T_m values of 49°C and 51°C for MB-1 in volatile fatty acids and control buffers respectively. This indicated that MB-1 is folded at the rumen temperature of 39°C.

The means and standard deviations of triplicate results are summarized in Table 2. No statistical comparison was done to see if the T_m values obtained under the volatile fatty acids buffer condition were different from the conditions in the control buffer because the slight difference of temperatures did not warrant statistical analyses.

Table 2 Thermal stability of MB-1 and the natural proteins

PROTEIN	T_m °C VFA buffer	T_m °C control buffer	Aggregates
MB-1	48.9 ± 0.7	50.6 ± 0.8	No
RNAse A	60.2 ± 1.1	62.5 ± 0.9	Yes
Cytochrome c	75.3 ± 1.3	76.4 ± 1.5	Yes
Urease	68.7 ± 0.5	70.9 ± 0.2	Yes

T_m: melting temperature

3.8 DISCUSSION

Under the conditions tested, the results indicated that MB-1 stays folded up to 50°C. This means that MB-1 has a stable structure at a the rumen temperature of 39°C, at a pH (6.5) and in physiological concentrations of the major volatile fatty acids. Currently, there are about 40 synthetically designed proteins (none of them intended for animal nutrition) but only three of them have been shown to undergo transition denaturation with the use of GuHCl (Ho and DeGrado, 1987; Regan and DeGrado; 1988; Hecht *et al.;* 1990; Goraj *et al.*, 1990). So far, there is no published data on thermal denaturation studies on these proteins, thus, this thermodynamic studies on MB-1 was the first on a synthetically designed protein.

Comparative studies with other proteins were necessary to have a thorough understanding of MB-1 and its potential for use. Currently, there are no synthetic proteins available on the market since those that were synthesized were found to be either unstable (Hagan and Warren, 1982) or had toxic effects (Maldague *et al.*, 1991). Furthermore, MB-1 could not be compared with other crude protein supplements available because these products have different purity than MB-1. As a consequence, pure proteins were selected for comparison with MB-1. The selection of these proteins was based on many factors: similar and different molecular weight proteins which are highly pure and inexpensive. Furthermore, the proteins selected should have been previously well characterized. RNAse A, cytochrome c and urease

were selected in order to have comparative results from representatives of different classes of natural proteins. RNAse and cytochrome c are well characterized monomeric proteins of similar molecular weight to MB-1 (i.e. within the range of 11 to 13 kDa). Urease was chosen because in comparison to RNAse A, cytochrome c and MB-1, it represents a different class of larger size proteins.

An important fact that was noted throughout these experiments was that all the natural proteins aggregated upon denaturation. Protein aggregation is believed to be due to conformations that expose hydrophobic clusters which promote protein association (Peters *et al.*, 1995). No aggregates were visually detected throughout the denaturation process of MB-1. This may indicate that MB-1 has a minimal exposure of hydrophobic surfaces in the denatured state. If indeed there was a minimal exposure of these surfaces, then MB-1 could have a high resistance to proteases.

The information obtained from this work suggested that the major rumen volatile fatty acids at pH 6.5 did not have much impact on structural stability of the proteins studied. Furthermore, in all these cases, the proteins were folded above 39°C. Based on these results, it could be inferred that MB-1 could be stable in this environment and may be as resistant to rumen proteases as are other natural proteins. At this stage, no major modification of MB-1 leading to a better folding is considered.

Chapter 4: *IN VITRO* DEGRADATION OF MB-1 BY PROTEASES OF MICROBIAL ORIGIN

4.1 Introduction

Protein degradation studies in ruminants are varied and complex. Several attempts have been made in the past to estimate protein degradation using *in vitro* and *in vivo* methods (Stern *et al.*, 1982; Broderick, 1982; Verite, 1980). The *in vivo* methods employ dacron bags in rumen-fistulated animals (Mehrez and Orskov, 1977; Nocek *et al.*, 1983). Although these methods are ideal for the study of protein degradation, the availability of surgically prepared animals is costly (Mahadevan *et al.*, 1987) and also has its own limitations. For instance, such an approach is not suitable for studying MB-1 because it is a small molecular weight protein and will diffuse through the pores of the bags which have to be large enough to allow the large protease molecules to pass through.

As an alternative to *in vivo* techniques, many workers have been trying to devise suitable methods to study protein degradation *in vitro* (Mahadevan *et al.*, 1987; Krishnamoorthy *et al.*, 1983). One approach would be to incubate feed proteins with freshly collected rumen fluid and then determine the rate of ammonia accumulation (Mahadevan *et al.*, 1987). However, the main drawback of this procedure is that since some of the ammonia is reutilized for microbial synthesis, the method gives a low rate

of degradation (Broderick, 1978). In order to overcome some of these difficulties, inhibitors of amino acid and ammonia utilization have been added to the incubation mixture (Broderick, 1978). However, the specificity of these inhibitors of amino acid deaminases are not well established (Wallace and Kopencny, 1983). Thus, other *in vitro* methods had to be considered.

The two other alternatives would be to purify rumen proteases and use them for degradation studies (Mahadevan *et al.*, 1987) or to obtain commercially available proteases of microbial origin (Poos *et al.*, 1985; Pichard and Van Soest, 1977). Currently, there are no rumen proteases commercially available and purifying rumen proteases is beyond the scope of this project.

Studies of protein degradation with non-rumen proteases (of microbial origin) monitored by the disappearance of the protein, may reveal information on the rate of degradation of protein in the rumen (Roe *et al.*, 1991). Commercial proteases of microbial origin are used as a standard procedure in feed evaluation laboratories to estimate feed degradation (Roe *et al.*, 1991; Krishnamoorthy *et al.*, 1983). The use of commercially available protease is also suitable for studying pure and soluble proteins. Thus, Pronase E and Neutrase were selected for proteolytic degradation studies in this work.

Pronase E (from *Streptomyces griseus*) is a broad spectrum protease that has both

exopeptidase and endopeptidase activity as do those proteases found in the rumen (Roe *et al.*, 1991). Neutrase (from *Bacillus subtilus*) is a protease with broad spectrum endopeptidic activity (Roe *et al.*, 1991). Neutrase was selected in this study since Assoumani *et al.* (1990) had reported that regression equations computed with Neutrase gave a highly significant correlation ($R^2 = 0.92$) with an *in vivo* nylon bag method. In their comparative studies of *in vitro* and *in situ* techniques for estimation of ruminal degradation of protein, Roe *et al.* (1991) also found similarly high correlation between the Neutrase and *in situ* methods.

The objective of this study was to predict MB-1's degradation in the rumen using commercially available enzymes, Pronase E and Neutrase. As was done in Chapter 3, comparative studies were also conducted using the natural proteins, cytochrome c, RNAse A and urease.

4.2 Material and Methods

4.2.1 Proteolytic enzymes

Pronase E (P-5147, type XIV *S. griseus* protease) was purchased from Sigma Chemical Co. (St. Louis, MO). Neutrase was a gift from Novo Nordisk Bioindustrials, Inc., Danbury, CT.

4.2.2 Buffer preparation

4.2.2.1 Borate-phosphate buffer

Borate-phosphate buffer was prepared by dissolving 7.60 g of sodium phosphate monohydrate (BDH Inc., Toronto, ON) and 13.17 g of sodium borate decahydrate (BDH Inc., Toronto, ON) in enough water to make up 1 L solution. The pH was adjusted to pH 6.8 using 0.1 N NaOH.

4.2.2.2 Citrate buffer

Sodium citrate buffer was prepared by dissolving 21.01 g of citric acid (BDH Inc., Toronto, ON) in water. The pH was adjusted to 6.5 using 200 mL of 1 N NaOH and the volume was then adjusted to 1 L using distilled water.

4.2.3 *In vitro* degradation by Pronase E

In the Pronase E studies, 1 mg each of MB-1, urease, cytochrome c and RNAse A were incubated in 160 μL of borate-phosphate buffer at pH 6.8. The solution temperature was equilibrated by incubating it in a 39°C bath for 15 minutes. Protease was added at a level of 6.6 units per g of sample protein. The reaction was allowed to proceed for two hr. Ten microlitre aliquots were withdrawn (each 20 minutes) and

added to 10 μL of loading buffer (2% w/v SDS). Samples were then heated for 5 minutes at 100°C to deactivate the protease and stored at -20°C.

4.2.4 *In vitro* degradation by Neutrase

In the Neutrase studies, 1 mg of each protein was dissolved in 160 μL of 0.1 M sodium citrate buffer and incubated for 15 minutes in a 39°C water bath. Neutrase was added at 9.6 units per g sample protein and 10 μL of samples were withdrawn each 10 minutes. These samples were added to 10 μL of loading buffer (2% w/v SDS), heated for 5 minutes at 100°C and then stored at -20°C.

4.2.5 SDS-PAGE

The thawed sample (10 μL) was loaded into SDS-PAGE, and electrophoresis was run as described in section 3.2 Gels were stained for 20 minutes using 0.25% Commassie blue (for natural proteins) and destaining was done (in 40% methanol and 10% acetic acid) overnight. For MB-1, staining with Commassie blue was not very successful therefore, silver staining was performed according to manufacturer's (Bio-Rad, Mississauga, ON).

4.2.6 Estimation of degradation

The amount of protein remaining undegraded was determined by measuring the density of each band on the gels using a densitometer Interpretation of the data was done by computing the percentage of initial crude protein that remained undegraded after each period of incubation for each enzyme. The results of duplicate test samples were averaged for analyses and a plot of percentage of undegraded protein versus time was constructed as described by Roe *et al.* (1991). Error bars were plotted by calculating the standard deviations of each test.

4.3 RESULTS

Some modifications of the original procedure were necessary for a number of reasons. The method which was developed by Krishnamoorthy *et al.* (1983) uses a larger protein sample (at least 0.5 g for each test). Because the current yield of MB-1 is very low (only 10 mg per litre of culture), a method that utilizes minimal amount of protein was required. As a result, gel electrophoresis was selected as a suitable method for these studies. The original method also suggested the use of time intervals up to 48 hr (0.5, 1, 2, 4, 8, 12, 24 and 48 hr), however, since the preliminary results indicated that MB-1 degraded in less than 2 hr, a modification of incubation time was required to facilitate the study. The results from each degradation experiment were plotted on the same graph for all proteins.

Figure 13 shows the rate of degradation of MB-1, RNAse A, cytochrome c and urease in the presence of Pronase E protease. In the first 40 minutes of the reaction, 70% of MB-1 was degraded. Experiments with natural proteins showed that 35% of cytochrome c, 30% of RNAse A and only 10% of urease degraded within 40 minutes. At the end of 2 hr, MB-1 was totally degraded wherease some percentages of the natural proteins were left undegraded.

Figure 14 shows the degradation of each protein by Neutrase. As shown in the graph, MB-1 degraded completely after 30 minutes. Experiments with the natural proteins showed that at the end of the 60 minutes, 55% of cytochrome c, 30% of urease and 10% of RNAse A were degraded. The results from these experiments indicated that the natural proteins were more resistant to degradation by Neutrase than MB-1.

4.4 DISCUSSION

In this study, the degradation of MB-1, RNAse A, cytochrome c and urease by two proteases of microbial origin was examined. In both cases, it was observed that MB-1 degraded faster than the natural proteins which may suggest that MB-1 has more exposed targets that are susceptible to protease attack.

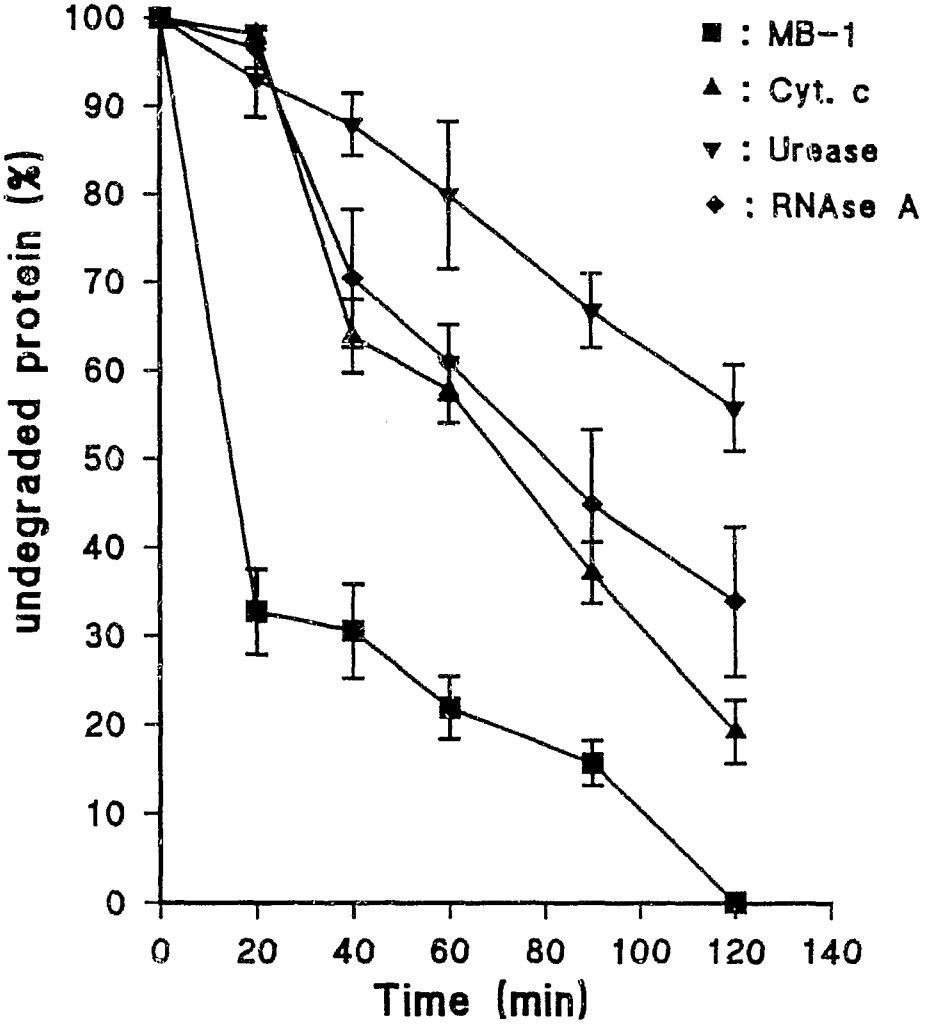

Figure 13. Protein degradation using Pronase E

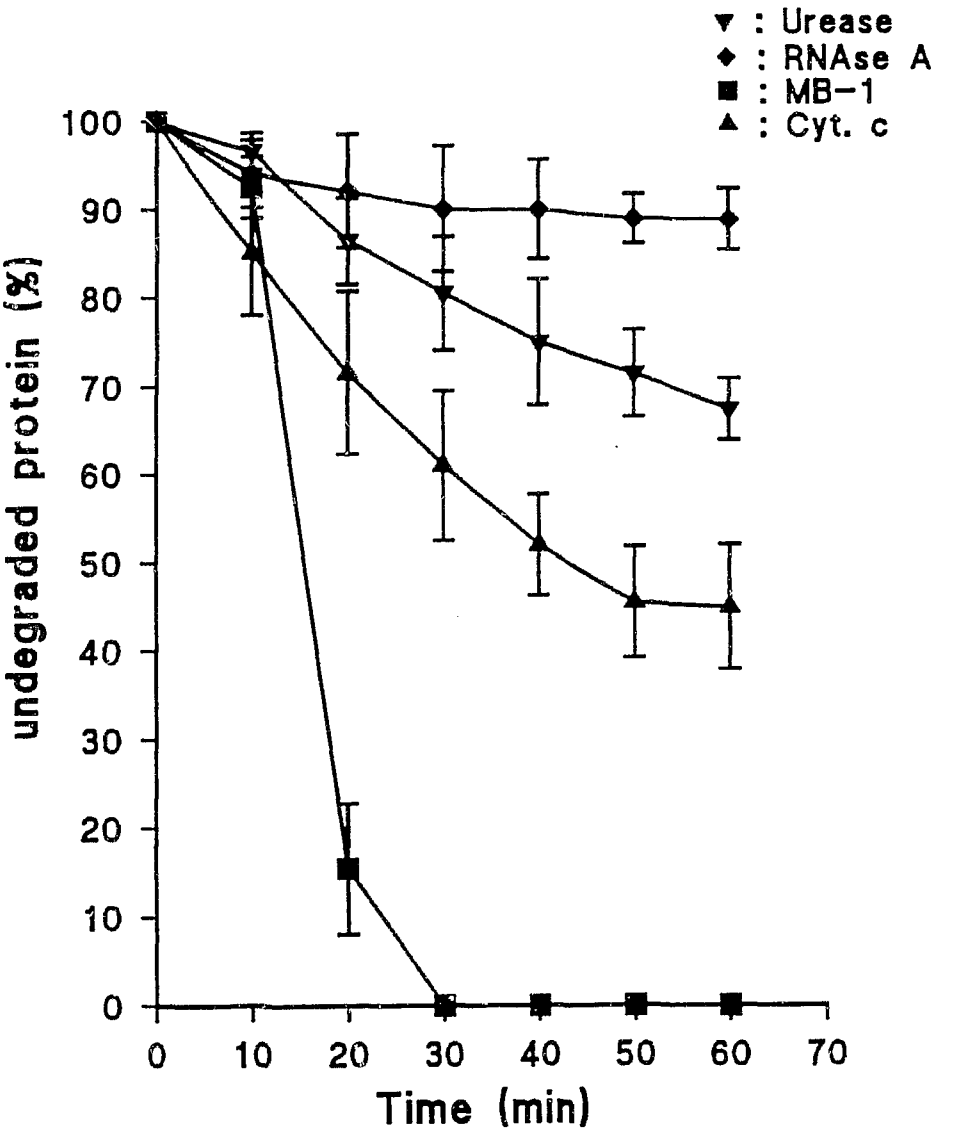

Figure 14. Protein degradation using Neutrase

From the earlier studies on thermal denaturation (Chapter 3), it was evident that the structure of MB-1 was folded and stable at 39°C and this may minimize its degradation. Thus, other factors may have influenced its proteolytic stability.

The rapid degradability of MB-1 may be explained as follows. Because of the wealth of information available on *Eschericia coli* proteases, MB-1 was designed to be stable in this bacteria (Beauregard *et al.*, 1995). To accommodate this, MB-1 was designed by avoiding potential *E.coli* targets such as aromatics and paired basic residues (Goldberg and Goff, 1986; Gottesman, 1989). Thus, no consideration was taken for the variety of microbial proteases such as those found in the rumen. In fact there is only little information available on their targets. As a consequence, MB-1 was not resistant to the proteases used in this study. Further explanation for the rapid degradation of MB-1 may be due to its high solubility. Solubility is generally assumed to be directly related to degradation of proteins by rumen microorganisms (Crooker *et al.*, 1978). Therefore, the highly soluble nature of MB-1 could have been positively correlated to its degradation.

Throughout these experiments, RNAse A, urease and cytochrome c showed the most resistance to protease attack. RNAse A has four disulphide bonds (Anfinsen, 1960), urease contains 1 disulphide bond per subunit (Riddles *et al.*, 1983) and cytochrome c also contains 1 disulphide bond. The presence of these linkages could be the major contributing factor for their high stability. This fact is further supported by Mahadevan *et al.* (1980) who suggested that the presence of these linkages have a

stabilizing effect on proteins.

This study has indicated that MB-1 is highly degradable which means that overprotection (which results in lower digestibility) would not be a problem. However, since MB-1 is degraded faster than the unprotected proteins studied here, it can not be used as a feed additive. Future consideration of MB-1 would have to include structural modification to make the protein less susceptible to protease. The summary of future directions will be the topic of the next chapter.

Chapter 5: SUMMARY AND CONCLUSION

In brief restatement of the purpose of the research, our group initiated a project to increase the nutritional quality of protein available to dairy cattle. In dairy cows, most of the protein available to be digested by the animals comes not from the feed but from microorganisms in its rumen (Hungate, 1966). Crops and rumen bacterial protein are however not high quality proteins and may be insufficient to meet the essential amino acid demands of lactating cows (Chalupa, 1975). The result of this is deficiency of certain amino acids such as methionine, threonine, lysine and leucine and a lesser deficit in other amino acids (King *et al.*, 1990). Naturally occurring proteins rich in these essential amino acids are rare. As a consequence, the cow has a limited pool of essential amino acids for high quality milk production. Thus, the particular interest of our laboratory was to design and produce an essential amino acid enriched protein to be used as a potential feed additive.

Recent advances in protein engineering have made the design of *de novo* proteins a reality. A protein can be "tailor-made" to have specific properties. Because the nutritional value of a protein depends on its essential amino acids composition, the quality can be changed by altering its primary structure. Therefore, protein engineering can offer the possibility of tailoring proteins to meet specific requirements.

Through recent advances in the knowledge of three-dimensional structure of natural proteins, the folding rules and restriction are beginning to emerge. *De novo* proteins have been designed using these folding rules, expressed in bacteria and have been characterized. Thus, it is feasible to encode information in the primary structure that promotes a given protein fold. Previous attempts to synthesize essential amino acids enriched proteins resulted in either unstable polypeptides with no tertiary structure (Jaynes *et al.*, 1985; Doel *et al.*, 1980) or polypeptides which are insolubie. These insoluble peptides were low in digestibility and were potentially toxic (Boebel and Baker, 1982).

Milk bundle-1 (MB-1) is a *de novo* protein designed to adopt a stable tertiary fold in spite of an artificially high content in four essential acids (Beauregard *et al.*, 1995). MB-1 was designed to resemble natural proteins, consisting of 15 types of amino acids which could have the potential of being successful in animal feeding.

Before MB-1 could be used as a feed additive, certain aspects of its stability had to be investigated. For a protein to survive the harsh environment of the rumen, its physical as well as proteolytic stability had to be assessed. In this work, both aspects were investigated in order to understand how MB-1 would behave in certain conditions. The physical stability studies were conducted to address the issue of protein folding in the rumen-like environment. The proteolytic degradation studies were conducted to estimate the degradation of MB-1 by rumen proteases.

These studies were restricted by two factors. Firstly, the rumen fluid obtained from fistulated cows hindered the possibility of stu ;ing folding stability by fluorescence spectroscopy technique. Because rumen buffer contains pigments, proteins and microbes, MB-1 could not be selectively studied. Thus, an artificial buffer that resembles the rumen fluid in terms of its pH, ionic strength and volatile fatty acids was prepared. The second restriction was that proteolytic degradation studies could not be conducted using rumen proteases due to the fact that there were no purified rumen proteases available on the market. Thus, the studies were done with non rumen proteases of microbial origin according to methodology currently used in feed evaluation laboratories. To our knowledge, this is the first time that a synthetic protein (intended for nutrition) has been investigated for both its folding and proteolytic degradation.

Fluorescence measurements were conducted to find the melting temperature of the proteins. Melting temperature indicates the temperature at which MB-1 will be half folded and half unfolded. Thermal stability studies of MB-1 has indicated that this *de novo* protein stays folded up to 50°C which is well above the rumen temperature. The presence of physiological level of volatile fatty acids (at pH 6.5) had no impact on MB-1's stability. Furthermore, under these conditions, MB-1 showed denaturation characteristics similar to the natural proteins studied here.

The detection of a denaturation transition of MB-1 indicates that MB-1 may be close

to a "natural" native state. Information on other synthetic proteins that had shown such denaturation transition has not been previously reported in the literature. This implies that MB-1 is the first *de novo* protein (intended for nutrition) which has been successfully produced and shown to have some true characteristics of a protein.

Proteolytic studies using protease of microbial origin indicated that MB-1 is very susceptible to rumen degradation. An important factor that should be noted is that MB-1 was designed by considering the protease targets in *E.coli* only (Beauregard *et al.*, 1995). If MB-1 is going to be successful as a feed additive, future work should consider identifying other protease targets. The first step towards this goal is to separate the cleaved peptide fragments using techniques such as reversed-phase chromatography and then identify the amino acid sequence of the end fragments. The information that is obtained from this will then be used to modify the cleavages sites of MB-1.

The proteolytic stability of MB-1 could also be improved. Firstly, MB-1 has to be protected to decrease its solubility by putting some hydrophobic residues on external side of helices. This would mean slightly changing the amino acid composition for example by adding more methionine (hydrophobic) residues. Secondly, introducing disulphide bridges to MB-1 could decrease its susceptibility to proteases. Disulphide bridges are formed between the side chains of two cysteine residues (Creighton, 1993) and have stabilizing effects (Mahadevan *et al.*, 1980). Thus, redesigning of MB-1

should include the incorporation of disulphide bridges (by inserting at least two cysteine residues at the proper end orientation).

The ultimate goal of the project will be to make MB-1 available as a high quality protein feed supplement. Apart from stabilizing the protein itself, several options have to be considered in the future. One option under current investigation by Agriculture and Agri-Food Canada is to introduce a recombinant rumen bacteria carrying MB-1 gene into the rumen population. The population of recombinant bacteria will then produce MB-1 intracellularly. A portion of this population, like every other rumen bacteria, will be digested by the cow and the intracellular protein will be made available to the cow. Substantial progress has been made in this respect and the introduction of MB-1 gene in the rumen bacteria vector will be possible soon. A second option could be introducing the MB-1 gene into a plant. MB-1 would have to be modified to promote the formation of aggregates probably by insertion of additional methionine residues. Feeding the animal with these new crops could be an effective means of providing dairy cows with this essential amino acid-enriched protein.

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