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The role of noncovalent forces in the micellization phenomenon using the globulin, legumin, as a study system.

BY

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THE ROLE OF NONCOVALENT FORCES IN THE MICELLIZATION PHENOMENON USING THE GLOBULIN, LEGUMIN, AS A STUDY SYSTEM

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ABSTRACT

The mild isolation technique developed in this study included solubilization of globulins in 0.5 M NaCl (pH 7.0), selective denaturation of the storage globulin vicilin followed by hydrophobic precipitation of legumin. Contaminating phenolic compounds were removed by gel filtration. Homogeneity of the protein fraction was established with isoelectric focusing. In terms of physicochemical parameters, the legumin was a multimer (M.W. 363,000; pI = 4.7) formed by the noncovalent association of two main subunits with molecular weights of 54,000 and 46,600 as determined by SDS-polyacrylamide gel electrophoresis. Under reducing conditions, two main subunits with molecular weights of 39,200 and 17,300 were apparent. The average hydrophobicity (819.46 cal/ A.A. residue) and the charge frequency (0.46) were determined for legumin from amino acid data.

The isolated legumin interacted to form micelle structures and elaborate protein networks under different environmental conditions. The role of noncovalent forces in this micellization phenomenon was investigated with environments varying in pH, electrolyte composition and the level of denaturant. Several molecular parameters were correlated with observed micelle interaction patterns; these included thermal properties as indicators of protein stability and surface hydrophobicity as an assessment for the potential of hydrophobic interactions. The optimal pH for micelle formation ranged from 5.5 to 6.5, values at which electrostatic repulsions were minimal and surface hydrophobici-

ties were adequate to allow attractive hydrophobic interactions.

The micelle response was affected by the anionic environment both in terms of concentration and identity of the anion. At lower concentrations (μ = 0.25 and 0.5), the exposure of a specific level of hydrophobic residues appeared critical. Exaggerated hydrophobicity values, as observed with the citrate anion, were detrimental to the micelle response. At higher concentrations (μ = 1.0), increased binding of the anion (chloride, acetate, thiocyanate) to the protein surface created an isoelectric situation unfavorable to the micelle phenomenon.

Gradual denaturation of legumin by increasing urea concentrations had a negative impact on the micelle response. This was attributed to decreasing surface hydrophobicity values. In all cases, a delicate hydrophobic-hydrophilic balance at both intra- and intermolecular levels appeared critical to micelle formation and subsequent interaction. The most appropriate environment for micelle formation was one in which legumin electrostatic repulsion was minimized to allow hydrophobic association.

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INTRODUCTION

The rapidly growing world protein requirement has directed attention to the use of plant seed proteins as alternatives to protein from animal sources. Refinements in protein recovery processes have overcome many of the past problems in the use of plant seed proteins as food. Finding appropriate uses for these isolates depends upon the functionality of the protein involved. Protein functionality in a food system is often related to the ability of the protein molecules to interact among themselves and with other compounds making up the food structure. Although the importance of such interactions is recognized, the exact mechanisms involved are poorly understood.

The purpose of this study was to establish a model protein system, containing one pure native protein, and to study specific noncovalent forces important to the self-association of this protein under different environmental conditions. The protein chosen for the present study was legumin, a seed storage globulin from Vicia faba var Diana. In the purification scheme for this protein, an initial protein isolate was prepared using a mild extraction procedure developed by Murray et al. (1978). This involved initial stabilization of the storage globulins in 0.3 M NaCl followed by massive protein precipitation with the introduction of a specific volume of cold tap water. The precipitated protein was characteristically in the form of microscopic spheres or micelles. Analytically, this protein isolate, referred to as protein micellar mass (PMM) contained approximately 96% protein

(N x 5.85) plus minimal traces of ash (Murray et al., 1981).

Using PMM as a starting material, a procedure was developed to isolate large quantities of the single protein, legumin. In addition to high yields, the method developed required minimal protein manipulation ensuring a product in its native form. The isolated legumin was subsequently characterized in terms of molecular weight, isoelectric point, subunit structure and amino acid composition.

LITERATURE REVIEW

Legumin Isolation

The seed of any plant is produced with a view to the future and although it contains a variety of functionally necessary proteins (e.g. zymogens, enzymes, inhibitors etc.) these represent but a small portion of the total protein packed into the seed. In the case of fababeans (Vicia faba), Boulter (1970) reported that two storage proteins, legumin and vicilin, make up 90% of the total seed protein. These proteins are called storage proteins since they are laid down at one stage of the life cycle for future use at a metabolically more active stage.

The idea to isolate and purify storage seed proteins such as vicilin and legumin is not new. Osborne and Campbell (1898) used salt precipitation plus repeated dilution and dialysis to separate globulins of legume seeds into the two major protein fractions, legumin and vicilin. Danielsson (1949) established a new procedure, now described as classical, to isolate a globulin fraction from Pisum sativum using protein solubility characteristics to achieve the separation. Legumin and vicilin, as globulins, are soluble in dilute salt solutions and are insoluble in water.

In Danielsson's (1949) method, legumin and vicilin were first extracted with sodium chloride and then precipitated with ammonium sulfate (70% saturation) to remove nonproteinaceous material. The resulting precipitate consisted of legumin and

vicilin, plus some contaminants. Gradual removal of salt by dialysis against water resulted in the precipitation of legumin and vicilin and the solubilization of other contaminating proteins (Bailey and Boulter, 1970). Although the method was simple, fractionation was usually incomplete and it was necessary to undertake further purification steps to achieve separation of legumin and vicilin. As a modification of this method, sodium borate at low ionic strength and alkaline pH was used to initially solubilize the proteins (Matta et al., 1981).

For further purification of the crude globulins, precipitation can be carried out by several procedures (Derbyshire et al., 1976). The most successful method of purifying legumin (11S protein) has been isoelectric point precipitation (Danielsson, 1949) leaving the other storage protein, vicilin (7S protein) in solution. For complete separation, this method must be repeated several times. Except for the time consuming factor the major disadvantage of this method was that some of the precipitated proteins would not redissolve when the pH was readjusted to the original value (Scholz et al., 1974). The formation of disulfide-bridged polymers has been connected with this phenomenon (Nash et al., 1971).

A modification of this method, termed zonal isoelectric precipitation, overcame these disadvantages. Zonal precipitation is a chromatographic procedure using a Sephadex column eluted with an ammonium sulfate gradient. The first approach in applying this method to seed proteins from legumes was made by Shutov and

Vaintraub (1966). They took advantage of the difference in the isoelectric points of vicilin (pH = 5.5) and legumin (pH = 4.8), replacing the ammonium sulfate salt gradient by a pH step between 5.0 and 7.9. Legumin was retarded in the column by its insolubility at pH 5.0 and eluted with the buffer at pH 7.9. This technique afforded relatively pure, readily solubilized legumin preparations, but the vicilin fraction obtained by this method was contaminated with legumin and other impurities (Derbyshire et al., 1976).

Studies on seed storage globulins indicated that legumin was more easily isolated from protein fractions than vicilin. Simard and Boulter (1978) isolated legumin (11S) from vicilin (7S) using a simple method based on the differential precipitation of these two in different salt environnments. Various concentrations of ammonium sulfate were used. They eventually succeeded in the isolation of legumin by precipitation in 60-70% saturated salt solution. In contrast, vicilin was precipitated in 70-85% saturated ammonium sulfate. Although legumin was well separated from vicilin, the legumin fraction was contaminated by other 16-17S proteins (Casey, 1979).

Immunoaffinity chromatography was also considered as a potentially rapid and efficient purification method (Derbyshire et al., 1976). The antiserum used as a source of IgG was raised in response to crude legumin; monospecific antibodies were purified from this by affinity chromatography on a legumin-Sepharose column. This approach had the disadvantage that an initial amount of pure legumin was required.

Theoretically legumin (molecular weight approximately 350,000, sedimentation coefficient 11S) can be separated from vicilin (molecular weight 180,000, sedimentation coefficient 7S) by gel filtration. However, experimental results showed incomplete separation of these two proteins (Derbyshire et al., 1976). The main reason for this ineffectiveness was that although the differences in molecular weights and sedimentation coefficients exist, both vicilin and legumin have similar Stokes' radii (132 A and 185 A, respectively) (Murray et al., 1981). They also have a tendancy to associate under certain environmental conditions (Ismond, 1984). In spite of these factors, gel filtration can still be used to remove low molecular weight proteins and nonproteinaceous contaminants such as phenolics during the protein purification procedure (Ismond, 1984).

Physicochemical Properties

Molecular Weight of Legumin from Vicia faba

There have been many physicochemical studies of purified legumin from a variety of plant seeds. Danielsson (1949) reported a molecular weight for legumin of 331,000 based on the measurement of the sedimentation coefficient and the diffusion coefficient in the ultracentrifuge. Since that time, higher values in the range of 390,000 to 420,000, depending on the legume source, have been reported with similar ultracentrifugal analyses (Casey, 1979). On the other hand, analytical gel filtration was used to

estimate the Stokes' radius (185 A) of legumin from Vicia faba (Murray et al., 1981). From that result, the approximate molecular weight of legumin was estimated to be about 330,000. Molecular weight values of legumin isolated from different plant seeds differ widely (Derbyshire et al., 1976).

Subunit Profile

Maplestone et al. (1985) extracted legumin from Vicia faba seeds and found that it was an oligomeric protein with a molecular weight of 328,000. The subunit profile of this complex protein has been investigated in various electrophoretic studies (Wright, 1973). In general, legumin appears to be composed of six pairs of subunits, each pair consisting of one "acidic" α (MW ca. 36,000) and one "basic" β (MW ca. 20,000) subunit linked by disulfide bonds (Wright, 1973). Within each subunit class, heterogeneity existed i.e. the α subunits contained two N-terminal amino acids, leucine and threonine, whereas the N-terminal amino acid sequence of the β subunits was variable (Derbyshire et al., 1976). In addition, β subunits showed multiple bands in different gel electrophoretic systems. Comparison of the SDS-PAGE patterns of reduced and nonreduced legumin by Wright (1973) indicated that two major components of molecular weights 49,500 and 50,000, found only in the absence of reducing agents, represented products of disulfide bond formation between α and β subunits. It is likely that the disulfide bonds existed in the legumin molecule itself, since random disulfide bond formation as a result of

disruption of secondary and tertiary structure by sodium dodecyl sulfate would have produced a whole range of molecular weight species (Utsumi and Mori, 1983). The molecular weights of these "intermediary subunits" can only be explained by postulating a structure in which one α and one β subunit are combined. The discrepancy in the apparent molecular weight of these proposed β intermediary subunits (49,500 and 50,000) and the combined molecular weight of an α and $\alpha\beta$ subunit (57,900 - 60,800 according to which β subunit is considered) probably reflects the increased secondary and tertiary structure of the intermediary subunit, produced as a result of disulfide bonding (Wright, 1973). The presence of inter- and intramolecular disulfide bonds in proteins has been shown to affect their electrophoretic mobilities in sodium dodecyl sulfate gels and thus can lead to erroneous estimates of molecular weights (Utsumi and Mori, 1983).

The subunit profile of legumin has been also investigated by isoelectric focusing (Gatehouse et al., 1980). The "basic" and "acidic" subunits of legumin from Pisum sativum were separated on a preparative scale by ion exchange chromatography using 50% v/v formamide as a dissociating medium. Formamide (50%) was also used as a dissociating factor for an analytical isoelectric focusing procedure. The isoelectric point values for the "acidic" subunits were from 5.0 to 5.3; those for the "basic subunits" were from 8.3 to 8.7. Both types of subunits were shown to be heterogeneous in charge and molecular weight by two dimensional analysis employing isoelectric focusing in the first dimension and SDS-PAGE in the second (Gatehouse et al., 1980).

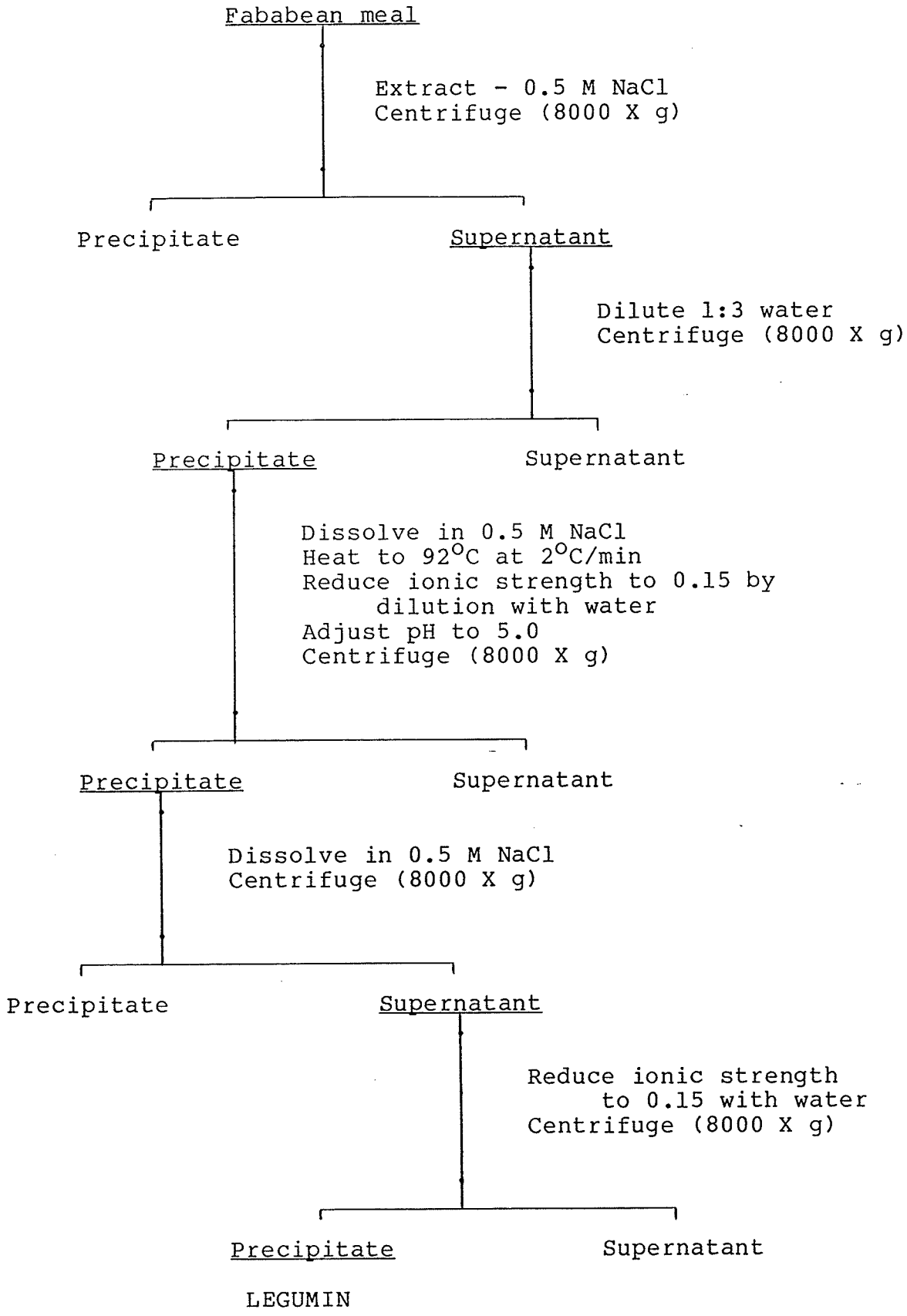
MATERIALS AND METHODS

Isolation and Purification of Legumin

A protein concentrate was prepared by air classification of dehulled pin milled fababeans (Vicia faba L. var. Diana) From this protein concentrate, the storage protein legumin was isolated using a modification of the methods of Dalinenko et al. (1985) and Murray et al. (1978) as summarized in Figure 1.1. Fababean meal was extracted for 30 minutes at 37°C with a ratio of 1 part meal to 10 parts sodium chloride (0.5 M). The suspension was centrifuged at 8,000 x g for 15 minutes in a RC2-B Sorvall refrigerated centrifuge at 4°C. The supernatant was diluted with 3 volumes of cold (4°C) tap water in order to precipitate the globulins. To remove these massively precipitated globulins, the suspension was centrifuged at 8,000 x g for 15 minutes. The precipitated proteins were referred to as a protein micellar mass (PMM) by Murray et al. (1978). This protein isolate, PMM, is predominantly composed of two structural proteins, legumin and vicilin (Murray et al., 1981) plus some contaminating phenolics (Ismond, 1984). The subsequent steps in the purification procedure were designed to selectively remove both vicilin and the phenolics from the legumin fraction.

In order to continue the purification, the PMM was redissolved in 0.5 M sodium chloride, pH 7.0, and stirred for 30 minutes at room temperature. The resulting suspension was heated at a constant rate of 2°C per minute from 22 to 92°C in a Haake-C

Figure 1.1. Isolation scheme for legumin from Vicia faba.


Fababean meal
Extract - 0.5 M NaCl
Centrifuge (8000 X g)
Precipitate
Supernatant
Dilute 1:3 water
Centrifuge (8000 X g)
Precipitate
Supernatant
Dissolve in 0.5 M NaCl
Heat to 92°C at 2°C/min
Reduce ionic strength to 0.15 by dilution with water
Adjust pH to 5.0
Centrifuge (8000 X g)
Precipitate
Supernatant
Dissolve in 0.5 M NaCl
Centrifuge (8000 X g)
Precipitate
Supernatant
Reduce ionic strength to 0.15 with water
Centrifuge (8000 X g)
Precipitate
Supernatant
LEGUMIN

water bath controlled by a Haake PG-20 automatic digital system. Reduction of the ionic strength (0.5 initially) of the suspension to 0.15 with cold (4°C) water and the adjustment of the pH to 4.7 with 0.1 N hydrochloric acid resulted in massive protein precipitation. The precipitate was then recovered by centrifugation at 8,000 x g for 15 minutes. The residue was dissolved in 20 volumes of 0.5 M sodium chloride, pH 7.0, and centrifuged at 8,000 x g for 15 minutes. Legumin was then precipitated using a sudden reduction of ionic strength by the addition of 3 volumes of cold (4°C) water. The legumin precipitate was collected by centrifugation at 8,000 x g for 15 minutes. The resulting protein isolate was freeze dried and stored at 4°C.

No evidence of vicilin contamination in legumin preparations was detected during subsequent analyses by isoelectric focusing. Contaminating phenolics were removed by gel filtration (Ismond, 1984). Lyophilized legumin (3 g) was dissolved in 0.5 M sodium chloride, pH 7.0. The solution was applied to a Pharmacia K26-100 gel filtration column containing Sephacryl S-300 as a matrix. A downward flow rate of 30 mL/h of the eluting solution [0.5 M sodium chloride, pH 7.0, with 0.02% (w/v) sodium azide] was maintained by an LKB Microperpex peristaltic pump. Fractions of 80 drops per tube (approximately 3.75 mL) were collected with an LKB 2112 Redirac fraction collector. An ISCO dual beam monitor followed the absorbance of the eluant at a wavelenth of 280 nm. The resulting chromatogram was characterized by two peaks, with the second corresponding to legumin. The first broader peak contained nonproteinaceous material as determined by a modified

Lowry procedure for the assay of proteins in the presence of interfering materials (Bensadoun and Weinstein, 1976). The eluant corresponding to legumin was dialysed against distilled water for 24 h. The resulting protein isolate was freeze dried and stored at 4°C for further use.

Physicochemical Characterization

Isoelectric Point Determination by Isoelectric Focusing

Isoelectric focusing on polyacrylamide gel was performed according to the method of Winter et al. (1977). Prior to isoelectric focusing, protein samples (approximately 1 mg/mL in 0.5 M sodium chloride, pH 7.0) were dialysed against 0.01 M sodium citrate, pH 8.0, in order to reduce the ionic strength as much as possible. Isoelectric focusing was carried out with an LKB 2117 Multiphor apparatus and an LKB 2197 Constant Power supply. A Haake D1 circulating water bath was used to control the temperature at 10°C. An ampholine Pag Plate (LKB) polyacrylamide gel with a potential pH range of 4.0-6.5 was used for the focusing. The anode electrode solution was 0.1 M glutamic acid in 0.1 M phosphoric acid and the cathode electrode solution was 0.1 M l-alanine. Legumin samples and Pharmacia narrow range protein standards (10 uL; Table 1.1) were applied near the anode. The gel was focused at a constant power of 30 W. After 2.5 h, the gel was fixed, destained, stained at 60°C and destained (Table 1.2). Gels were photographed after two days of destaining. A calibration

Table 1.1. Isoelectric points of protein standards used for isoelectric focusing.

Protein	pI (24±1.5°C)
Amyloglucosidase	3.50
Glucose oxidase	4.15
Soybean trypsin inhibitor	4.55

Table 1.2. Staining procedure for isoelectric focusing.

Solution	Time	Solution composition
Fixative	1 h	17.3 g sulfosalicylic acid (Fisher Chem. Co.), 57.5 g trichloroacetic acid (Fisher Chem. Co.), 500 mL distilled water.
Destaining	5 min	500 mL ethanol, 160 mL acetic acid, distilled water to make 2000 mL.
Stain	20 min at 60°C	0.460 g Coomassie Brilliant Blue R-250 (Sigma Chem. Co.) 400 mL destaining solution. Stain filtered through Whatman No. 1 filter paper.
Destaining	1-2 days with frequent solution changes.	As given above.
Preserving	1 h, after destaining.	300 mL ethanol, 100 ml acetic acid, 100 mL glycerol, 500 mL distilled water.

curve for the relationship between distance of migration from the anode and isoelectric point of the standards was established (Appendix I). The isoelectric point was extrapolated from this relationship.

Molecular Weight Determination by Gel Filtration

A lyophilized sample of legumin and a mixture of molecular weight markers (Bio-Rad; Table 1.3), were dissolved in 0.5 M sodium chloride, pH 7.0. This solution (3 mL; 36 mg/mL) was applied to a Pharmacia K26-100 gel filtration column containing Sephacryl S-300 as a matrix. A downward flow rate of 30 mL/h of the eluting solution [0.5 M sodium chloride, pH 7.0, with 0.02% (w/v) sodium azide] was maintained by an LKB Microperpex peristaltic pump. Fractions of 80 drops per tube (approximately 3.75 mL) were collected with an LKB 2112 Redirac fraction collector. An ISCO dual beam monitor followed the absorbance of the eluant at a wavelenth of 280 nm. The molecular weight of legumin was estimated from the linear relationship between log molecular weight and elution volume of the standard proteins (Appendix II).

Subunit Analysis with SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to approximate the molecular weight of legumin subunits. This technique was applied according to the method

Table 1.3. Molecular weights of protein standards for calibration of the gel filtration column.

Protein	Molecular weight	Reference
Vitamin B-12	1,350	Merck Index, 10th Edition
Myoglobin (horse)	17,000	Schellenberg (1967)
Ovalbumin (chicken)	44,000	Edsell (1953)
Gammaglobulin (bovine)	158,000	Merck Index, 10th Edition
Thyroglobulin (bovine)	670,000	Steiner *et al*. (1961)

of Fehrustrom and Moberg (1977). Legumin samples (2 mg/mL) in 0.5 M sodium chloride, pH 7.0, were dialysed prior to electrophoresis, against 0.01 M phosphate buffer, pH 7.1 containing 1.0% (w/v) SDS. After dialysis the samples were heated in a 100°C water bath for 2 minutes. The reducing agent 2-mercaptoethanol, 1% (w/v) (Aldrich Chem. Co.), was included in the original buffer of some samples. Each sample (250 uL) was mixed with 10 uL of bromophenol blue dye (0.25% w/v in phosphate buffer). The polyacrylamide gel (Table 1.4) was preelectrophoresed for 30 minutes at 150 mA. After preelectrophoresis, 10 uL of the sample plus specific protein standards (Table 1.5) were applied onto the horizontal gel slab of an LKB 2117 Multiphor apparatus with an LKB 2197 Constant Power supply. Temperature was controlled at 10°C by means of a Haake-D1 water bath. Electrophoresis was carried out at 10 mA for 10 minutes followed by 195 mA for 4.5 h. The gel was placed in the fixative solution (Table 1.6) immediately after electrophoresis to minimize diffusion effects. It was then stained with Coomassie Brilliant Blue R-250 stain solution for 2 h, destained and preserved (Table 1.6). The following measurements were made from the stained gel: a) height of the gel before fixing and after drying, b) distance of migration of the protein band, and c) distance of migration of the bromophenol blue dye. This information was used to calculate the relative mobility:

$$Rf = \frac{\text{distance of the protein migration X length before fixing}}{\text{length after drying X distance of dye migration}}$$

From the linear relationship of relative mobility as a function

Table 1.4. Composition of polyacrylamide gel for SDS-PAGE.

Solution	Volume of solution for a final acrylamide concentration of 7.5%
Distilled water	7.5 mL
Buffer stock solution [1]	33.0 mL
Acrylamide solution [2]	22.2 mL
Ammonium persulfate [3]	3.2 mL
N,N,N',N' tetramethyl ethylene diamine (TEMED)	0.1 mL

[1] Buffer stock solution (0.2 M, pH 7.1): 39.0 g $NaH_2PO_4 \cdot H_2O$ to 258.0 g $Na_2HPO_4 \cdot 12H_2O$. Adjust to 5.000 L with distilled water.

[2] Acrylamide solution: 22.2 g acrylamide (Eastman Kodak Co., Enzyme Grade), 0.6 g bisacrylamide, adjust to 100 mL with distilled water.

[3] Ammonium persulfate solution: 15 mg/mL in distilled water. Prepared fresh daily.

Table 1.5. Molecular weights of protein standards used for SDS-PAGE.

Protein	Molecular weight
Lysozyme	14,300
B-lactoglobulin (subunit)	18,400
Pepsin	34,700
Ovalbumin	45,000
Bovine serum albumin	66,200

Table 1.6. Staining procedure for SDS-PAGE using Coomassie Brilliant Blue.

Solution	Time	Solution composition
Fixative	1 h	17.0 g sulfosalicylic acid (Fisher Chem. Co.), 57.0 g trichloroacetic acid (Fisher Chem. Co.), 150 mL methanol, 350 mL distilled water.
Stain	2 h	1.25 g Coomassie Brilliant Blue R-250 (Sigma Chem. Co.) 227 mL methanol, 46 mL acetic acid, 227 mL distilled water. Stain filtered through Whatman No. 1 filter paper.
Destaining	Several days with frequent solution changes.	1500 mL ethanol, 500 mL acetic 3000 mL distilled water.
Preserving	1 h, after destaining.	300 mL ethanol, 100 ml acetic acid, 100 mL glycerol, 500 mL distilled water.

of log molecular weight of the standards, the molecular weights of the legumin subunits were determined (Appendix III).

Amino Acid Analysis

High performance liquid chromatography (HPLC) analysis of amino acid composition of the protein was used. Sample preparation, including hydrolysis and derivatization was performed according to the method of Bidlingmeyer et al. (1984). Legumin samples (0.5 to 10 µg) were dissolved in 0.1 N HCl (20 µg/mL). A sample volume corresponding to 0.1 to 5.0 µg was pipetted into a 50x60 mm tube; the tube was subsequently placed into a vacuum vial. The vial was then attached to the workstation (Waters) manifold and the solvent was removed under vacuum. After drying, 6 N HCl (1 mL) with liquid phenol (10 µL) was pipeted into the bottom of the vacuum vial. Following evacuation and sealing of the vacuum vial, samples were hydrolysed at 105°C for 48 h.

After hydrolysis, the sample was derivatized with phenylisothiocyanate (PITC). The derivitizing reagent, prepared fresh daily, consisted of ethanol, triethylamine (TEA), water and PITC (7:1:1:1). The PITC amino acids were prepared by adding derivitizing reagent (20 µL) to the dried samples and sealing them in vacuum vials for 20 min at room temperature. Following the derivitization period, the reagents were removed under vacuum using the workstation.

The chromatographic system (Waters Millipore Assoc.) con-

sisted of two pumps, M45 for aqueous solvent and M510 for organic solvent, and an M441 fixed wavelength ultraviolet (U.V.) detector (254 nm). A U6K manual injector was used for sample injection. The Pico.Tag column (15 cm x 39 mm) was maintained at 38± 1°C by a Waters temperature control module. The solvent system consisted of two eluents: an aqueous buffer (A) and 60% acetonitrile in water (B) (Bidlingmeyer et al., 1984). Chromatograms were plotted by a Shimadzu CR34 plotter.

Method for Tryptophan Determination

To avoid the loss of tryptophan during acid hydrolysis, tryptophan levels in legumin were assessed by the spectrophotometric method of Messineo and Musarra (1972) for the determination of free and bound tryptophan.

Protein Assay

In general, protein concentration of the various extracts was determined by the Lowry et al. (1951) method and the Coomassie method (Pierce Chem. Co., 1983) using bovine serum albumin (Sigma) as a standard.

RESULTS AND DISCUSSION

Legumin Isolation

Of a variety of plant protein sources considered, fababeans (*Vicia faba* var. *Diana*) proved to be a suitable protein source for this study because of the lack of a number of interfering substances [*i.e.* compounds that unnecessarily complicate the various analytical techniques employed (Murray *et al.*, 1978)].

In general, two main factors, temperature and ionic strength, were used in the isolation of the protein, legumin. Initially, fababean meal was extracted with 0.5 M sodium chloride, pH 7.0. Soluble globulins, legumin and vicilin, were solubilized in this medium due to the "salting-in" effect of the salt; other insoluble compounds remained in the pellet. The ionic strength of the suspension was reduced suddenly; as a result, the globulins precipitated through massive hydrophobic interactions to form a viscous, gelatinous protein micellar mass (PMM; Murray *et al.*, 1978). The isolated protein micellar mass (PMM) was composed primarily of the two storage proteins, legumin and vicilin (Murray *et al.*, 1981).

In order to separate the two proteins, the principle of selective denaturation was applied. The denaturation temperature of legumin (105^{o}C) is approximately 20^{o}C higher than that for vicilin (85^{o}C) when both proteins are exposed to 0.5 M NaCl (Arntfield *et al.*, 1986). With this in mind, the resolubilized proteins were heated at a constant rate of 2^{o}C/min up to a temperature of 92^{o}C. Under these conditions, vicilin was denatured

while legumin remained native. Both the native legumin and the denatured vicilin were precipitated in 0.15 M sodium chloride, pH 4.7. In addition to the low ionic strength, pH 4.7 is an approximation of the isoelectric point for legumin. At this point, the separation of native legumin from thermally denatured vicilin was easier due to the increased hydrophobic accessible surface area of the denatured molecules (Voutsinas et al., 1983). As a result, the legumin was selectively dissolved in 0.5 M sodium chloride; the denatured vicilin remained insoluble. To complete the purification, legumin was precipitated through hydrophobic associations caused by a sudden reduction of ionic strength.

Separation of legumin from contaminating phenolic compounds was based on the differences in their molecular weight. In this respect, gel filtration on a Sephacryl S-300 matrix, with 0.5 M sodium chloride, pH 7.0, as an eluting solution (Ismond, 1984) was used to purify further the legumin fraction.

A difficult parameter to establish for any protein fraction is the chemical purity of the isolated product. Basically two physical attributes of the protein molecules are exploited in purity studies, particle size and particle charge. In terms of the first paramter, the legumin isolated in this study showed a symmetrical peak during gel filtration. However, this is not adequate evidence to indicate homogeneity. On the other hand, the presence of a single distinct band during analytical isoelectric focusing of legumin at a pI value of 4.7 appeared to confirm the presence of a single protein. (Figure 1.2). If vicilin were

Figure 1.2. Isoelectric profile of isolated legumin.


4.70
4.55
4.15
3.50

present in the legumin sample, there would be an additional band at a pI of 5.3 (Ismond, 1984).

Determination of Isoelectric Point

In addition to purity assessment, isoelectric focusing was used to determine the isoelectric point of legumin from Vicia faba var. Diana prepared with the above method. Gatehouse et al. (1980) noticed that undissociated legumin could not be focused to its isoelectric point in polyacrylamide gels. The protein usually precipitated in the gel before it had reached its isoelectric point resulting in an ill-defined smear of material in the pH region from 5 to 6. However, these problems did not appear in the present study. Legumin focused as a single band at a pI value of 4.7. However, this band was broader than those of the standard proteins (Figure 1.2). In addition there was a band of precipitated protein at the origin. The pI value from this study agrees with those referred to by other authors (Derbyshire et al., 1976; Casey, 1979; Gatehouse et al., 1980) as shown in Table 1.7.

Determination of Molecular Weight

In addition to its use for purification, gel filtration was used for the approximation of the molecular weight of legumin. The value of 363,000 calculated from gel filtration data, agrees with values approximated by different authors (Table 1.7). Most of these authors estimate the molecular weight to be between 300,000 and 400,000 although others (Matta et al., 1981; Derby-

Table 1.7. Physicochemical characteristics of legumin from different *Vicia faba* cultivars.

MW	N[1]	Subunit MW range	pI	Reference
320,000	3	23,000-56,000	4.7	Bailey and Boulter (1970)
ND[2]	3	23,000-56,000	ND[2]	Wright and Boulter (1974)
380,000	6	18,000-40,000	4.7	Gatehouse *et al*. (1980)
300,000 to 400,000	7	21,000-58,000	4.6	Matta *et al*. (1981)
380,000	5-9[3]	30,000-80,000	ND[2]	Maplestone *et al*. (1985)
363,000	5	19,100-58,000	4.7	Present study

1. Number of subunits, nonreducing conditions.

2. Not determined.

3. Depends on the cultivar.

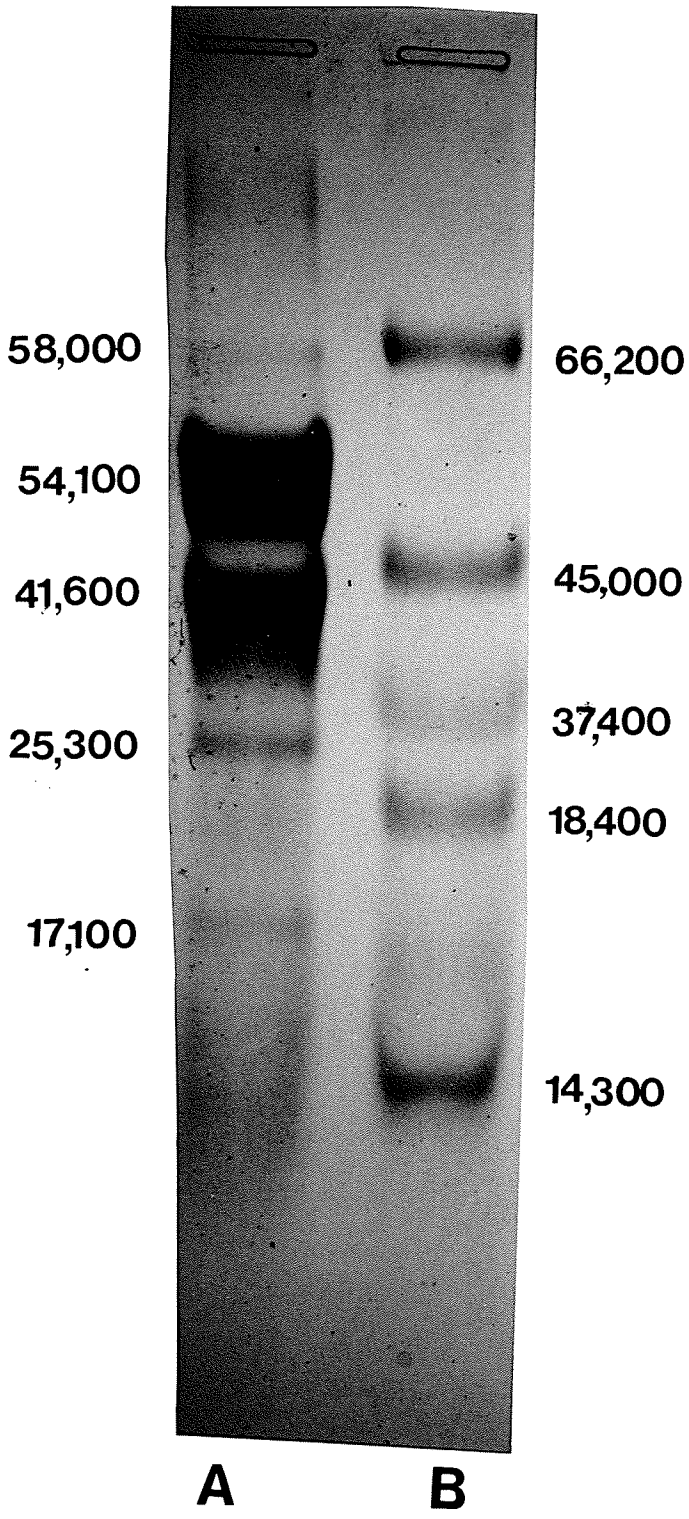
shire et al., 1976) refer to even higher values. This variety of results among different studies appears to be due to both different plant sources and different methods used for molecular weight estimation.

Subunit Determination

Subunit analysis of legumin with SDS-PAGE under nonreducing conditions revealed five subunits having molecular weights of approximately 58,000, 54,100, 41,600, 25,300 and 19,100 (Figure 1.3). These were not all present in equal amounts; the electrophoreogram was dominated by two major bands at 54,100 and 41,600. The currently accepted model of Vicia faba legumin is that each molecule is a hexamer consisting of six pairs of disulfide-bonded large (acidic,) and small (basic,) subunits of molecular weights approximately 37,000 and 20,000, respectively. The two major bands observed in this study may correspond to pairs of units. The variation in molecular weight of the combined structures may be the result of heterogeneity in the molecular weight of the large and small subunits (Wright and Boulter, 1974). In addition, the discrepancy between total molecular weight (eg. 41,600) and the individual subunits (37,000, 20,000) may be due to the presence of inter- and intrachain disulfide bonds. These may prevent complete unwinding of the nonreduced polypeptides resulting in an apparent molecular weight lower than the actual one (Matta et al., 1981).

The small bands at 25,300 and 19,100 in the nonreduced electrophoreogram may represent dissociated subunits. The band

Figure 1.3. SDS electrophoreograms of legumin (A) and protein standards (B) under nonreducing conditions.


58,000
54,100
41,600
25,300
17,100
66,200
45,000
37,400
18,400
14,300
A
B

observed at 58,000 may correspond to a 56,000 molecular weight subunit observed originally by Bailey and Boulter (1970).

Under reducing conditions with SDS-PAGE, three subunits were apparent. These had molecular weights of approximately 39,200, 28,300 and 17,300 (Figure 1.4). The major bands at 39,200 and 17,300 may represent individual α and β subunits. In addition, the remaining minor band may signify a small α unit (28,300). However, limitations of the technique followed (one-dimensional SDS-PAGE) do not permit the exact identification of the specific reduced substances which make up the nonreduced structures. Two-dimensional SDS-PAGE with isoelectric focusing in the second dimension may overcome this problem (Matta et al., 1981).

The final assembly of the different subunit pairs to form hexameric ($\alpha_6\beta_6$) legumin still remains an unresolved question (Horstmann, 1983). Various molecular species of legumin exist which differ in their subunit composition, but it is unknown whether all conceivable combinations of subunit pairs are possible or whether certain restrictions exist at the molecular level favoring distinct combinations.

Amino Acid Analysis

The amino acid composition of legumin is presented in Table 1.8. High contents of glutamic acid, aspartic acid and arginine are reflective of the protein's storage role (Derbyshire et al., 1976).

Figure 1.4. SDS electrophoreograms of legumin (A) and protein standards (B) under reducing conditions.

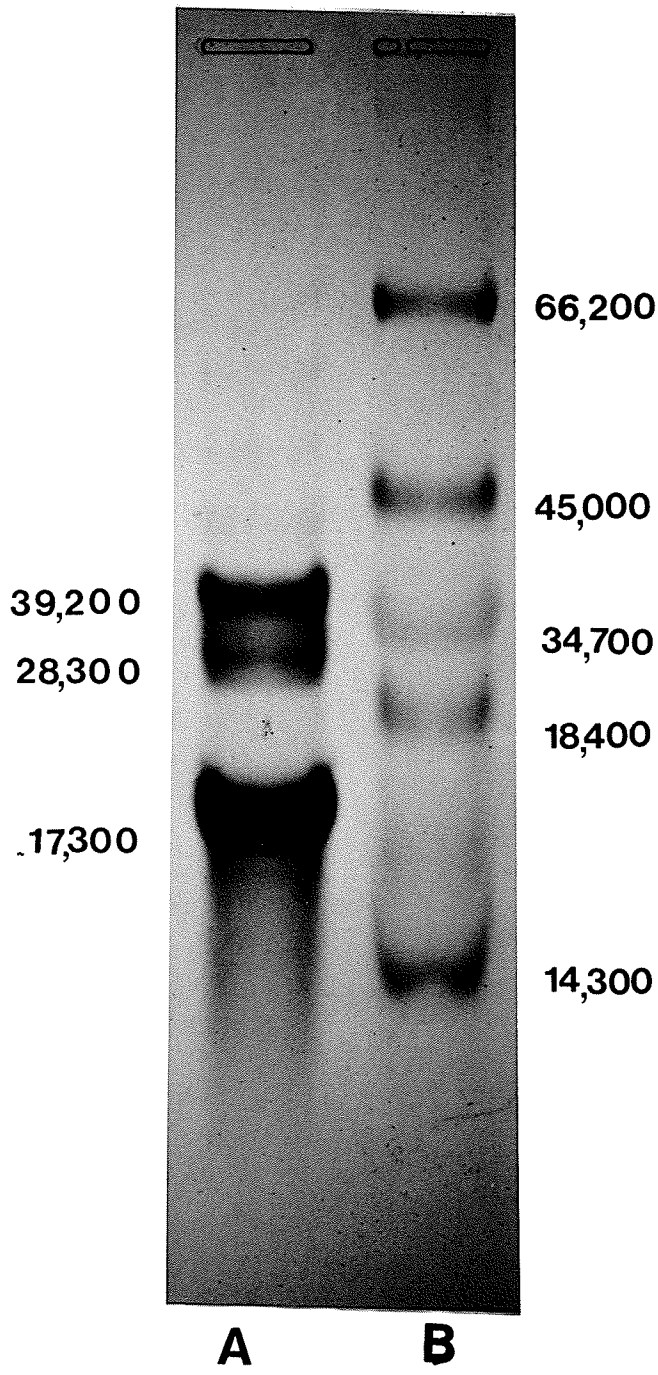

66,200
45,000
39,200
28,300
34,700
18,400
17,300
14,300
A
B

Table 1.8 Amino acid composition of legumin isolated from Vicia faba.

Amino Acid	Level (moles AA/100kg protein)
Glu	78.08
Asp	37.53
Ser	18.86
Thr	12.26
Arg	38.95
Lys	9.55
His	8.84
Tyr	11.58
Trp	0.36
Phe	10.99
Pro	20.54
Gly	16.92
Ala	15.71
Val	13.72
Ile	9.03
Leu	19.50
Met	1.66
1/2 Cystine	1.50

The amino acid data were used to calculate values for average hydrophobicity (HΦ), positive and negative charge potential plus frequency of charged groups. The calculation procedure for these parameters is as follows:

1. Average Hydrophobicity (HΦ)

$$H\Phi = \frac{\sum_{n=i} (\text{Moles of Amino acid})_i (\text{Amino acid hydrophobicity value})_i}{\text{Total Moles of Amino Acids/100 kg protein}}$$

$$= \frac{\text{Total Hydrophobicity}}{\text{Total Moles Amino Acids/100 kg Protein}}$$

Hydrophobicity values to calculate the total legumin hydrophobicity were determined by Bigelow (1967) from the transfer free energy (ΔFt) of the amino acid side chains as derived from Tanford (1973). The transfer free energy is defined as the free energy required to transfer one mole of amino acid from water to ethanol. Ethanol was considered to be representative of the hydrophobic interior of the molecule.

2. Charge Frequency

Bigelow (1967) also considered the frequency of charged groups within a protein - that is, the total number of charged residues considering complete ionization of all possible side chains. The charge frequency was defined as:

$$\text{Charge frequency} = \frac{\text{Negative + Positive Potential}}{\text{Total Moles of Amino Acid/100 kg Protein}}$$

where Negative Potential = Total moles of glutamic acid + aspartic acid - amide groups

and Positive Potential = Total moles of lysine + arginine + histidine.

In terms of these parameters, legumin isolated in this study

had a lower HΦ and a higher charge frequency than was originally anticipated (Table 1.9). Bigelow (1967), in a survey of more than 150 proteins, suggested that most globular proteins exhibited an HΦ within the narrow range of approximately 1000 to 1200 cal/amino acid residue and a charge frequency from 0.20 to 0.35. In order to determine if the low HΦ and high charge frequencies were similar to those for other legumin molecules, these values were calculated for legumin from five different cultivars of Vicia faba (Utsumi et al., 1980; Table 1.9). In this comparison, legumin from this study had a slightly lower HΦ and a higher charge frequency than legumin from the different cultivars. However all values calculated were outside the limits of globular proteins as suggested by Bigelow (1967). This would suggest that legumin in general is a less hydrophobic, more highly charged structure than was initially speculated in this study.

In summary, a relatively mild method has been developed to isolate an apparently homogeneous legumin fraction from Vicia faba. The legumin isolated was a multimer formed by the noncovalent association of two main subunits with molecular weights of 54,100 and 46,100. In terms of physical parameters, legumin was characterized by a molecular weight of 363,000 and an isoelectric point of 4.7.

Table 1.9. Total hydrophobicity, negative and positive potentials plus charge frequency as determined from the acid analysis of legumin from *Vicia faba*.

Vicia faba cultivar	H⓪ (cal/AA residue)	Negative potential[1]	Positive potential[1]	Charge frequency
Sanuki-Nagasaya[2]	876.63	226.56	109.79	0.38
S-N-Wase[2]	847.99	234.43	110.53	0.40
Gifu-Wase[2]	848.64	236.65	104.43	0.39
Kumamot-Churyu[2]	865.94	232.49	113.55	0.40
Issun[2]	862.52	211.44	180.02	0.38
Diana[3]	819.46	812.76	345.87	0.46

1. Moles/100 Kg protein.

2. Amino acid composition taken from Utsumi *et al*. (1980).

3. Present study.

CHAPTER TWO

IMPLICATIONS OF ENVIRONMENTAL MANIPULATION ON MICELLE FORMATION BY LEGUMIN

INTRODUCTION

With the recognition of micelle formation as a valuable method for the isolation of native protein (Murray et al., 1978) it becomes apparent that more in depth analysis of this type of protein association is warranted. In order to predict and possibly manipulate this protein response, the specific forces promoting intermolecular associations are of fundamental concern. A study system involving a single protein was established to assess the types of micelle related interactions that could occur in a variety of environments.

As discussed in the first chapter of this study the storage globulin legumin was isolated, purified and characterized. This protein was used as a study system for micelle assessment. The micelle forming capacity of legumin was investigated under different pH, electrolyte and denaturing conditions. The different types of micelles formed in these media were observed by light microscopy and grouped into subjective categories according to their microscopic characteristics. Several molecular parameters were correlated with observed micelle interaction patterns; these included thermal properties, as indicators of protein stability and surface hydrophobicity as an assessment of the potential for hydrophobic interactions. The ultimate goal was to show that the phenomenon of micellization can be predicted and possibly manipulated by controlling electrostatic and hydrophobic noncovalent interactions.

Noncovalent Forces

Noncovalent interactions between atoms in a gas or a regular solid are fairly well understood. This is not the case in liquids, owing to the complexity of the constantly changing interactions between ensembles of molecules (Bull and Breese, 1974). This is especially important with macromolecules, such as proteins, because their folded conformations, three-dimensional structures and associations are usually studied in aqueous environments.

The most important characteristic of all intra- and intermolecular forces in aqueous solutions is that they are generally more dependent upon the properties of this environment than on the nature and strength of the forces themselves. The interactions of water with ions, dipoles and hydrogen bond donors or acceptors are strong enough to cause a leveling or disappearance of most of the forces that would produce a strong intra- and intermolecular interaction between such groups in a nonpolar environment (Bull and Breese, 1974). The various types of noncovalent bonds were reviewed by Scheraga (1963) and Nemethy (1969) using model systems in water or mixtures of proteins in an aqueous environment. Noncovalent forces can be categorized as electrostatic or ionic associations, hydrogen bonds, van der Waals forces and hydrophobic interactions.

Electrostatic interactions between molecules are forces that may be involved in protein-protein interactions. Two principal types of molecular electrostatic interaction can occur in protein-water solutions. The salt linkage or ion-pair bond is an attractive interaction that occurs between closely spaced, fixed charges of opposite sign on a protein molecule. This is similar to the interaction of sodium and chloride ions in a sodium chloride crystal. There is also a longer range repulsive force resulting from the net charge on a protein molecule (Singer, 1962). Acidic and basic amino acid residues in proteins are charged at different pH values. The pK values of each group can vary considerably depending on the local environment; as a result net charge is not constant.

The extent to which electrostatic interactions stabilize protein structure and protein interactions is not clearly understood. It is frequently suggested that only marginal stabilization energy can be expected from these forces (Scheraga, 1963) because even a relatively low ionic strength environment should provide a sufficient concentration of counter ions to dilute any electrostatic interactions (Ryan, 1977). While this may be true in general, electrostatic interactions can play important roles in the stabilization of protein structures and protein-water systems.

One specific type of electrostatic interaction between polar molecules is the hydrogen bond. In this type of association a hydrogen atom is covalently bonded to an electronegative atom

(eg. nitrogen, oxygen or sulfur) and interacts with another electronegative atom (Pauling, 1960). Because of their composition, proteins are capable of hydrogen bonding. The oxygen and nitrogen atoms of proteins participate in forming significant hydrogen bonds. Hydrogen bond donors such as -OH and -NH can interact with hydrogen bond acceptors such as oxygen atoms or nonprotonated nitrogen atoms. Protein secondary structures such as α-helix and β-sheet are stabilized by hydrogen bonds which are maximized during folding (Pauling et al., 1951). The net contribution of hydrogen bonding to the stability of intra- and intermolecular structures has been questioned. In aqueous environments, hydrogen bonded structures are thermodynamically unstable (Kollman and Allen, 1972).

Van der Waals forces are the most general and nonspecific electrostatic interactions which contribute to protein-protein interactions. These are very weak short-range attractive forces between groups of atoms. These forces arise as a result of the polarization of atoms by the formation of momentary induced dipoles in the rapidly fluctuating electrical field (Ryan, 1977). The magnitude of van der Waals forces is limited by the fact that these forces are individually extremely weak and short range. Atoms which form van der Waals interactions in the interior of a folded protein molecule would also form van der Waals interactions with water molecules in the denatured protein molecule. They have no special committment to the native protein structure (Damodaran and Kinsella, 1982). The net contribution to the

stability of the folded structure is the difference between the energy of the intra- and intermolecular interactions and for that reason may be very small. In spite of these considerations, the apparent maximization of these interactions suggest that some constraints on protein-protein and protein-water interactions are imposed by van der Waals interactions (Scheraga, 1963).

Hydrophobic interactions form among the nonpolar side-chains of proteins in water. Kauzmann (1959) predicted that interactions of nonpolar residues in proteins would be energetically favored. Stable protein conformations might contain a significant proportion of nonpolar side-chains buried in the interior of the molecule away from the surrounding aqueous solvent. Approximately 35-40% of the amino acids in most proteins either have nonpolar side-chains (alanine, valine, isoleucine, leucine, proline, phenylalanine) or relatively nonpolar groups (methionine, cysteine, tryptophan). These nonpolar groups can interact with each other and with the solvent by means of van der Waals forces (Waugh, 1954). However, Kauzmann (1959), Nemethy (1969) and Tanford (1962; 1978) emphasized that changes in the structure of water surrounding the nonpolar groups must play an important role in the formation of hydrophobic interactions. It has been frequently suggested that hydrophobic interactions are the predominant contributors to the stability of protein structure but there is not general agreement on this point (Tanford, 1973).

The existence of nonpolar regions in the interior of proteins has been established by X-ray crystallography (Tanford,

1973). The three dimensional structure of several proteins shows that the interior contains largely nonpolar amino acids whereas both nonpolar and polar residues occur on the surface (Brant et al., 1967). The driving force for nonpolar interactions does not arise in the inherent attraction of nonpolar side-chains for each other through van der Waals forces but in the energetically unfavorable effect they have on the structure of the water around them. When nonpolar groups associate in an aqueous system, water is transferred from the vicinity of the hydrocarbon to the bulk solvent phase. This process is regarded to be energetically favored at low temperatures, although there is disagreement about the mechanism (Shinoda, 1977).

The driving force of hydrophobic interactions is entropic and an enthalpy change (ΔH) acts in opposition to this tendency. When a hydrophobic side-chain is removed from water there may be a change in the overall hydrogen bonding of the water (Pashley et al., 1985). In thermodynamic measurements on simple systems at low temperatures, it was shown that the transfer of hydrophobic groups from water to nonpolar solvents is not accompanied by a negative ΔH (Frank and Evans, 1945). On the other hand, the overall free energy change $\Delta G = \Delta H - T\Delta S$ for the process is favorable ($\Delta G < 0$). The driving force is a large positive entropy change (ΔS) which is a result of a change in water structure from the relatively ordered nature in the hydration layer surrounding the solute molecule (iceberg structure) to the less ordered structure of the bulk liquid water (Nemethy and Scheraga, 1962).

Quantitative estimates of the stability of hydrophobic association in proteins is not a simple task (Ryan, 1977). The most important factors for protein stability in an aqueous solution are the values of ΔH and ΔS but these values are very difficult to measure in such a complicated system. In order to assess the strength of hydrophobic interactions, Tanford (1962) introduced an empirical scale measuring the hydrophobicity of unionized amino acid side-chains in proteins. This scale measured the apparent free energy of transfer (ΔG_{tr}) of the side-chains to water from a nonpolar solvent, generally ethanol or dioxane. This was measured by the relative solubilities of the free amino acids in aqueous and nonpolar solvents, designated S_w and S_{np}, respectively:

$$\Delta G_{tr} = RT \ln (S_{np}/S_w)$$

Amino acids are very insoluble in nonpolar solvents, as a result of the polar amino and carboxyl groups, so the value of S_{np} was generally obtained by extrapolation of the solubilities measured in various mixtures of water and nonpolar solvents to the pure nonpolar solvent. The measured values were then made relative to that of glycine, which was assigned $\Delta G_{tr} = 0$ because the side-chain consists of a single hydrogen atom.

All noncovalent forces should be taken into consideration when amphiphilic macromolecules like proteins are dissolved in an aqueous environment. Each group of these noncovalent forces (hy-

drogen bonds, electrostatic interactions, van der Waals forces, hydrophobic associations) has its own contribution to the protein arrangement in solution. The importance of the thermodynamic parameters, derived from the noncovalent forces, is referred to by several authors (Privalov and Khechinashvili, 1974; Rose et al., 1985). From their studies, we can conclude that a) the only contributions to positive entropy and enthalpy changes arise from ionic and hydrophobic interactions and b) the only source of negative enthalpy and entropy changes arises from van der Waals interactions and hydrogen bond formation. The values $\triangle G^{O}$, $\triangle H^{O}$, $\triangle S^{O}$ and $\triangle Cp$ are often all of negative sign and therefore it is not possible to account for the stability of an associated complex of proteins on the basis of hydrophobic interactions alone.

Micelle Formation

When amphiphilic macromolecules, like globular proteins, with an abundance of nonpolar groups are dissolved in an aqueous environment, they can actively segregate their hydrophobic (nonpolar) portions from the polar solvent by self-aggregation. In general these aggregates are of fixed structure (Tanford, 1973) formed by the self assembly of monmeric protein subunits into larger aggregates (Simons et al., 1978). The aggregated products may be in the form of micelles, although not all proteins can form micelles (Tanford, 1973). The casein micelle system in bovine milk, for example, is unique in that protein aggregates of similar sperical shape but extreme variability of size are formed

by the self-assembly of three major nonidentical subunits (Payens, 1965). Electron microscopy reveals that a_{s1}-, α- and k-casein subunits are associated into spherical soap micelle-like particles with the k-casein segregated into one portion, giving these particles an amphiphilic nature (Payens, 1965). The a_{s1}- and α-caseins are hydrophobic while the k-casein portion of the particle surface is hydrophilic.

The casein micelle system described above does not seem to typify the characteristic structure of all protein micelles for at least two reasons. First, even though these micelles place casein in the virus size class or larger, self-assembly occurs only with three major nonidentical subunits, a_{s1}-casein, α-casein and k-casein. Secondly, the formation of casein micelles is dependent on the undefined action of calcium, phosphate and citrate ions (Griffin and Anderson, 1983). This dependence on ionic interactions makes the casein protein arrangement somewhat different from the traditional description of a micelle.

In order to identify proteins with a micelle forming capacity it is necessary to take into consideration their amino acid compostition. Proteins with an abundance of hydrophobic amino acid side chains can generally form micelles under certain conditions (Ismond, 1984). The amount and kind of these hydrophobic side chain groups are critical factors (Tanford, 1973). The aromatic side chains are usually larger contributors than the aliphatic ones (Tanford, 1973). The protein concentration itself is also important. At very low concentration in aqueous solution,

the protein is dispersed mostly as "monomers", although "dimer" and "trimer" units may exist. At a critical micelle concentration (CMC) the monomers may assemble (Reynolds, 1979) in an aggregate to form micelles. This process can be described with the following equation:

$$mD = Dm$$

where m is the average association number, D is the concentration of monomeric protein and Dm is the concentration of micelles. The association constant is given as:

$$K = [Dm] / [D]^m$$

As the concentration of the protein is increased above the initial micelle concentration, more micelle assemblies are formed with the amount of free "monomers" remaining approximately constant and equal to the CMC values (Reynolds, 1979).

The presence of large numbers of hydrophobic residues is not necessarily indicative of the capacity of a protein to form micelles. The amount and type (aliphatic, aromatic) of nonpolar side chains plus the nature of the environment surrounding the protein determine the concentration, micelle size, aggregation number and structure. In an aqueous environment micelle formation is believed to be the result of three primary forces namely: hydrophobic associations between nonpolar side chains as a result of their interaction with the aqueous environment, charge repulsion of ionic groups and van der Waals attractions. Environmental

parameters such as electrolytes, temperature, pH, organic solvents and denaturants mainly affect the hydrophobic and electrostatic interactions responsible for micelle formation. It is entropically favorable if the hydrophobic surface residues of proteins can be removed from the aqueous environment (Nakai, 1983) through intermolecular associations. This is not always possible because of the protein's folded structure. As a result, some hydrophobic side chains are still exposed to the aqueous environment. Consequently, for thermodynamic reasons, proteins aggregate and form a spherical micelle structure, characterized by minimal exposure of hydrophobic residues and maximum exposure of hydrophilic side chains to the aqueous environment (Murray et al., 1978).

Although hydrophobic interactions are considered to be responsible for micelle formation, electrostatic forces may influence the result of aggregation (Murray et al., 1981). Hydrophobic interactions are stronger than electrostatic ones, especially at low ionic strength. Electrostatic parameters result from the overall charge of the protein as determined by the degree of amino acid ionization. This ionization is also affected by the environmental pH and the solubilized electrolytes. If electrostatic repulsions are greater than hydrophobic attractions, aggregation is not possible and consequently micelle formation cannot occur (Ismond, 1984). A delicate balance between noncovalent forces is apparently fundamental to protein micelle formation, micelle shape and size. The regulator, therefore, for micelle existance is both the protein itself and the environment.

Environmental Impacts on Micelle Formation

Proteins in aqueous solutions not only change their folding properties but also change the structure of the surrounding aqueous solvent, mainly because of hydrophobic interactions. These hydrophobic interactions are sensitive to the other compounds added to the aqueous solution. Generally globular proteins are soluble in some specific salt environments, but as the ionic strength is decreased, micelle formation and precipitation may occur. To explain the different behavior of globular proteins in various salt environments, Hatefi and Hanstein (1969) divided neutral salts into two different groups, chaotropic and nonchaotropic, depending on the stability of the protein-salt solution. Nonchaotropic salts tend to have an influence on the order of the water in the protein environment. Because of this influence only nonpolar group interactions are thermodynamically favorable and as the ionic strength of the nonchaotropic salt solution is increased, precipitation occurs. This phenomenon is well known as "salting-out" (Hatefi and Hanstein, 1969). An example of a nonchaotropic salt is ammonium sulfate. Chaotropic salts, on the other hand, are those which do not promote hydrophobic interactions. As the ionic strength of the chaotropic salt solution increases, electrostatic interactions have solubilizing effects on the protein. This phenomenon is known as "salting-in". An example of a salt in this group is sodium thiocyanate.

Hofmeister (1888), over 90 years ago, categorized some neutral salts according to their effectiveness in the precipitation of serum glubulins from aqueous solutions. The effect depends on the nature of the ions, according to the following hierarchy (Creighton, 1984):

Most Effective Least Effective

Cations: $Mg^{++} > Li^{+} > Na^{+} > K^{+} > NH_4^{+}$

Anions: $SO_4^{2-} > HPO_4^{2-} >$ acetate > citrate > tartarate > $Cl^{-} > NO_3^{-} > ClO_3^{-} > I^{-} > ClO_4^{-} > SCN^{-}$.

The same hierarchy has been observed in the effectiveness of hydrophobic interactions, affecting the protein micelle formation (Ismond, 1984). It is also likely that all these effects on hydrophobic interactions arise indirectly from the effects on the structure of water. For example the Hofmeister series generally correlates best with the effects of the salts on the surface tension (σ) of the water (Melander and Horvath, 1977). Those salts that increase hydrophobic interactions also increase the surface tension of the water.

According to the theory of "cavity" formation (Arakawa and Timasheff, 1982), nonchaotropic salts promote a preferential hydration of the protein surface meaning that the immediate area of the protein molecule is occupied by water molecules while salt ions remain further away from the protein structure. The formation of this structure requires a certain amount of free energy,

dependent upon the nature and the concentration of the salt. Therefore, in nonchaotropic salt environments, a lower level of free energy would be required for the micelle structured protein, than the "monomer" one. Micelle structured protein has a smaller "cavity" than the individual "monomers" and consequently a smaller contact surface area within the solvent. Hydrophobic interactions are promoted and micelle formation is thermodynamically favorable.

Chaotropic salts, according to Arakawa and Timasheff (1982), have a destabilizing effect on the protein structure. They do not cause preferential hydration but, in fact, they tend to bind to the surface of the protein. A charged "imaginary" sphere is therefore formed immediately on the protein surface. Because of the electrostatic charge of these protein-salt spheres, electrostatic repulsive forces do not encourage intermolecular aggregation (micelle formation is poor) and solubility is increased.

Determination of Protein Hydrophobicity

Many early studies were concerned mainly with the total number of hydrophobic side chains making up the protein structure (Waugh, 1954; Tanford, 1962; Fisher, 1965). This hydrophobicity was accordingly referred to as "total " hydrophobicity (Bigelow, 1967). However, the "effective" hydrophobicity i.e. true surface hydrophobicity (Keshavarz and Nakai, 1979) has greater significance in elucidating protein function (Kato and Nakai, 1980). Although many hydrophobic residues are buried in the interior of

most native proteins to avoid contact with the polar aqueous environment, some hydrophobic groups may remain exposed at the molecular surface. This "surface" hydrophobicity does not directly correlate with the "total" hydrophobicity (Nakai, 1983) especially in the case of native globular proteins (Ponnuswamy et al., 1980; Rose and Roy, 1980; Rose et al., 1985).

Hydrophobic interaction chromatography (Er-el et al., 1972) is one technique used for the determination of the surface hydrophobicity of proteins. This technique is based on the capacity of a protein to interact with a hydrophobic ligand associated with an agarose gel column. The extend to which the protein interacts with the ligand depends on the number of hydrophobic residues located on the surface of the protein (Hayakawa and Nakai, 1985). Proteins with a low surface hydrophobicity (hydrophilic) are eluted first due to their reduced capacity to react with the hydrophobic ligand whereas others with a high surface hydrophobicity are more strongly bound to the gel and consequently are eluted last.

Another method used to determine surface hydrophobicity of proteins is the two phase aqueous polymer system used by Shanbhag and Axelson (1975). This system is composed of two polymer phases: a polyethylene glycol phase and a dextran phase. The polyethylene glycol phase contains a small percentage of a hydrophobic polyethylene glycol derivative such as polyethylene glycol palmitate. A sample protein is introduced into the system, followed by vigorous mixing. The degree to which a protein parti-

tions itself into the polyethylene glycol phase containing the hydrophobic groups is compared to its partitioning into the polyethylene glycol phase without hydrophobic derivatives. The difference in partitioning due to the hydrophobic groups is used as an indicator of protein surface hydrophobicity (Keshavarz and Nakai, 1979).

Surface hydrophobicity (So) of proteins can also be assessed through the use of fluorescent probes (Sklar et al., 1977). These probes are not inherently fluorescent; however, they become fluorescent when bound to specific active groups on proteins. One of these fluorescent probes is cis-parinaric acid (CPA) introduced by Sklar et al. (1977). The interaction of this natural polyene fatty acid with proteins has a similarity to a natural lipid-protein interaction. In addition to CPA which binds to aliphatic hydrocarbon side-chains of proteins, 1-anilino-naphthalene-8-sulphonate (ANS; Hayakawa and Nakai, 1985; Wicker et al., 1986) is also widely used due to its capacity to bind with aromatic side-chain residues. For both probes, the net fluorescence intensity (FI) is determined by subtracting the fluorescence intensity of the protein itself without probe from that with the probe, at specific excitation and emission wavelengths, characteristic for each probe (i. e. 325 and 420 nm for CPA, 390 and 470 nm for ANS). The initial slope of a plot of fluorescence intensity as a function of protein concentration is an index of protein surface hydrophobicity (Hayakawa and Nakai, 1985).

Use of Differential Scanning Calorimetry to Follow Protein Conformational Changes

Most proteins in their native states are folded into well defined, but flexible three dimensional structures. For many proteins this structure is compact and globular. However, a change or disruption of this ordered structure may occur with variations in the environment. In this regard, a number of methods have been used to evaluate protein conformational changes with fluctuations in the surrounding environment. These have included assessments of variations in spectra determined by circular dichroism (CD) or optical rotary dispersion (ORD), patterns of intrinsic fluorescence, sedimentation coefficients, electrophoretic profiles, elution patterns during gel filtration and enzyme activity. Some methods such as CD appear to be superior due to their overall sensitivity. However, the choice of the method is determined mainly by the characteristics of the protein itself and its respective environment. An alternate approach to following conformational changes is to assess the thermal stability of the protein using differential scanning calorimetry (DSC). The use of DSC as a conformational probe has been well outlined by a number of authors (Privalov and Pfeil, 1979; Biliaderis, 1983; Murray et al., 1985).

In general, differential scanning calorimetry (DSC) is a technique for obtaining reasonable calorimetry data within a reasonable time frame (Donovan, 1984). It is called scanning calorimetry because measurements are usually made under dynamic

conditions with the sample being heated or cooled at a precise rate. It is called differential scanning calorimetry because the basis of the measuring system is to compare the rate of heat flow to the sample with that of an inert reference material. Both sample and reference are heated or cooled at the same rate. Events in the sample like phase transitions (Biliaderis, 1983) or protein denaturation (Arntfield and Murray, 1981) which involve absorption or evolution of heat cause a change in the differential heat flow.

As a specific example, when a protein undergoes a transition from a native to a denatured state, the change in heat flow or heat capacity as a function of temperature results in an endothermic peak reflecting the interconversion of the two forms. Both the temperature corresponding to the largest change in heat capacity, or denaturing temperature (Td), and the heat required to effect this conversion reflect the stability and conformation of the original protein. Thermal parameters such as Td and the enthalpy of denaturation (ΔH) are characteristic of the unfolding process. As a result, any changes in these thermal parameters should reflect conformational differences in the structure of the original or native state of the protein (Chlebowski and Williams, 1983).

MATERIALS AND METHODS

Assessment of Legumin Thermal Stability with Differential Scanning Calorimetry

Differenial scanning calorimetry (DSC) was used to assess the thermal properties of legumin in different environments (Table 2.1). A DuPont 9900 computer/thermal analyser with a 910 differential scanning calorimeter cell base was used. The sample (10 uL), containing 2 μg of lyophilized legumin in appropriate buffer (Table 2.1), was sealed hermetically in DuPont aluminum pans, coated on the interior with an inert polymer. The net weight of the solution was determined by a Mettler AE163 analytical balance. Both the reference, an empty sealed pan, and the sample pan were placed in the DSC cell under nitrogen at a constant pressure of 300 PSI. Silicon heat sink compound (Dow Corning Corp.) was used for better thermal conductivity. Plots of heat flow as a function of temperature, referred to as thermal curves (Arntfield and Murray, 1981), were established at a heating rate of 10°C/min over a temperature range of 25 to 150°C with sensitivities of 0.016 or 0.032 mW/cm. From the thermal curves, the denaturation temperature (Td), representing the temperature at which there is maximum heat flow into the sample, and the enthalpy of denaturation (ΔH), a function of the area of the endothermic heat flow curve, were determined. The thermal stability of legumin under different pH conditions (Group A, Table 2.1) and in different electrolyte media (Group B, Table 2.1) was examined by DSC. Destabilizing conditions were applied by using

Table 2.1. Legumin environments used for differential scanning calorimetry, fluorescence spectroscopy and micelle assessment.

Solution	Environment
Group A	
	pH values
phosphate buffer (0.25 M)	5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0
Group B (in 0.01 M phosphate buffer, pH 6.5)	Electrolyte conc. (M)
Sodium chloride[1]	0.25, 0.5, 1.0
Sodium acetate	0.25, 0.5, 1.0
Sodium thiocyanate	0.25, 0.5, 1.0
Sodium citrate[2]	0.04, 0.08, 0.16
Group C (in 0.01 M phosphate buffer, pH 6.5)	Urea conc. (M)
Urea	0.5, 1.0, 2.0, 3.0, 6.0, 8.0

[1] Sodium chloride not used in fluorescence experiments due to quenching by the anion.

[2] Sodium citrate values correspond to equivalent ionic strengths for other electrolyte media.

different concentrations of the denaturant, urea (Group C, Table 2.1). The protein concentration of each sample was determined by the Coomassie method (Pierce Chem. Co., 1983) using bovine serum albumin (Sigma) as a standard. All values of Td and ΔH were determined using a minimum of three samples; mean values and standard deviations of the mean are given for each.

Surface Hydrophobicity (So) Determinations of Legumin by Fluorescence Spectroscopy

A fluorescence probe method, first introduced by Kato and Nakai (1980) and modified by Ismond (1984), was used to determine the surface hydrophobicity of legumin. Two fluorescence probes were used: 1-anilino-8-naphthalensulfonate (ANS, Sigma Chem. Co.) and cis-parinaric acid (CPA, Calbiochem.). The ANS solution was prepared according to the method of Hayakawa and Nakai (1985); specifically, 8 mM magnesium ANS was dissolved in 0.02 M phosphate buffer, pH 7.4. A solution of CPA was prepared according to Kato and Nakai (1980); this consisted of equimolar (3.6 mM) CPA and butylated hydroxytoluene in ethanol. Each protein sample was serially diluted with appropriate buffer (Table 2.1) to obtain a range of protein concentrations from 0.04 to 0.40 µg/mL. The ANS or CPA (10 µL) was added to 2 mL of each sample. Relative fluorescence intensity (RFI) was measured with a Perkin-Elmer LS-5 fluorescence spectrophotometer using a slit width of 0.5 nm and a fixed scale of 1.0. Temperature was controlled at 20°C with a Haake-G water bath. Wavelengths of excitation and emission were

390 and 470 nm for ANS and 325 and 420 nm for CPA. The net fluorescence intensity (FI) at each protein concentration was determined by subtracting the fluorescence intensity of each solution without probe from that with the probe. The initial slope of a plot of fluorescence intensity as a function of protein concentration was used as an index of protein surface hydrophobicity (So). This initial slope was determined by standard linear regression analysis. The surface hydrophobicity of legumin was examined in different pH regimes (Group A, Table 2.1), in different electrolyte media (Group B, Table 2.1) and under denaturating conditions, using different concentrations of urea (Group C, Table 2.1). Protein concentration was determined by the Coomassie method (Pierce Chem. Co., 1983) using bovine serum albumin (Sigma) as a standard. All values of So were determined using a minimum of four samples; mean values and standard deviations of the mean are given for each.

Micelle Observation by Light Microscopy

Light microscopy was used to assess micelle formation of legumin in a number of different environments (Table 2.1). Sample preparation included extensive dialysis of the legumin fraction eluted from the gel filtration column (approximately 1 mg/mL in 0.5 M sodium chloride, pH 7.0) against the media described in Table 2.1. Legumin in each experimental environment was then concentrated using an Amicon-B15 macrosolute concentrator to a concentration of approximately 75 mg/mL. The capacity of legumin

for micelle formation in each experimental medium was assessed using light microscopy. Protein samples (20 µL) were examined initially without a coverslip using a Zeiss Universal Research Microscope. Distilled water (20 µL) was then added to the protein solution. Protein precipitation was observed initially by the unaided eye. After precipitation, a coverslip was added. Micelle structures were photographed with a C35M Carl Zeiss camera. The micelle response was rated from 0 to 5 according to a scheme by Ismond et al. (1986a; Table 2.2; Figures 2.1, 2.2). The protein concentration of each sample was determined by the Coomassie method (Pierce Chem. Co., 1983) using bovine serum albumin (Sigma) as a standard. A minimum of two samples were observed for each environmental condition.

Statistical analysis

A multiple Student-Newman-Keuls test was used to evaluate the differences in thermal parameters (Td, ΔH) and So values for various concentrations within a specific environment.

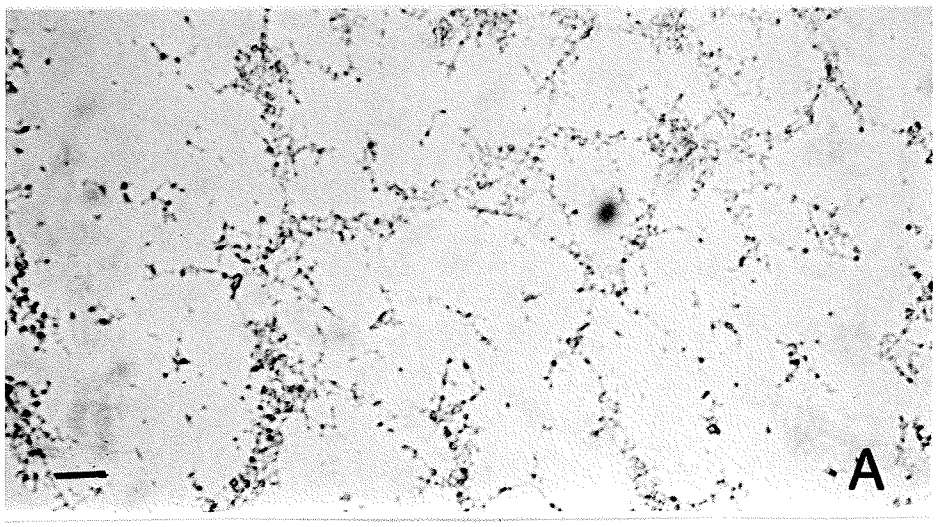
Table 2.2. Characteristics of the various types of micelle formations observed with legumin in different environmental conditions. Each type of formation has been assigned a numerical value, referred to as a micelle rating.[1]

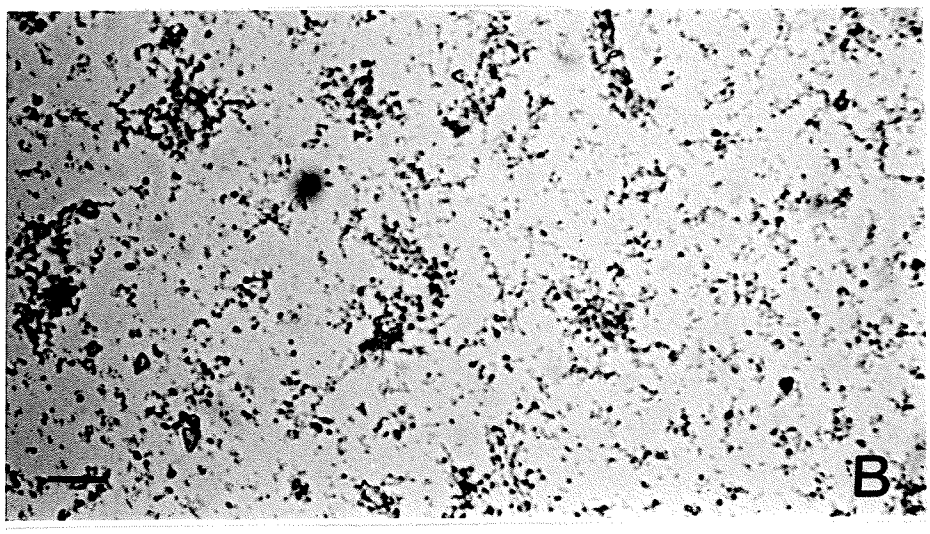
Characteristics	Micelle rating
No micelle formation	0
Small single micelles (<2 μm in diameter)	1
Small micelles in aggregates Possible granular network	2
Small, intermediate and large discrete micelles (2-20 μm in diameter)	3
All micelle sizes; homogeneous coalescence	4
Extensive coalescence of all micelle sizes to homogenous networks and protein sheets	5

1. From Ismond *et al.* (1986a).

Figure 2.1. Photomicrographs of micelle responses corresponding to ratings 1, 2 and 3. Bar represents 25 μm.

A. Small single micelles, rating 1.
B. Small micelles in aggregates, rating 2.
C. Small, intermediate and large micelles, rating 3.


A


B

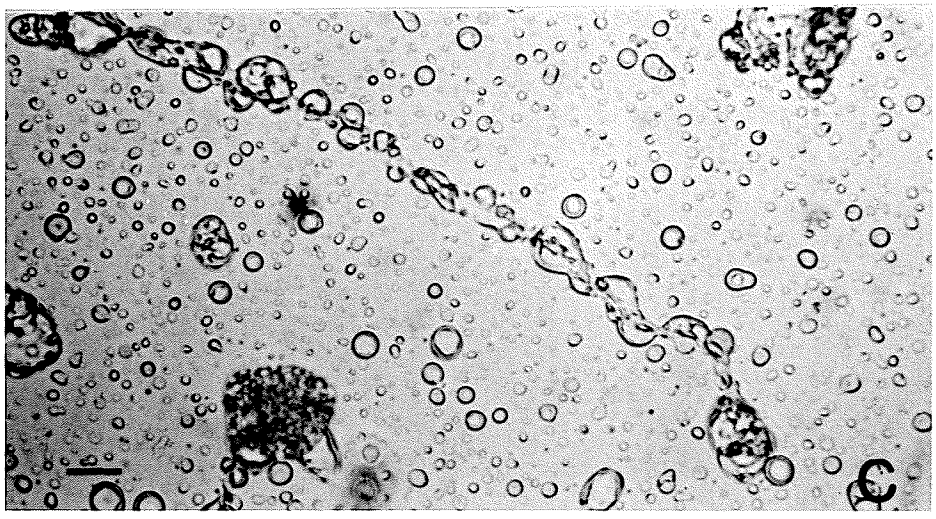
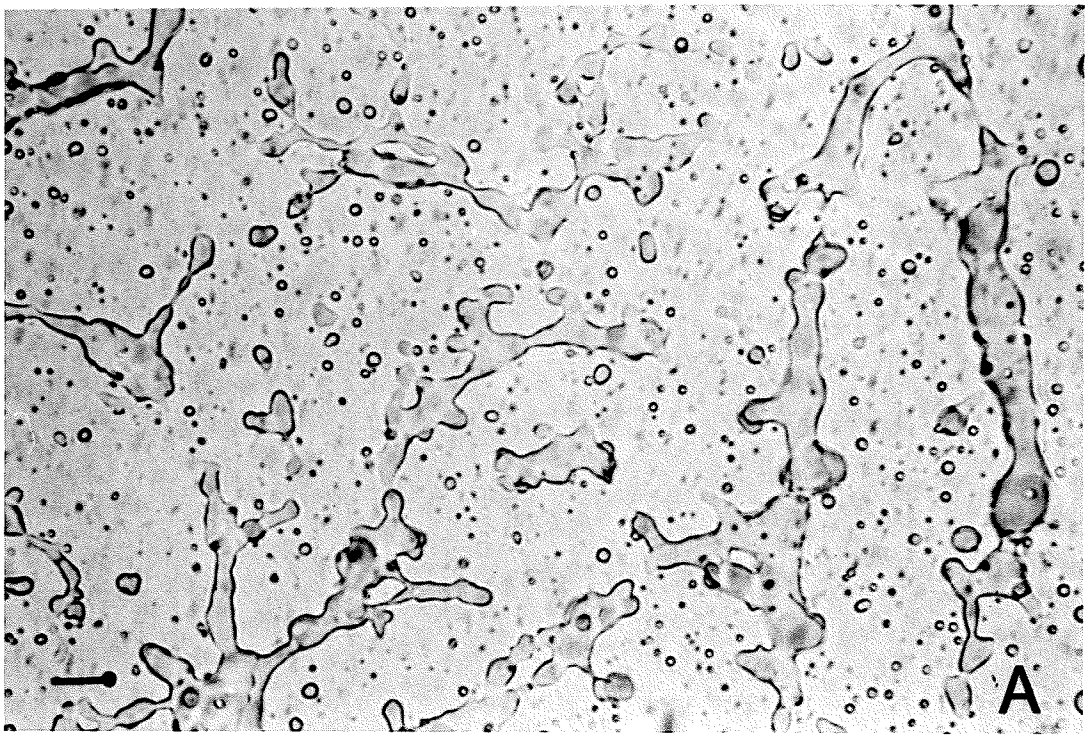
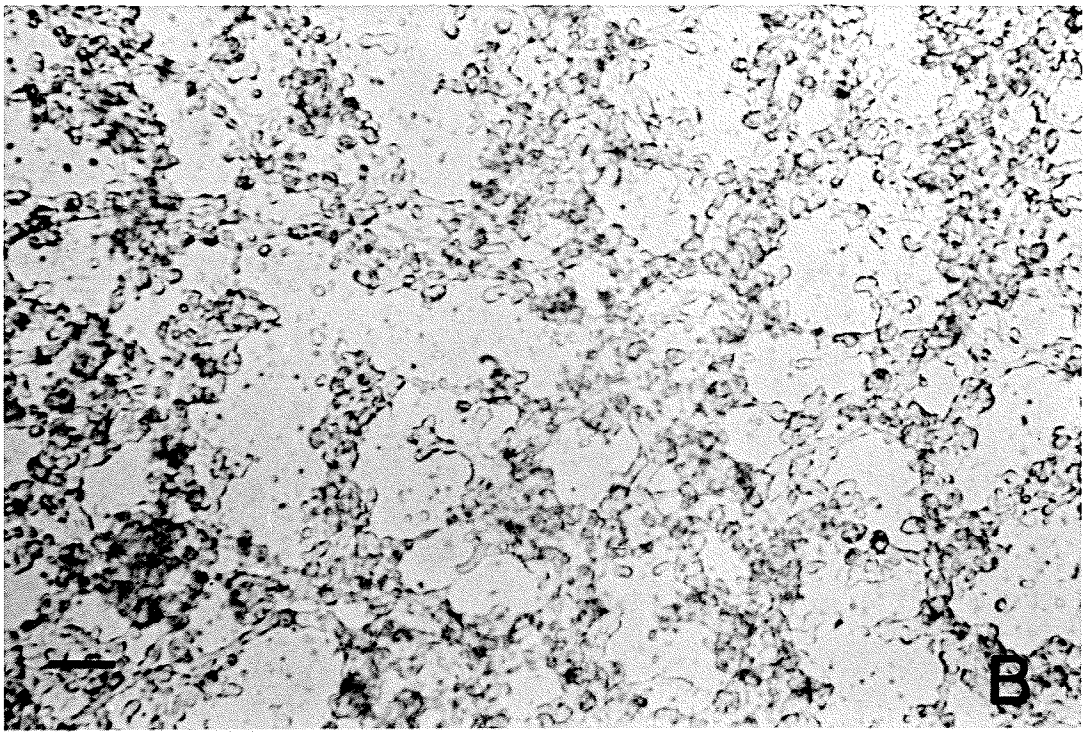

C

Figure 2.2. Photomicrographs of micelle responses corresponding to ratings 4 and 5. Bar represents 25 μm.

A. Homogeneous coalescence, rating 4.
B. Extensive coalescence, rating 5.


A


B

RESULTS

Micelle Assessment and Classification

Protein micelles are not stoichiometric compounds but aggregates capable of existing over a wide range of micelle sizes, depending upon the environmental conditions (Ismond, 1984). Tanford (1973) categorized micelles into three major groups; small with aggregation number less than 100, large with aggregation number more than 1,000 and intermediate with aggregation number between 100 and 1,000. Previous studies on protein micelles (Murray et al., 1978; Ismond, 1984) showed that specific micelles may be characterized by a heterogeneity of different structures including single micelles, protein networks or amorphous protein masses. Ismond et al. (1986a) classified the various types of micelles observed in different environmental conditions for vicilin, a fababean storage protein, according to micelle structures shown by a numerical value from zero to five referred to as a micelle rating (Table 2.2; Figure 2.1, 2.2). Using this micelle rating system the capacity of legumin to form micelles was assessed in different environments.

Effect of pH on Conformational Paramters and Micelle Formation by Legumin

In order to assess the effect of pH on micelle formation by legumin, the protein was exposed to a series of 0.25 M phosphate buffer media ranging in pH from 5.5 to 9.0. Each environment differed by 0.5 pH units. The micelle structures in these dif-

ferent environments were related to certain molecular parameters, specifically surface hydrophobicity and thermal properties (Table 2.3). A strong dilution response occurred at pH 5.5 and 6.0. Small intermediate and large micelles were observed with homogeneous coalescence and protein sheets (micelle rating 5; Figure 2.2). A visible dilution response in terms of all micelle sizes (especially small micelles) was observed at pH 6.5. Some limited coalescence was also observed; as a result, the overall response was given a micelle rating of 4 (Figure 2.2). No micelle formation was observed at pH values of 7.0 to 9.0.

In terms of molecular parameters, both aliphatic (S_c, for CPA) and aromatic (S_a, for ANS) surface hydrophobicity values initially followed the micelle response (Table 2.3; Figure 2.3). At pH values of 5.5 and 6.0 the micelle rating was 5. This was paralleled by an increase in S_o values from 115.3 (pH 5.5) to 130.8 (pH 6.0) for S_c and from 156.9 to 192.9 for S_a. At pH 6.5 with a micelle rating of 4.0, the surface hydrophobicities decreased significantly - to 109.3 (S_c) and 119.6 (S_a). Micelles were not formed at pH 7.0 (i.e. micelle rating 0); this was related to the lowest S_o values observed, specifically 76.7 for S_c and 105.8 for S_a. Although the micelle rating remained 0 at pH 7.5, the S_c value increased to 114.6 whereas the S_a value reached the value of 561.6. At pH values of 8.0 and 8.5 (micelle rating 0 for both), S_c values decreased from 134.2 to 100.5 whereas S_a values decreased from a value of 537.6 (pH 8.0) to 471.0 (pH 8.5). Finally the absence of micelles at pH 9.0 was related to an S_c value of 100.5 and an S_a of 356.4.

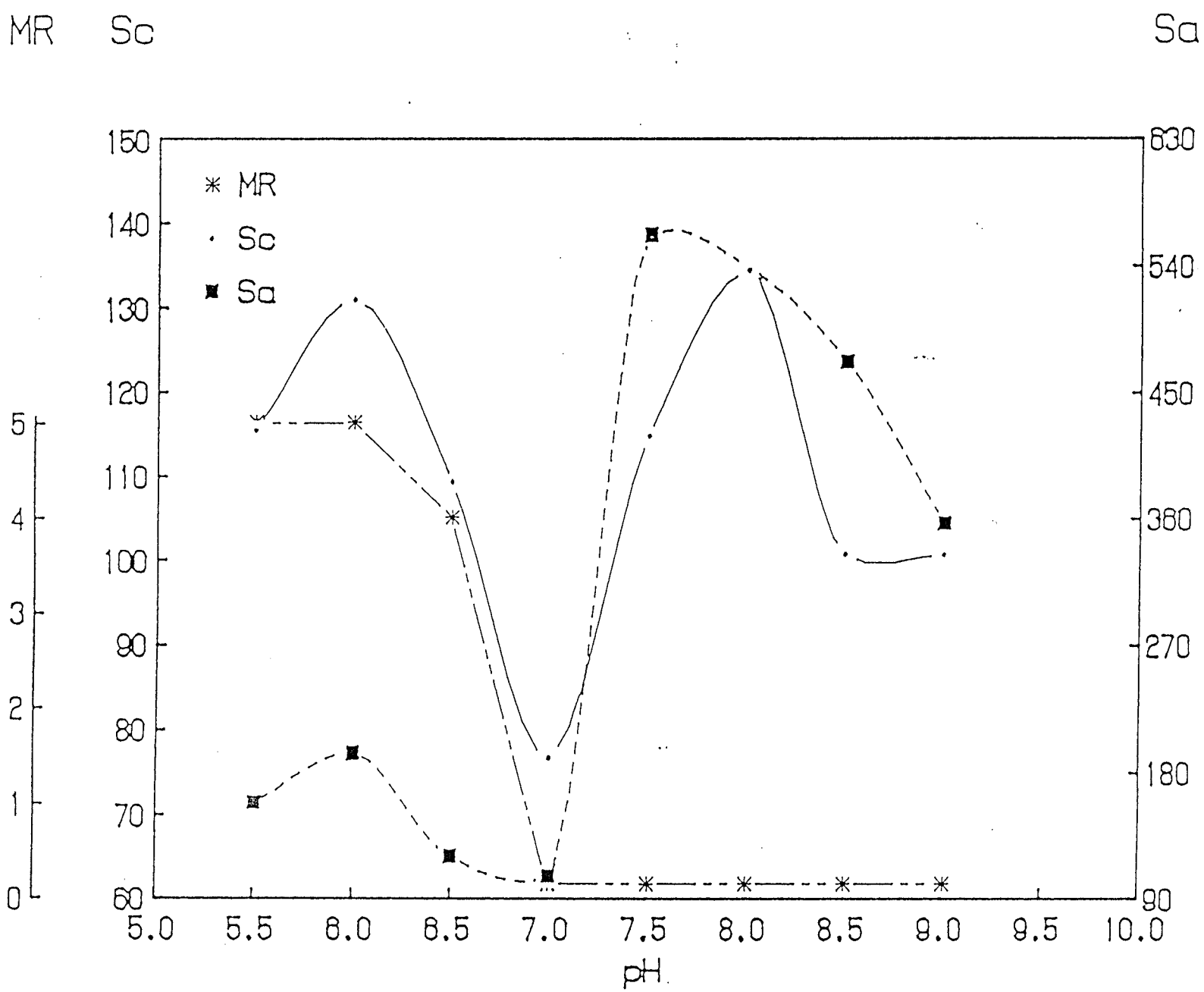
Table 2.3. Micelle rating (MR), surface hydrophobicities (S_c, S_a), denaturation temperature (Td) and enthalpy of denaturation (ΔH) for legumin in 0.25 M phosphate buffer of different pH values.

		So			
pH	MR[1]	S_c[2]	S_a[2]	Td(°C)[2]	ΔH(cal/g)[2]
5.5	5	115.3± 9.0[a]	157.0±10.6	106.9±0.6[a]	3.30±0.21[a]
6.0	5	130.8± 8.6[b]	193.0±11.8	106.2±0.7[ab]	2.78±0.11
6.5	4	109.3± 8.0[ac]	119.6± 7.9[a]	106.3±0.7[ab]	3.34±0.48[a]
7.0	0	76.7± 9.5	105.9± 8.9[a]	107.7±0.3[ab]	3.27±0.67[a]
7.5	0	114.6± 5.2[a]	561.7±30.4[b]	108.3±1.0[b]	3.46±0.38[a]
8.0	0	134.3±11.6[b]	537.7±29.8[b]	106.1±0.6[ab]	3.32±0.40[a]
8.5	0	100.5± 5.3[ac]	471.0±89.9[bc]	105.2±1.1[ab]	3.15±0.30[a]
9.0	0	100.5± 9.8[ac]	356.4±37.6[c]	105.1±3.2[a]	3.06±0.19[a]

1. Description of micelle ratings is given in Table 2.2, Figures 2.1 and 2.2.

2. Column values followed by the same lettter are not significantly different as determined by the Student-Newman-Keuls test ($P \leq 0.05$).

Figure 2.3. Micelle ratings (MR) and surface hydrophobicity values (S_c, S_a) for legumin exposed to different environmental pH levels.


MR
Sc
Sa
* MR
· Sc
Sa
150
140
130
120
110
100
90
80
70
60
630
540
450
360
270
180
90
5
4
3
2
1
0
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
9.5
10.0
pH

Although pH strongly influenced the So values for legumin, no dramatic changes occurred in the protein's thermal characteristics (Table 2.3). There were no significant differences in either the Td or ΔH values for legumin among the different pH environments. (Table 2.3).

Effect of Specific Anions on Conformational Parameters and Micelle Formation by Legumin

Different sodium salt environments of comparable ionic strengths were used to assess the micelle forming capacity of legumin. The salts chosen represented three categories of environments - highly stabilizing (sodium citrate), moderately stabilizing (sodium acetate) and destabilizing (sodium thiocyanate) (von Hippel and Wong, 1964). For each salt, the concentrations investigated ranged from a minimum ionic strength of 0.25 to a maximum of 1.0. These concentrations represented two levels of salt action. Specifically, concentrations at which salt effects on legumin were primarily electrostatic (μ = 0.25) were contrasted with concentrations at which salt effects were more lyotropic, or related to interactions with the solvent (μ = 0.5, 1.0). As in the pH study, micelle ratings were related to the molecular parameters of surface hydrophobicity and thermal stability.

In terms of thermal parameters, legumin showed interesting conformational changes when exposed to different salts or to different concentrations of the same salt. These changes were reflected primarily in the Td values. For example, the Td values

for legumin in sodium thiocyanate were significantly lower than those for legumin exposed to sodium chloride, sodium acetate and sodium citrate environments (Table 2.4; Figure 2.4) The effect of concentration differences of a particular salt on the Td values of legumin were significant. In sodium chloride, acetate and citrate, there was an increase in Td values when the salt concentration was increased (Table 2.4; Figure 2.4). The greatest increase in legumin Td values occurred when the protein environment was changed from 0.25 M sodium chloride (Td value 105.83^{o}C) to 1.0 M of the same salt (114.14^{o}C). This was an interesting result, as citrate is generally considered to be a more stabilizing anion than chloride. For sodium chloride, sodium acetate and sodium citrate, there was a significant positive correlation between Td values and salt concentration (M) as given by the relationships:

sodium acetate: $Td = 0.117\ M - 12.307,\ r = 0.9959$

sodium chloride: $Td = 0.090\ M - 9.292,\ r = 0.9999$

sodium citrate: $Td = 0.255\ M - 26.058,\ r = 0.9401.$

Salts like these for which there is a positive correlation between concentration and Td values are considered to be "stabilizers" of the protein's structure. However, when legumin was exposed to sodium thiocyanate, an increase in salt concentration resulted in a decrease of the Td values (Table 2.4; Figure 2.4). There was a significant negative correlation between Td

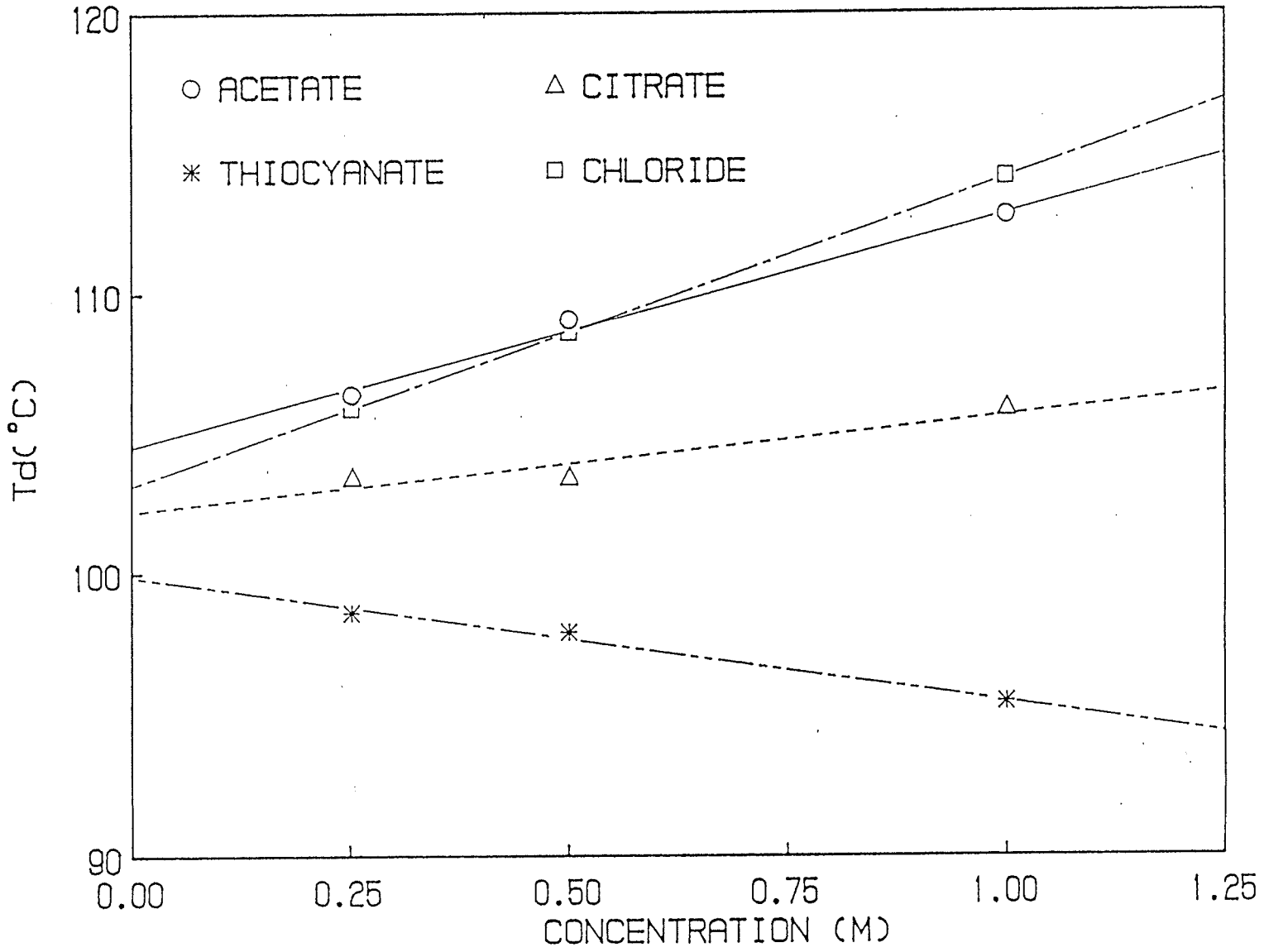
Table 2.4. Temperature of denaturation (Td) and enthalpy of denaturation (ΔH) values for legumin in various concentrations of different sodium salts.

Salt environment	Concentration (M)		
	0.25	0.5	1.0
	Td(^{o}C)		
sodium thiocyanate	98.6±2.9^{al}	97.9±1.3^{1}	95.4±1.4^{1}
sodium acetate	106.3±2.5^{bl}	109.0±0.7^{al}	112.7±0.1^{a}
sodium chloride	105.8±0.1^{b}	108.6±0.4^{a}	114.1±0.3^{a}
sodium citrate	103.3±0.4^{abl}	103.3±0.1^{1}	105.7±0.2
	ΔH(cal/g)		
sodium thiocyanate	3.62±0.05^{1}	3.67±0.15^{bl}	2.43±0.12
sodium acetate	4.22±0.09^{al}	4.22±1.22^{abl2}	5.45±0.18^{2}
sodium chloride	4.56±0.46^{a}	5.19±0.13^{a}	6.43±0.17
sodium citrate	3.18±0.21^{1}	2.89±0.40^{b}	3.31±0.10^{1}

1. Vertical column values followed by the same letter are not significantly different as determined by the Student-Newman-Keuls test ($P \leq 0.05$). Statistical comparisons apply to a single parameter at one concentration level.

2. Horizontal values followed by the same number are not significantly different as determined by the Student-Newman-Keuls test ($P \leq 0.05$). Statistical comparisons apply to a single parameter in one specific salt environment.

Figure 2.4. The effect of increasing the concentration of various sodium salts on the denaturation temperature (Td) of legumin.


120
110
100
90
Td(°C)
○ ACETATE
△ CITRATE
✳ THIOCYANATE
□ CHLORIDE
0.00
0.25
0.50
0.75
1.00
1.25
CONCENTRATION (M)

values and sodium thiocyanate concentration (M), defined by the relationship:

$$Td = -0.222\ M + 22.22, \quad r = 0.9923.$$

Salts like NaSCN for which there is a negative correlation between concentration and Td values are referred to as "destabilizing" salts.

In the destabilizing medium, sodium thiocyanate (0.25 M, 0.5 M), legumin formed all sizes of micelles resulting in extensive coalescence and protein networks (micelle rating 5; Figure 2.2). When the salt concentration was increased to 1.0 M, small micelles in aggregates (micelle rating 2; Figure 2.1) were observed. With respect to So values of legumin in this medium, there was an increase in both surface hydrophobicity values (S_c for CPA and S_a for ANS) with increasing thiocyanate concentration (Table 2.5; Figure 2.5). For example, the S_c value increased from 50.9 at 0.25 M to 124.7 at 1.0 M salt; in addition, the S_a values also increased from 42.6 (0.25 M) to 123.63 (1.0 M). For highly stabilizing salts like sodium citrate the situation was the opposite. An increase in salt concentration caused a decrease of both S_c and S_a values (Table 2.5; Figure 2.5). For example the S_c value decreased dramatically from 0.25 M (107.7) to 0.5 M (59.5) whereas the largest decrease for the S_a value occurred from 0.5 M (423.1) to 1.0 M (264.2). Micelle formation by legumin in sodium citrate followed the results from the So values. A granular network consisting of small micelles in aggregates (micelle rating 2; Figure 2.1) was observed at 0.25 M and 0.5 M. No micelles

Table 2.5. Micelle rating (MR) and surface hydrophobicity (S_c, S_a) values for legumin in various concentrations of different sodium salts.

Salt environment	Concentration (M) 0.25	0.5	1.0
		MR[3]	
sodium thiocyanate	5	5	2
sodium acetate	5	5	2
sodium chloride[4]	5	5	2
sodium citrate	2	2	0
		S_c	
sodium thiocyanate	50.9± 7.6	99.0±20.4[a]	124.7±33.7[a]
sodium acetate	142.0±35.0[a1]	89.7±33.4[ab12]	74.8±29.4[ab2]
sodium citrate	107.7±15.7[a]	59.5±11.8[b1]	44.7±13.7[b1]
		S_a	
sodium thiocyanate	42.7± 4.1[1]	82.4±10.9[a]	123.6±14.9[1]
sodium acetate	115.1±20.6[1]	111.5±26.5[a1]	76.3±23.9[1]
sodium citrate	466.0±71.9[1]	423.1±30.0[1]	264.2±41.3

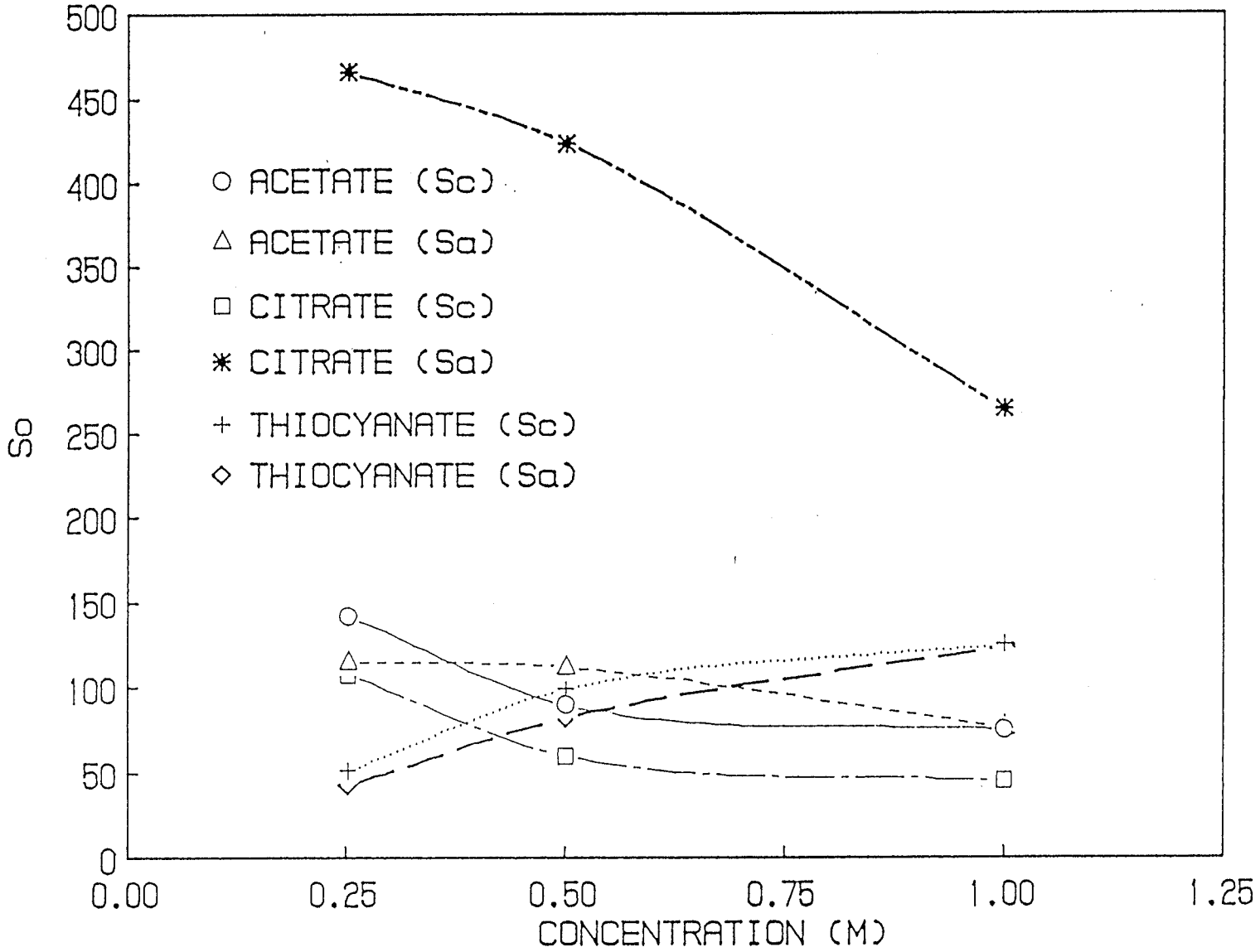
1. Vertical column values followed by the same letter are not significantly different as determined by the Student-Newman-Keuls test ($P \leq 0.05$). Statistical comparisons apply to a single parameter at one concentration level.

2. Horizontal values followed by the same number are not significantly different as determined by the Student-Newman-Keuls test ($P \leq 0.05$). Statistical comparisons apply to a single parameter in one specific salt environment.

3. Description of micelle ratings is given in Table 2.2, Figures 2.1 and 2.2.

4. S_c and S_a values not determined due to quenching effect of the anion.

Figure 2.5. The effect of increasing the concentration of various sodium salts on the aliphatic (S_c) and aromatic (S_a) surface hydrophobicities of legumin.


500
450
400
350
300
250
200
150
100
50
0
So
ACETATE (Sc)
ACETATE (Sa)
CITRATE (Sc)
CITRATE (Sa)
THIOCYANATE (Sc)
THIOCYANATE (Sa)
0.00
0.25
0.50
0.75
1.00
1.25
CONCENTRATION (M)

were formed in 1.0 M sodium citrate.

An impressive micelle response was observed in both the sodium acetate and sodium chloride media. At 0.25 and 0.5 M salt levels, homogeneous networks of extensive coalescence occurred (micelle rating 5; Figure 2.2). These structures were replaced by small aggregated micelles when the salt concentration was increased to 1.0 M (micelle rating 2; Figure 2.1). The So values for sodium acetate also decreased with an increase in salt concentration but not as extensively as in the case of sodium citrate (Table 2.5; Figure 2.5).

Effect of Urea on Conformational Parameters and Micelle Formation by Legumin

The capacity of legumin to form micelles was also assessed under denaturing conditions. Different urea concentrations (Table 2.1) were used. Micelle formation by legumin was related to the conformational parameters of surface hydrophobicity and thermal properties.

With dilution, legumin samples in 0.5 M urea showed immediate precipitation characterized by massive coalescence and network formation (micelle rating 5; Figure 2.2). When the urea concentration was increased to 1.0, 2.0 and 3.0 M, some precipitation with dilution was also observed. The micelle rating for all of these was 2, characterized by fine granular networks (Figure 2.1). At 6.0 and 8.0 M urea, the micelle response deteriorated to a minimum reaction described as rating 1 (Figure 2.1).

In terms of molecular parameters, the So values for legumin in different urea concentations (Table 2.6) showed parallel results to those of the micelle response. Specifically, at a urea concentration of 0.5 M, legumin exhibited maximum So values of 139.4 and 360.4 for CPA and ANS probes (S_c, S_a), respectively (Figure 2.6). Minimum So values were observed at 8.0 M urea (31.7 for S_c and 12.0 for S_a). The relationships defining the correlation between urea concentration (M) and So values were:

$$\text{CPA:} \quad S_c = 136.60 - 14.30\ M, \quad (r = 0.9519)$$

$$\text{ANS:} \quad S_a = 374.70 - 50.23\ M, \quad (r = 0.9727).$$

With respect to thermal properties, there were significant decreases in Td and ΔH values with an increse in urea concentration (Table 2.6; Figure 2.7, 2.8). A high Td value of 100.61^{o}C was observed at 0.5 M urea (Figure 2.7). The linear relationship between Td and urea concentration (M) was defined by the equation:

$$Td = 110.30 - 2.80\ M, \quad (r = 0.9917).$$

The progressive denaturation of legumin with increasing urea levels was further indicated by the dramatic decrease in ΔH values from 3.40 joules/g for legumin in 0.5 M urea to 0.02 joules/g at 8.0 M (Table 2.6; Figure 2.8). The relationship describing the correlation between urea concentration (M) and ΔH was given by the linear expression:

$$\Delta H = 4.17 - 0.53\ M; \quad (r = 0.9448).$$

Table 2.6. Micelle rating (MR), surface hydrophobicity (S_c, S_a), denaturation temperature (Td) and enthalpy of denaturation (ΔH) for legumin in different urea concentrations (M).

M	MR[1]	So		Td(°C)	ΔH(cal/g)
		S_c	S_a		
0.5	5	139.5± 1.1[a]	366.5±27.6[a]	108.6±0.1	3.40±0.33[a]
1.0	2	130.7±23.7[ab]	336.5±27.1[a]	106.1±0.3[a]	3.35±0.18[a]
2.0	2	108.1±21.6[b]	229.9±45.1[b]	106.2±0.1[a]	3.87±0.07
3.0	2	67.5± 5.6	175.9±14.0[b]	102.4±0.2	3.06±0.16[a]
6.0	1	49.0± 9.9	33.7± 9.8	94.3±0.6	0.38±0.08
8.0	1	31.7± 8.4	12.0± 4.9	87.1±0.8	0.02±0.00

1. Description of micelle ratings is given in Table 2.2, Figures 2.1 and 2.2.

2. Column values followed by the same letter are not significantly different as determined by the Student-Newman-Keuls test ($P \leq 0.05$).

Figure 2.6. The effect of increasing the concentration of urea on the aliphatic (S_c) and aromatic (S_a) surface hydrophobicities of legumin.

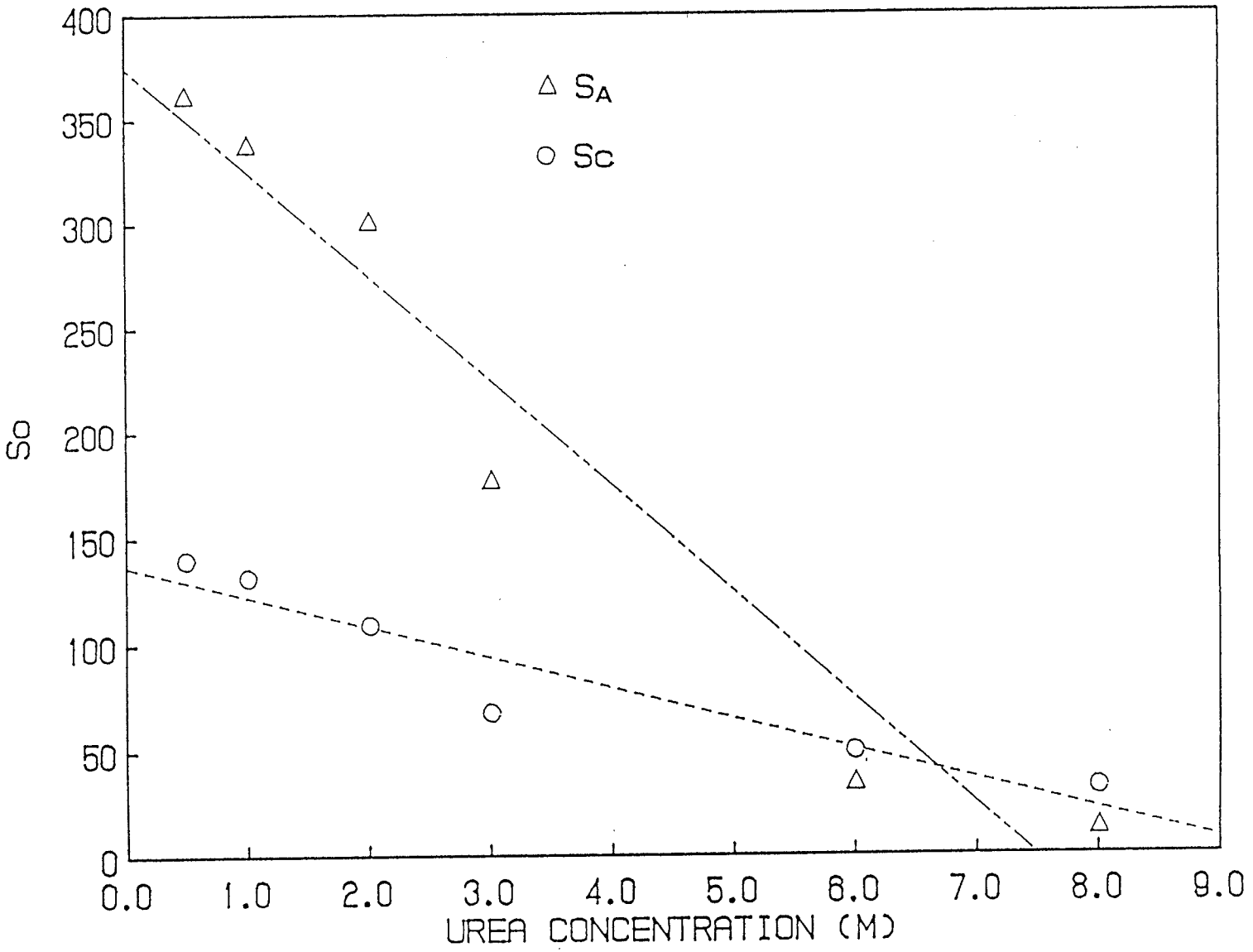

400
350
300
250
200
150
100
50
0
So
△ SA
○ Sc
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
UREA CONCENTRATION (M)

Figure 2.7. The effect of increasing the concentration of urea on the denaturation temperature (Td) of legumin.

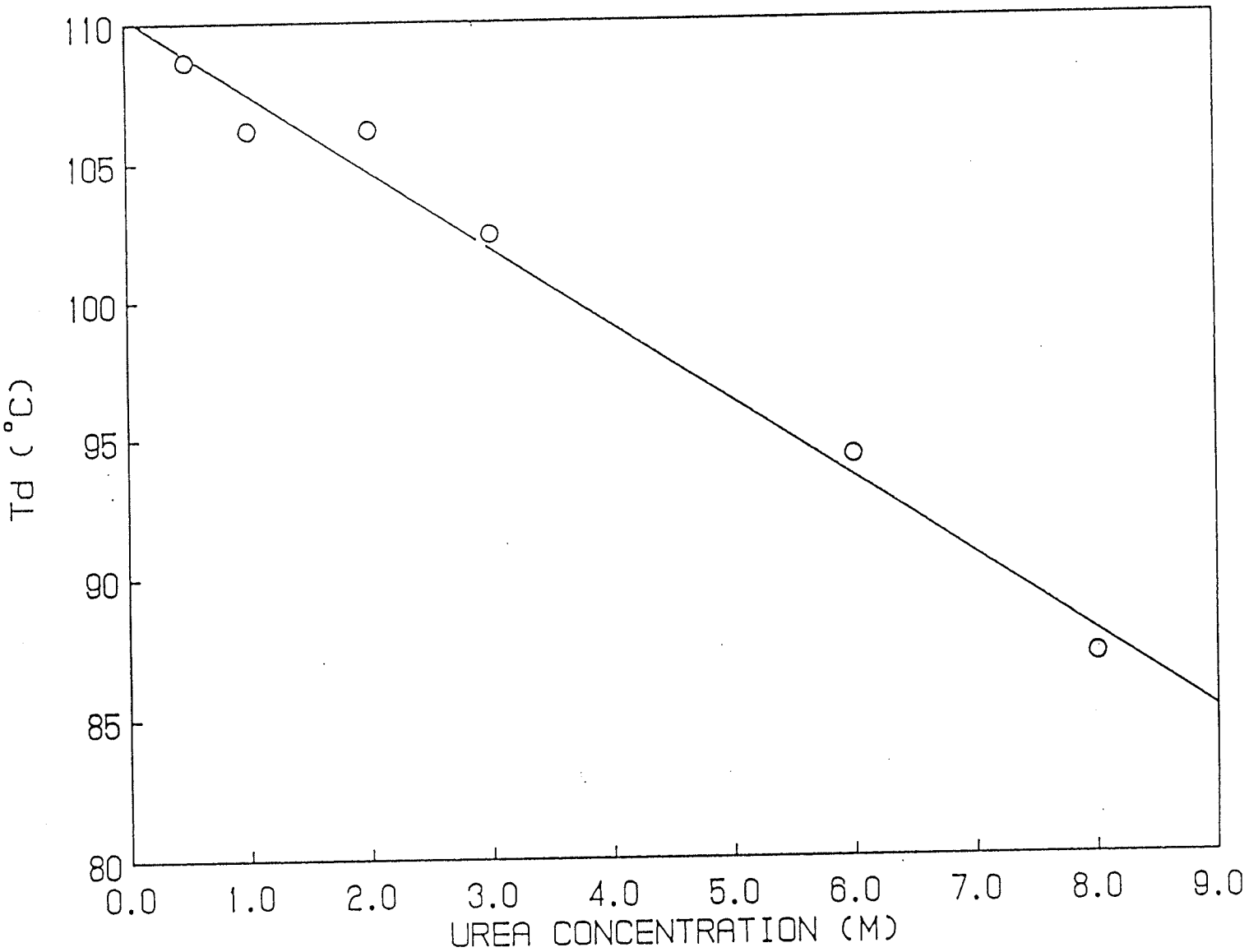
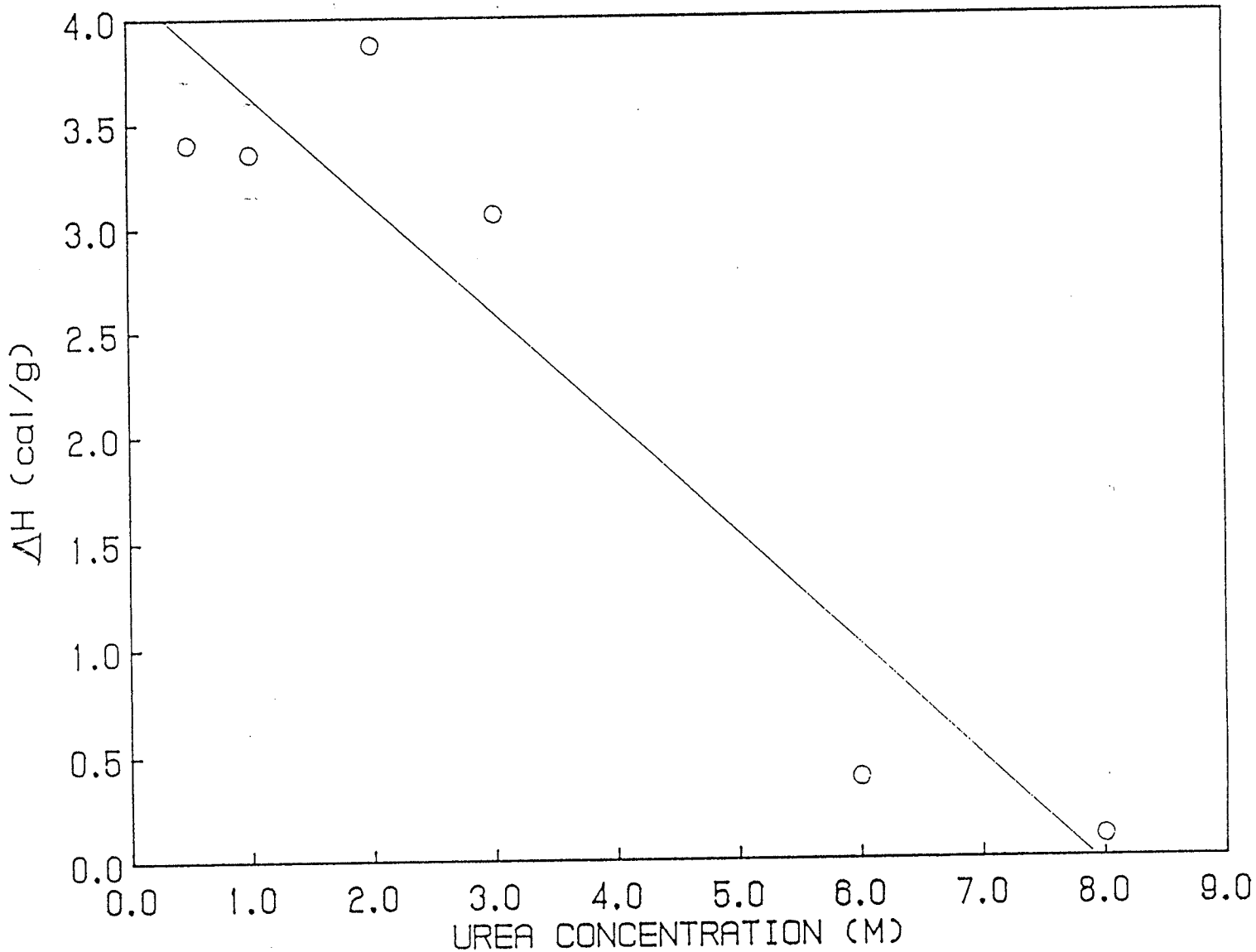

110
105
100
95
90
85
80
Td (°C)
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
UREA CONCENTRATION (M)

Figure 2.8. The effect of increasing the concentration of urea on the enthalpy of denaturation (ΔH) of legumin.


4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
ΔH (cal/g)
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
UREA CONCENTRATION (M)

DISCUSSION

Specific proteins, in spite of conformational limitations, have the capacity to self-associate into a micelle arrangement under certain environmental conditions (Murray et al., 1978; Simons et al., 1978; Evans and Philips, 1979; Ismond et al., 1986a,b). A major factor in this micelle response is the environmental surroundings. Amphiphilic protein molecules are dynamic and flexible; conformational changes can occur with even minor environmental fluctuations (Cooper, 1980). In order to achieve a micelle arrangement, it would appear that a specific intermolecular hydrophilic-hydrophobic balance is required (Tanford, 1973). Slight environmental modifications may alter this balance such that micelle formation no longer can occur.

Earlier studies have indicated that self-association into a micelle arrangement may be attributed to protein hydrophobic interactions (Murray et al., 1978; Ismond et al., 1986a,b). With this recognition, it was initially assumed that the self-associating molecules would be highly hydrophobic in nature. However, in comparison with other globular proteins described by Bigelow (1967), legumin, a molecule capable of extensive micelle formation, exhibited a low Bigelow hydrophobicity (819.46 cal/a.a. residue; Table 1.9) and a high charge frequency (0.46, Table 1.9). This was similar to the situation with vicilin, another fababean storage protein (Ismond, 1984). In addition, legumin was characterized by a relatively moderate surface hydrophobicity - both in terms of aliphatic (S_c = 44.7 to 142.0) and aromatic (S_a

= 42.6 to 561.6) values. Although the low Bigelow hydrophobicity values for legumin seem to be related to the moderate So values, there is not a correlation between these two parameters for globular proteins in general (Nakai, 1983).

As a result of these low hydrophobicity values, it would appear initially that legumin is not an ideal protein for micelle formation. However, it must be pointed out that a definite intramolecular hydrophilic-hydrophobic balance is necessary for micelle formation (Tanford, 197?). For example, Simons et al. (1978) suggested that bacteriorhodopsin, an integral membrane protein, would not aggregate into a micelle structure as a result of its high molecular hydrophobicity. With extreme hydrophobicity, protein molecules cannot form spherical aggregates with an extenstive enough hydrophilic surface to retain a micelle arrangement. From this, it would appear that an appropriate molecule for micelle formation would be one similar to legumin in which there is a specific intramolecular balance of hydrophilic and hydrophobic residues. However, this surface balance depends on the nature of the environment; the flexible conformation of a dynamic protein means that the critical balance may be disrupted with minimal environmental changes.

Types of Micelle Responses with Legumin

With the examination of micelle formation by legumin in a number of different environments, it was apparent that legumin

could self-aggregate into a variety of forms from single micelles to extensive molecular networks. As a result, micelle formation by legumin was "rated" according to a scale developed originally for vicilin from fababeans (Ismond et al., 1986a).

The general concept of a variety of different end-points from the self association phenomenon warrants some consideration. Original self-aggregation into a population of discrete micelles is considered to be a thermodynamically favorable event, resulting from intermolecular hydrophobic interactions (ratings 1, 2 and 3). The stabilization of a static population of discrete micelles is the result of a cooperative interaction of noncovalent forces. According to Tanford (1973), attactive forces must be dominant for the original formation of a micelle whereas surface repulsive forces must be dominant in order to restrict the micelles to a specific size. For legumin, the environmental media had a definite impact on the development of this surface repulsive phenomena.

In some situations, it was possible to not have the development of a major repulsive situation (rating 5). Interaction occurred until there were extensive amorphous masses of protein with complete phase separation. In order for this type of interaction to occur, there had to be a microenvironment established around the individual micelles with minimized repulsive forces and maximized attractive interactions.

In other cases, there was an intermediate association pheno-

menon (rating 4). Discrete micelles were followed by some coalescence which reached a static end-point without continuing on to the development of amorphous protein masses. At some point in the interaction, repulsive electrostatic forces dominate such that possible hydrophobic associations are minimized.

In order to examine electrostatic and hydrophobic contributions to the various types of micelle interactions, the capacity of legumin to form micelles was examined in a variety of environments. The impact of the environment was considered from two viewpoints - firstly, the influence of a specific environment on selected conformational aspects of legumin and its general capacity to form micelles; secondly, the effect of the environment on the degree and nature of intermicelle association. In both of these approaches, several types of environments were examined. Charge effects were assessed through changes in pH. Various electrolyte effects were examined from an electrostatic ($\mu < 0.5$) and a lyotropic ($\mu > 0.5$) viewpoint. In addition, the stabilizing and destabilizing effects of the salts were considered, basically from the perspective of relative position in the Hofmeister (1888) series. Finally, the influence of protein denaturation on micelle forming ability was examined.

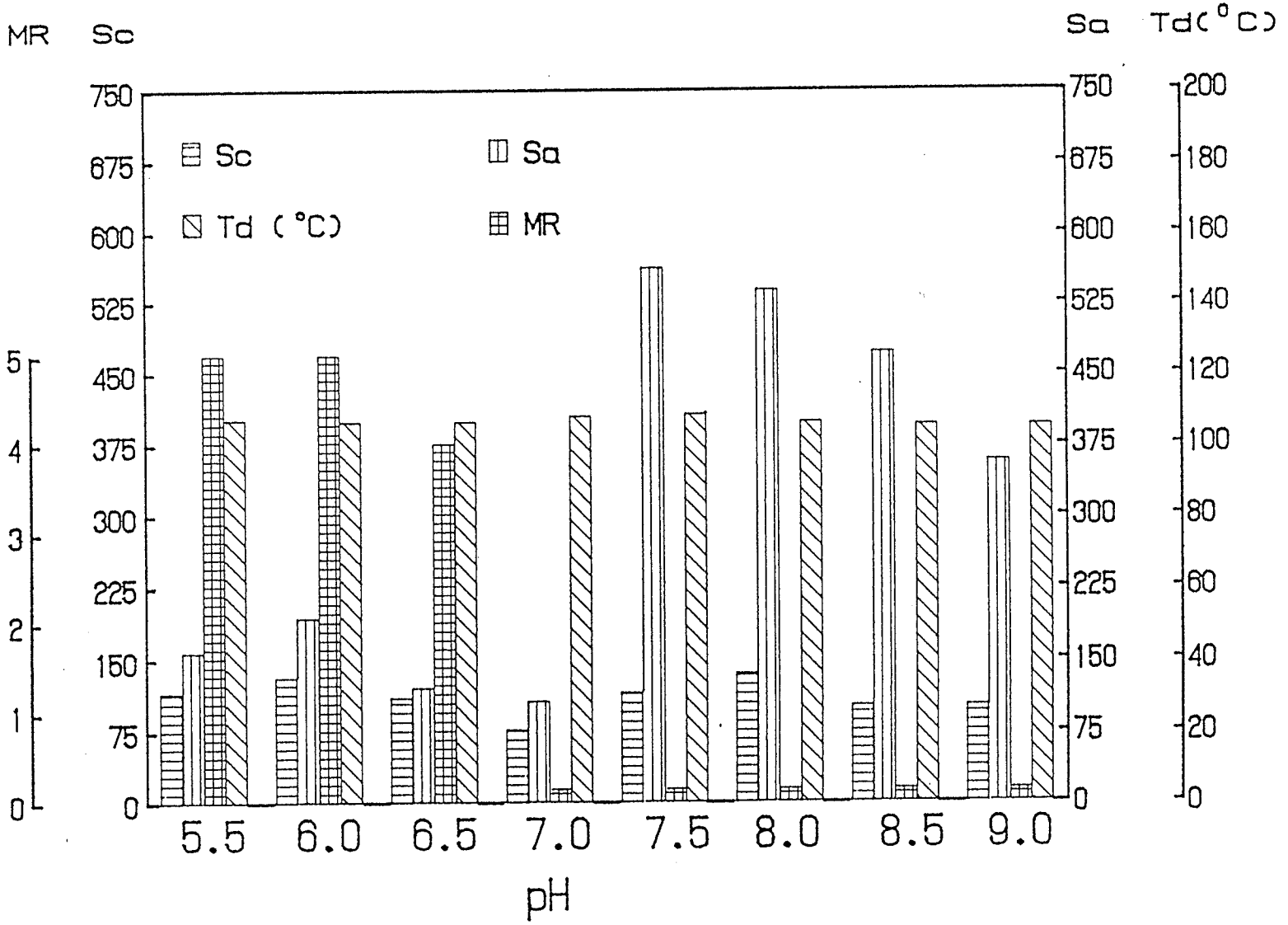
The Effect of pH on Micelle Formation by Legumin

The electrostatic properties of proteins have been recognized for many decades and protein precipitation at electrostatic

neutrality (i.e. the apparent isoelectric point) has been employed in different applications. Moreover, it is known that if the pH of the environment surrounding the protein is changed to either acid or alkali, the protein will assume a net positive or negative charge as a result of the ionization of reactive groups. In addition, it is probable that interprotein hydrophobic interactions will be suppressed due to the increased molecular distances. With these aspects in mind, specific legumin conformational parameters and micelle forming capacities were assessed at different pH levels. Extreme pH values were avoided due to potential denaturation.

From the micelle observations, the optimum structural characteristics of legumin for micelle formation and interaction existed from pH 5.5 to 6.5, with some deterioration in response at pH 6.5 (Figure 2.9). This maximum micelle response occurred near the isoelectric point (pH 4.7) where the net surface charge is effectively neutralized and hydrophobic interactions are maximized. From pH 7.0 to 9.0, however, there was a complete absence of micelle response. At pH 7.0, both aliphatic and aromatic hydrophobicity values (S_c and S_a) decreased significantly from the values at the lower pH levels. From these results it would appear that legumin conformation is not ideal for micelle formation if hydrophobic interactions are suppressed due to a decrease in S_c and S_a values. The observed importance of the surface hydrophobicity in general is supportive of the original premise by Murray et al. (1978) that these micelle structures are pro-

Figure 2.9. Micelle ratings (MR), surface hydrophobicities (S_c, S_a) and denaturation temperatures (Td) for legumin exposed to a series of a 0.25 M phosphate buffers ranging in pH from 6.0 to 9.0.


MR
Sc
Sa
Td(°C)
Sc
Sa
Td (°C)
MR
750
675
600
525
450
375
300
225
150
75
0
5
4
3
2
1
0
200
180
160
140
120
100
80
60
40
20
0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
pH

ducts of hydrophobic associative forces.

In contrast to the surface hydrophobicity values at pH 7.0, there was a significant increase in both S_c and S_a values at pH 7.5. In fact, the aromatic surface hydrophobicity (S_a) reached the highest value observed in the pH study. The absence of the micelle response at this pH may be explained in terms of a critical hydrophobic-hydrophilic balance. As the pH is increased, the protein becomes more electronegative; as a result of increased electrostatic repulsion, the protein undergoes conformational change. Interestingly, this change is not sufficient to be reflected in the protein thermal parameters (Td, ΔH). In fact, the stable Td and ΔH values over the entire pH range studied preclude any major structural alterations. However, the conformational change occurring at pH 7.5 did result in an exaggerated aromatic hydrophobicity; this, in turn appeared to be detrimental to micelle formation. This observation does not represent a new concept. Simons et al. (1978) suggested that bacteriorhodopsin, an integral membrane protein, would not self-associate into a micelle structure as result of its high molecular hydrophobicity. With extreme hydrophobicity, protein molecules cannot form spherical aggregates with an extensive enough hydrophilic surface to retain a micelle arrangement. For micelle formation it would appear that a specific intra- and interprotein hydrophobic-hydrophilic balance is critical (Ismond, 1984).

As the pH was raised from 7.5 to 9.0, the micelle response was still not observed. Several phenomena can be commented on at

these pH values. Firstly, the aromatic hydrophobicity (S_a) values were still high, possibly resulting in an unfavorable shift in the hydrophilic-hydrophobic balance. Secondly, the overall electronegativity became increasingly important in the micelle response. In addition to increasing the overall protein negative charge, there was a change in the electrolyte environment with an increase in pH. As the pH was changed from 7.5 to 9.0, the balance between the univalent phosphate ion ($H_2PO_4^-$) and the divalent phosphate (HPO_4^{-2}) ion would be expected to shift with an increase in pH from a predominant $H_2PO_4^-$ at pH 6.0 to a predominant HPO_4^{-2} at pH 8.0. As a result of the presence of the divalent anion, the entire system of protein and solvent becomes more electronegative as the pH is increased to pH 9.0. This extensive electrostatic repulsion is not conducive to hydrohobic associations and subsequent micelle formation. The pH induced surface hydrophobicity results were not a product of the impact of the environment on the probe protein relationship. There was no change in the association of tryptophan with ANS and leucine with CPA as the pH was varied from 6.0 to 9.0 (Ismond, unpublished results).

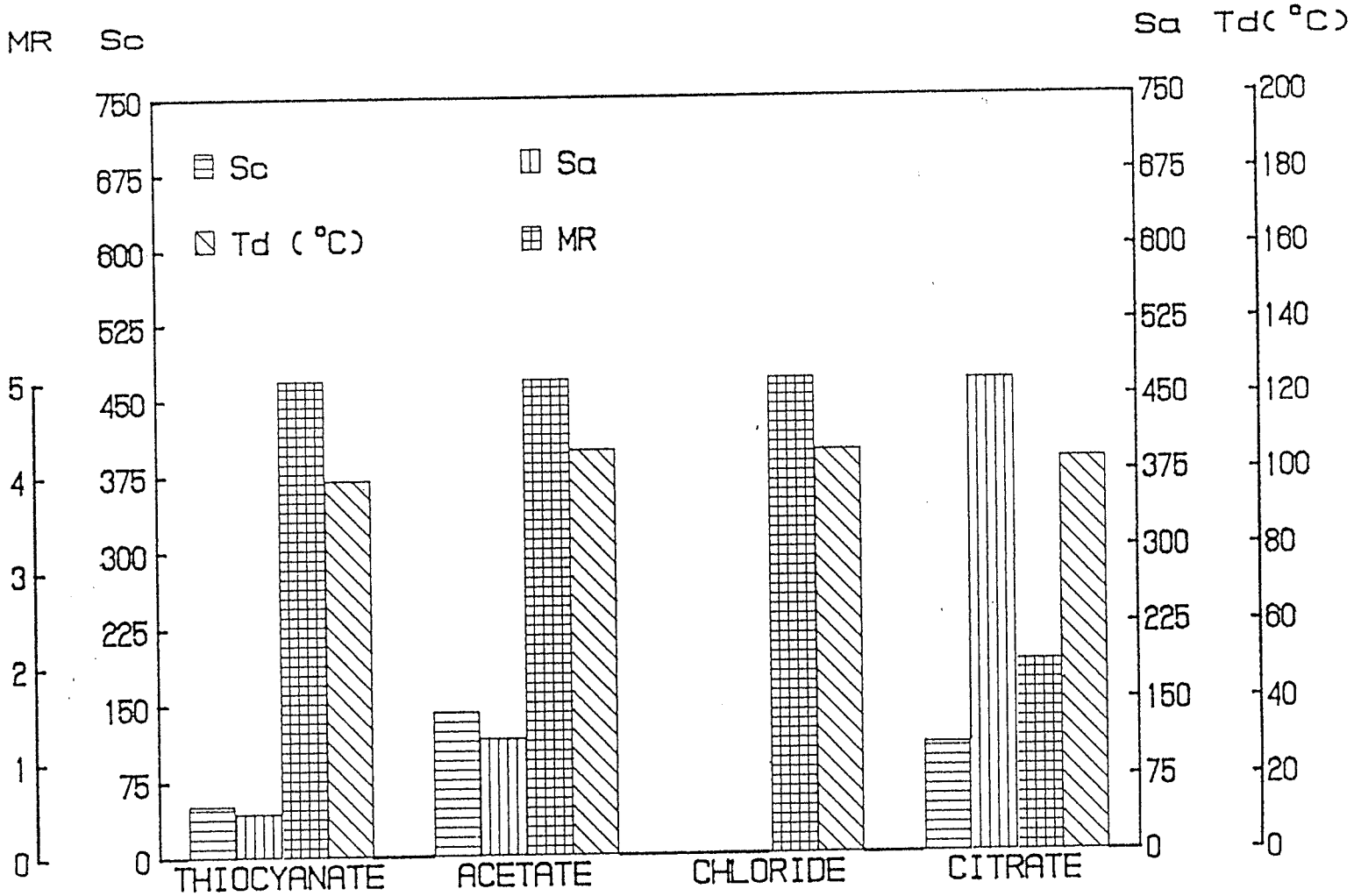
Effects of Specific Salts on Legumin Stability and Micelle Forming Capacity

In general, the effects of different electrolytes on protein-protein interactions have been attributed to variations in the anion component (Robinson and Jenks, 1965). This is a result

of the different hydration phenomena. With cations, the extensive hydration shells result in their almost complete exclusion from the water layer at the protein interface. In contast, anions are quite variable in their extent of exclusion due to their limited hydration spheres (Eagland, 1975). With respect to the anions alone, it is acknowledged that both the identity and the concentration of the anion are important in assessing protein conformational parameters. At low concentrations ($\mu<0.5$), anions may be considered as a collective group with their effects attributed to electostatic interactions related to the polar polyionic nature of the protein (von Hippel and Schliech, 1969). Most anions, therefore, at low concentrations have a salting-in effect with the extent of the electrostatic influence related to the ionic strength of the salt as well as the distribution and density of charged groups on the protein surface (von Hippel annd Schliech, 1969).

In this study, however, at μ = 0.25, there was a variation in the impact of the different anions on both conformational properties and micelle forming capabilities (Figure 2.10). The Td and ΔH values for legumin in 0.25 M sodium thiocyanate were significantly lower than those for legumin in sodium chloride or sodium acetate (Table 2.4, Figure 2.10). The destabilizing influence of thiocyanate, apparent even at low concentraions of the anion, may be related to its capacity to exhibit a high degree of nonspecific binding to a number of exposed protein polar sites (Arakawa and Timasheff, 1982).

Figure 2.10. Micelle ratings (MR), surface hydrophobicities (S_c, S_a) and denaturation temperatures (Td) for legumin exposed to a series of sodium salts at $\mu = 0.25$.


MR
Sc
Sa
Td(°C)
Sc
Sa
Td (°C)
MR
750
675
600
525
450
375
300
225
150
75
0
5
4
3
2
1
0
200
180
160
140
120
100
80
60
40
20
0
THIOCYANATE
ACETATE
CHLORIDE
CITRATE

For the three anions, thiocyanate, chloride and acetate, the micelle reaction was extensive (rating 5; Figure 2.10). In these situations, the sudden dilution of the concentrated legumin solutions may have resulted in a physical disturbance of the electrical double layer associated with the protein molecules. Therefore, intermolecular association of legumin by hydrophobic associations would be thermodynamically favorable in response to the aqueous environment if the protein surface hydrophobicity were adequate. As a further consequence of the dilution, electrostatic effects on the micelle surface may have been minimized to reduce intermicelle repulsive forces such that further interactions were favored.

For sodium citrate (μ = 0.25), the micelle response was reduced (rating 2) in comparison with the other three media (Figure 2.10). This may be related to the high aromatic hydrophobicity (S_a) observed - a value of 466.0 for citrate as compared to 115.0 and 42.6 for acetate and thiocyanate, respectively. The exaggerated hydrophobicity may not be favorable for extensive micelle formation, as discussed previously in conjunction with the pH results. The extensive exposure of originally internal hydrophobic side-chains may disturb the orientation of the hydrophobic-hydrophilic residues such that micelle arrangement is more difficult.

As the salt concentration is increased to higher levels (μ > 0.5), the identity of the anion becomes more important whereas

the protein charge status becomes less significant. With respect to charge at high salt levels, electrostatic interactions between charged residues are effectively neutralized by extensive ionic shielding (Kirkwood, 1943). As a result, the protein behaves as a neutral dipole; general anionic electrostatic influences become less important. In terms of the identity of the anion, at high concentration levels, anions in general can be divided into two categories - stabilizing and destabilizing anions. Those identified as destabilizing tend to promote protein solubility with increasing destabilization as salt concentrations are increased. On the other hand, stabilizing anions tend to stabilize conformational properties; at high concentrations, these anions cause protein precipitation as a consequence of hydrophobic associations (von Hippel and Wong, 1964).

The mechanism of action of the two types of salts has received considerable discussion. In general the effect of a stabilizing anion on protein structure seems to be related to its ability to cause preferential hydration of the protein surface (Arakawa and Timasheff, 1982) in addition to its capacity to increase the surface tension of the protein environment (Melander and Horvath, 1977). Therefore, as the concentration of the salt is increased, the salt-water interaction results in a gradual elimination of the salt from the protein surface. This effect results in an environment in which it is thermodynamically unfavorable for the protein to unfold and expose hydrophobic residues. In general, this is referred to as a lyotropic effect. In this study, citrate, chloride and acetate anions fall into the

stabilizing category.

In contrast, destabilizing anions are characterized by a reduced capacity to increase the surface tension of water (Melander and Horvath, 1977) and consequently, by a lower ability to induce protein preferential hydration (Arakawa and Timasheff, 1982). In fact, destabilizing anions appear to remain bound to the protein surface even at high concentration levels (Bull and Breese, 1970; Arakawa and Timasheff, 1982). This preferential binding results in increased electrostatic disturbances followed by conformational destabilization. In this study, thiocyanate was considered to be a destabilizing anion.

For the stabilizing salts in this study, there was a positive relationship between thermal stability for legumin, as assessed by Td values, and the concentration of various anions (Table 2.4). Within the stabilizing group there was some variation in the capacity of the anions to affect the overall thermal stability. Using Td and ΔH values as guidelines, the relative effectiveness of the anions could be described as:

chloride, acetate > citrate

This differs from the original Hoffmeister series (1888) and that put forward by more recent researchers (Robinson and Jencks, 1965). In these studies, citrate is recognized as a highly stabilizing anion with acetate and chloride as moderately stabilizing anions.

Thiocyanate, as expected, functioned as a destabilizing salt. There was a significant decrease in both Td and ΔH values as the salt concentration was increased to μ = 1.0 (Table 2.4; Figure 2.4). In addition, there was a significant increase in surface hydrophobicity (both aromatic and aliphatic values) with an increase in thiocyanate concentration. This may reflect the increased exposure of hydrophobic residues as a result of extensive electrostatic disturbances with thiocyanate binding to the surface of legumin.

In addition to conformational parameters, the high salt levels impacted the capacity of legumin to self-associate into a micelle arrangement. At μ = 0.5, micelle interactions were identical to those observed at μ = 0.25 (Figure 2.11); in contrast, there was a significant deterioration in the individual responses at μ = 1.0 (Figure 2.12). Although decreases in the micelle response were observed for each salt, the actual mechanisms causing the decrease may vary with the individual environments. With thiocyanate, the anion remains bound to the protein surface (Arakawa and Timasheff, 1982); this binding may persist after dilution to cause electrostatic repulsion or it may resume after dilution to create a charge repulsion among established micelles. In relation to previous results, the surface hydrophobicity of legumin in thiocyanate (μ = 1.0) appeared to be appropriate for micelle formation.

For the two moderately stabilizing salts, acetate and chloride, there was a decrease in the micelle response to rating 2 at

Figure 2.11. Micelle ratings, surface hydrophobicities (S_c, S_a) and denaturation temperatures (Td) for legumin exposed to a series of sodium salts at $\mu = 0.5$

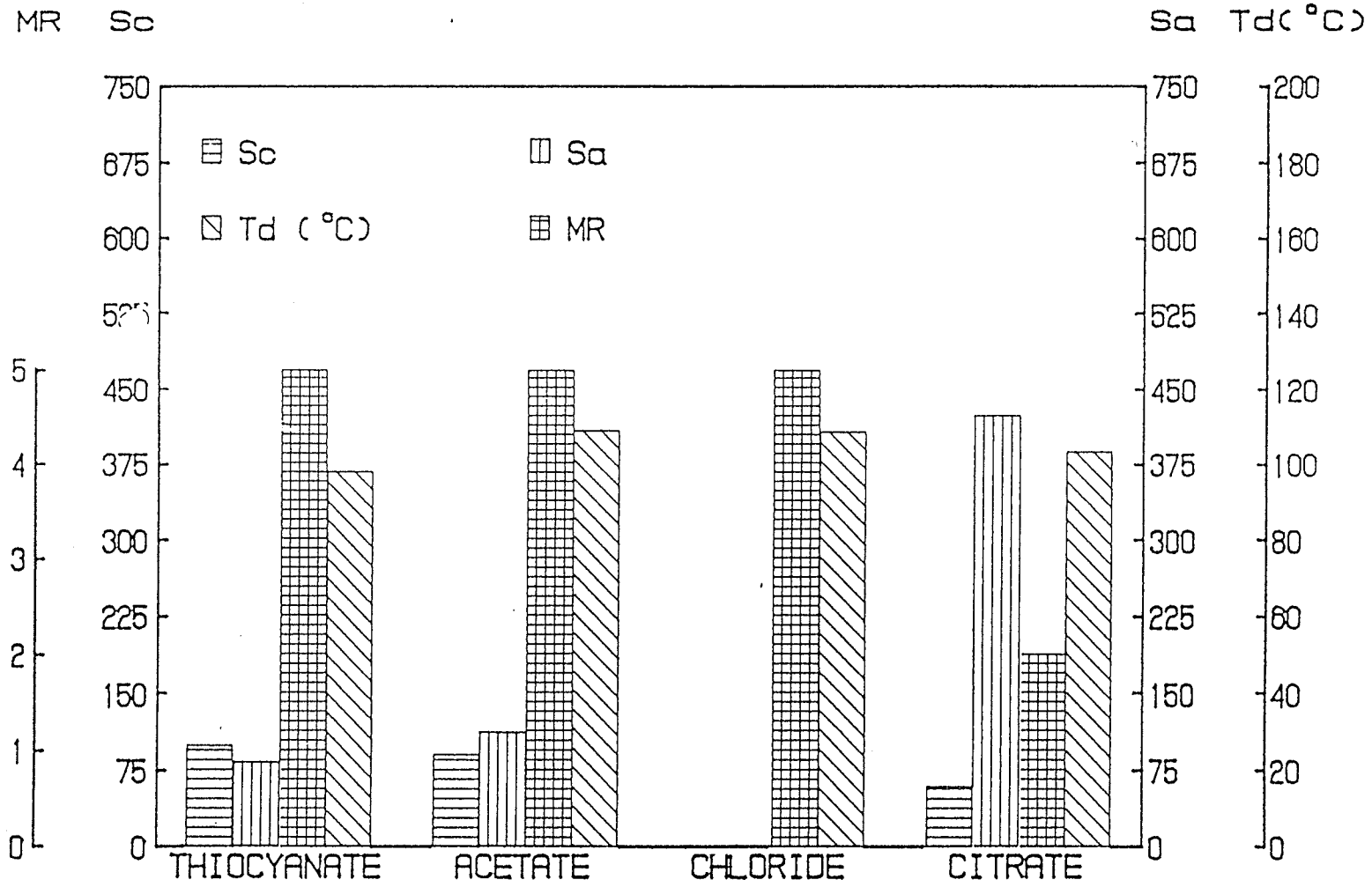
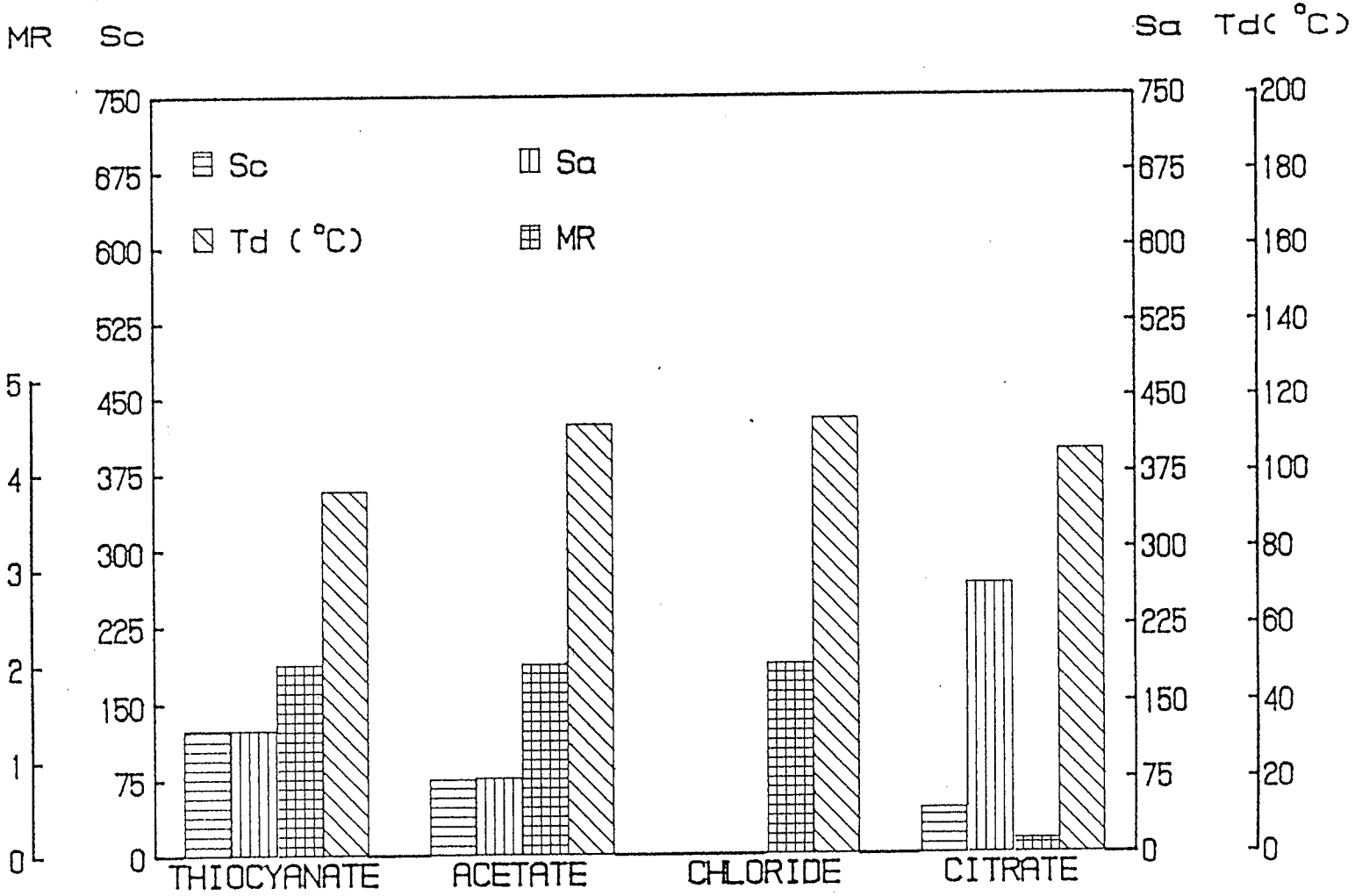

MR
Sc
Sa
Td(°C)
Sc
Sa
Td (°C)
MR
750
675
600
525
450
375
300
225
150
75
0
5
4
3
2
1
0
200
180
160
140
120
100
80
60
40
20
0
THIOCYANATE
ACETATE
CHLORIDE
CITRATE

Figure 2.12. Micelle ratings (MR), surface hydrophobicities (S_c, S_a) and denaturation temperatures (Td) for legumin exposed to a series of sodium salts at $\mu = 1.0$.


MR
Sc
Sa
Td(°C)
Sc
Sa
Td (°C)
MR
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675
600
525
450
375
300
225
150
75
0
5
4
3
2
1
0
200
180
160
140
120
100
80
60
40
20
0
THIOCYANATE
ACETATE
CHLORIDE
CITRATE

μ = 1.0 (Figure 2.11). Both salts are known to promote some predilution preferential hydration of the surface of proteins in general (Arakawa and Timasheff, 1982). For legumin, this phenomenon was reflected by the significant decrease in surface hydrophobicity (S_a and S_c) with increasing acetate concentations. This hydration effect, however, may have been reduced by specific binding of the anions to the protein surface (Arakawa and Timasheff, 1982). As a result, the reduced surface hydrophobicity did not allow extensive molecular association. Secondly, postdilution binding of acetate and chloride to the protein surface may have created prohibitory electrostatic effects that did not encourage massive association of the formed micelles.

Sodium citrate as a stabilizing salt also caused a decrease in micelle response to a zero rating at μ = 1.0 (Figure 2.12). This was accomplished by a significant decrease in the surface hydrophobicity (both S_c and S_a) with increasing salt concentration, presumably as a result of preferential hydration of the protein surface. Although the aromatic hydrophobicity was still high in relation to the other salts, the skewed hydrophobicity balance may still not be appropriate for micelle formation and interaction. The anion induced surface hydrophobicity results were not a product of the impact of the environment on the probe-protein relationship. In preliminary studies, there was no change in the association of tryptophan with ANS and leucine with CPA with the electrolyte media used in this study.

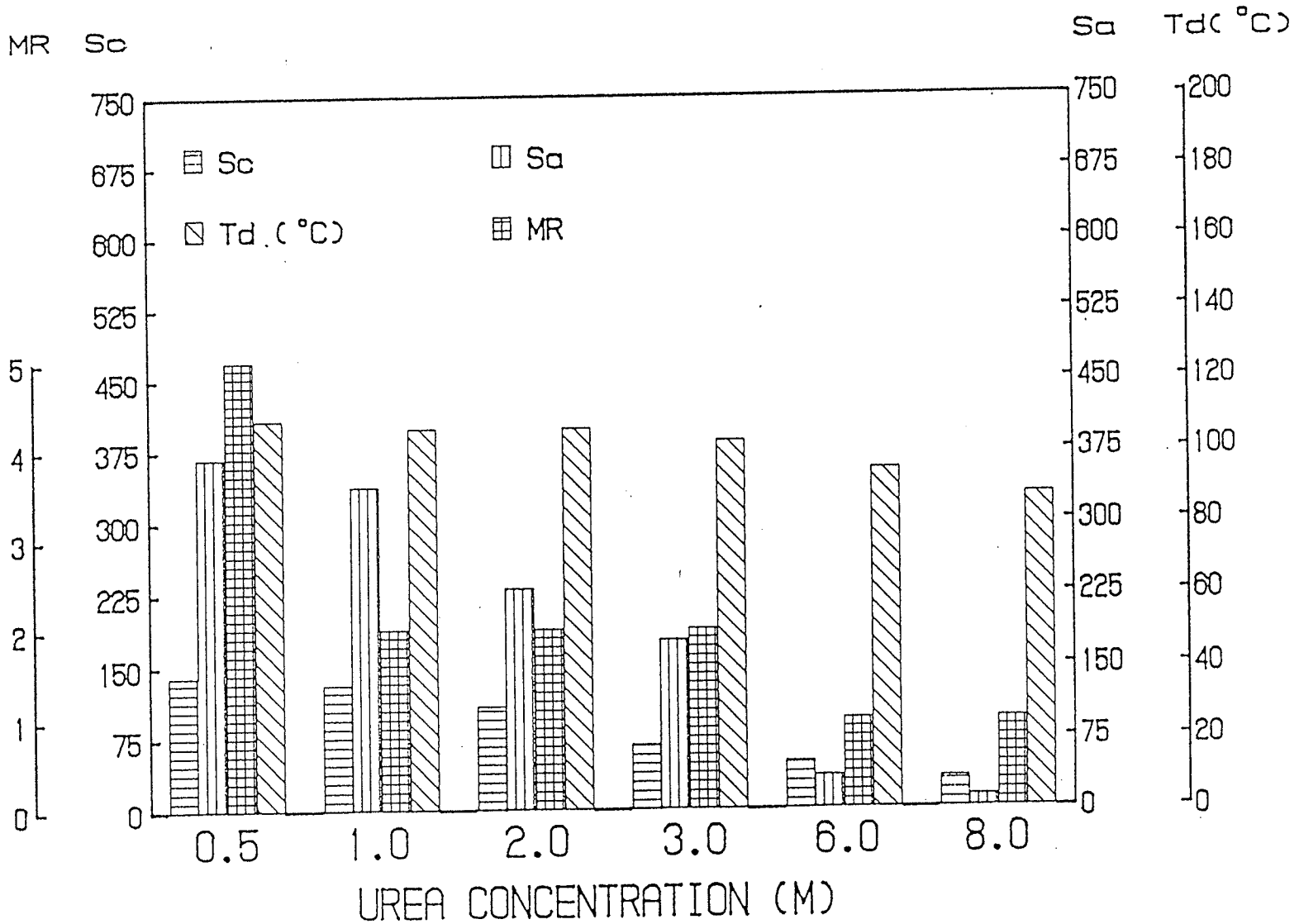
The Effect of Urea on Micelle Formation by Legumin

The last environment chosen for assessing the capacity of legumin to form micelles included the denaturant, urea. The rational for this experimentation was to investigate the micelle phenomenon, in terms of the delicate hydrophilic-hydrophobic balance required, with environments of different denaturing capacities. The impact of disrupting noncovalent interactions was monitored in terms of the conformational stability and the micelle response of legumin as a function of varying levels of urea concentration.

An initial extensive micelle response (rating 5) was observed with legumin exposed to 0.5 M urea (Figure 2.13). With the increase in concentration to 1.0 M urea, the micelle response deteriorated significantly (rating 2). Conformational disturbances were evident in terms of a significantly decreased Td value; however, other parameters (ΔH, S_c, S_a) were constant. Although the conformational changes appeared minimal, there was significant change in the molecular interactive capacity to result in a reduced micelle response. Sensitivity to low urea concentrations has been reported for other proteins. For example, Yao et al. (1984) observed that creatine kinase was inactivated at urea levels that had no influence on protein conformation as monitored by UV spectroscopy, fluorescence and exposure of sulfhydral groups.

The micelle response deteriorated further as the urea level

Figure 2.13. Micelle ratings (MR), surface hydrophobicities (S_c, S_a) and denaturation temperatures (Td) for legumin exposed to a series of urea concentrations ranging from 0.5 to 8.0 M.


MR
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Td(°C)
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Td.(°C)
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375
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225
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75
0
5
4
3
2
1
0
200
180
160
140
120
100
80
60
40
20
0
0.5
1.0
2.0
3.0
6.0
8.0
UREA CONCENTRATION (M)

was increased from 1.0 (rating 2) to 8.0 M (rating 1). This was parallelled by linear decreases in S_c, S_a, Td and ΔH. In terms of the thermal parameters, a reduction in Td is usually correlated with molecular destabilization. Complete denaturation is reflected by low ΔH values. Privalov and Khechinashvili (1974) stated that the denaturation enthalpy for globular proteins is the result of two opposite thermodynamic effects - an exothermic contribution resulting from the disruption of hydrophobic associations with denaturation and an endothermic contribution resulting from the dissolution of hydrogen bonds. Any decrease in the ΔH value reflects a change in the original conformation of the protein prior to the calorimetric denaturation.

The decrease in both aromatic and aliphatic hydrophobicity values was unexpected; deterioration of conformation to a random coil should be parallelled by an increase in the exposure of hydrophobic residues. When a globular protein undergoes denaturation, hydrophobic groups buried on the inside of the globular structure are exposed to the outside environment. However, the decreased hydrophobicity values observed here may reflect intermolecular aggregation as denaturation progressed. Other studies have shown noncovalent associations of various proteins with denaturant-induced unfolding (Prakish and Nandi, 1977; Fish et al., 1985).

On the other hand, urea molecues may have oriented themselves around exposed hydrophobic groups in the protein to create

a urea hydration sphere. This may have resulted in stearic hindrance such that the moderately bulky probes, CPA and ANS, were excluded from the protein hydrophobic residues. As the level of urea was increased, the exclusion of the probes was also increased. However, this possibility is unlikely from the results of preliminary studies - fluorescence values using tyrosine and tryptophan with ANS plus valine and leucine with CPA were constant over the entire range of urea concentrations (i. e. 0.5 to 8.0 M).

With the complete denaturation of legumin by urea, the micelle response deteriorated considerably. This may be attributed to a critical decrease in exposed hydrophobic residues as a result of intermolecular aggregation prior to the induction of the micelle response. This is a reasonable conclusion if micelle formation is considered to be a hydrophobically driven event (Murray et al., 1978).

In summary, micelle formation and subsequent interaction was impacted by the environment. Firstly, the pH of the surrounding medium had to be low enough to minimize electrostatic repulsion yet high enough to allow protein solubilization. In this respect, a pH range of 5.5 to 6.5 was identified as appropriate. Secondly, the concentration and identity of the environmental anions affected micelle formation. At lower salt concentrations (μ = 0.25 and 0.5), the exposure of a specific level of hydrophobic residues appeared critical. Exaggerated hydrophobicity values, as observed with the citrate anion, were detrimental to the micelle response.

At higher concentrations (μ = 1.0), increased binding of the anion (chloride, acetate and thiocyanate) to the protein surface created an electrostatic environment unfavorable to the micelle phenomenon. Thirdly, gradual denaturation of legumin by increasing urea concentrations resulted in low hydrophobicity values. This molecular aggregation prior to the introduction of an aqueous environment had a negative impact on the establishment of micelle-based structures. In all cases, a delicate balance of hydrophobic-hydrophilic noncovalent forces appeared critical to micelle formation and subsequent interaction.

CONCLUSIONS

A study system involving a single protein species was established by the selective isolation of the seed storage protein legumin from fababean, *Vicia faba* var. *Diana*. The ultimate purpose of this study was to examine the noncovalent forces involved in the self-association of legumin into a micelle arrangement. However, prior to this consideration, some appreciation was derived for the physical properties of the protein. In general, legumin was found to be a multimer (MW 363,000, pI = 4.7) formed by the noncovalent association of two main subunits with molecular weights of 54,100 and 41,600. Under reducing conditions, two main subunits with molecular weights of 39,200 and 17,300 were apparent.

This multimeric legumin appeared to be an appropriate protein for micelle formation on the basis of molecular surface properties, with specific reference to the distribution of hydrophilic and hydrophobic residues. Distortion of this hydrophobic-hydrophilic balance by charge manipulation, changes in the electrolyte environment, and progressive denaturation impacted micelle formation. In general, for micelle formation and interaction to occur, noncovalent intermolecular attractive forces had to predominate over repulsive interactions. The main attractive forces appeared to be hydrophobic in nature; the sudden introduction of a controlled volume of water was essential for micelle responses. As further evidence for this supposition, the exposure of a certain number of surface hydrophobic residues appeared to be critical for intermolecular association. For

example, both extensive hydrophobic exposure as occurred in the citrate environment and reduced hydrophobic residues as seen with increasing urea concentrations decreased intermolecular associations. In contrast, the main repulsive force appeared to be electrostatic with the magnitude of the repulsion related to the relative ionization of various amino acid residues and/or interactions of specific ions with individual protein molecules at the micelle surface. As a result, a balance of noncovalent forces was integral to micelle formation and subsequent interaction.

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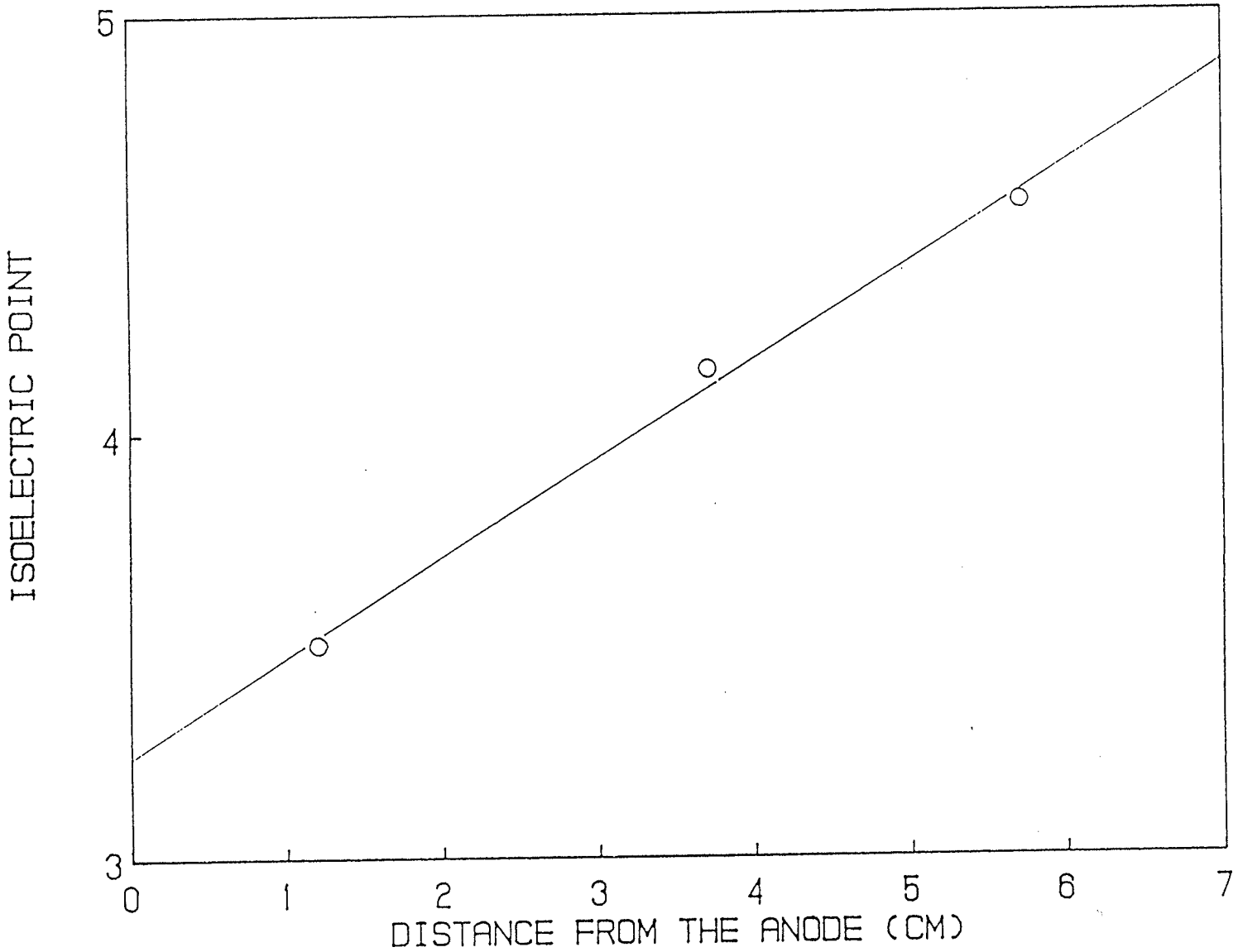
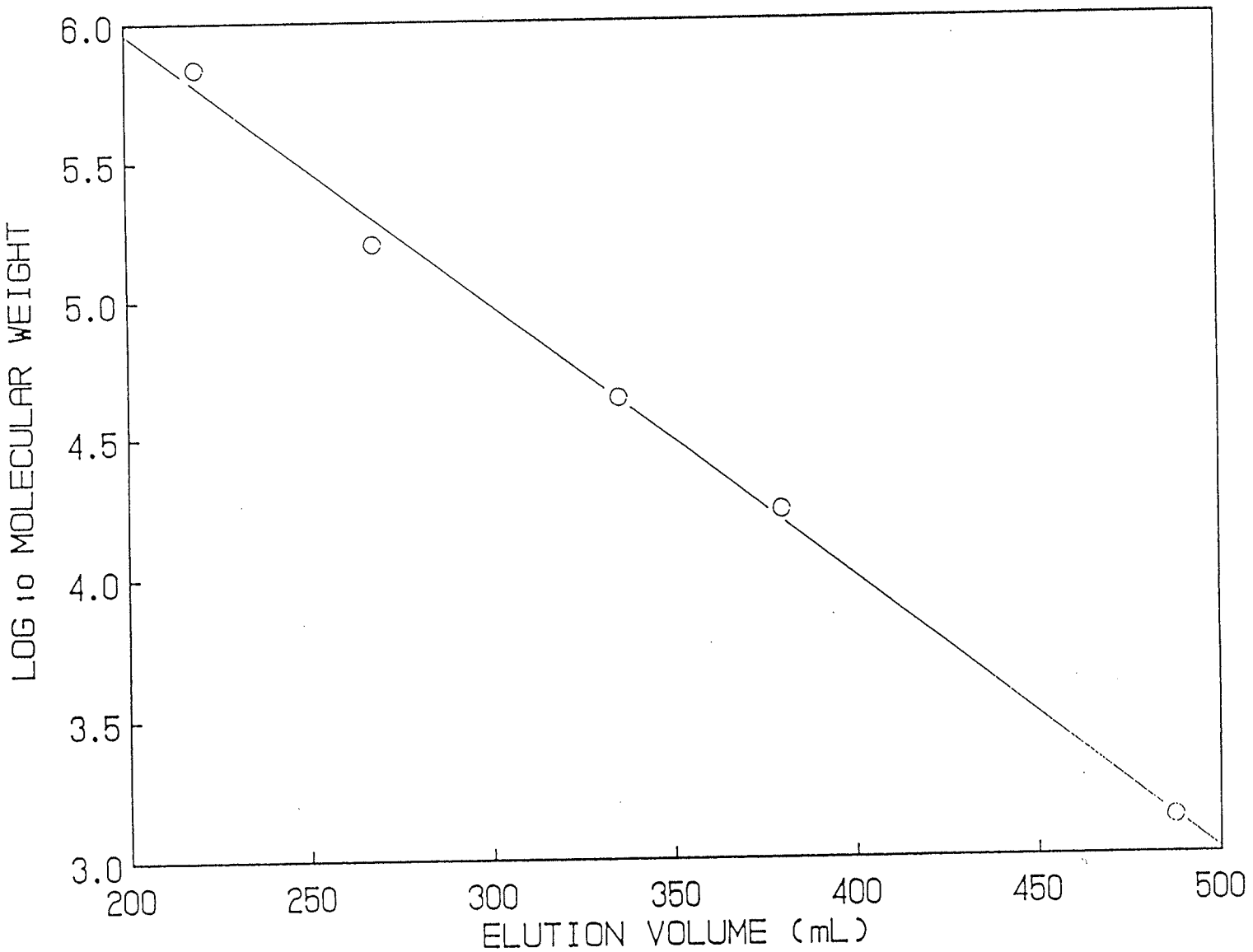
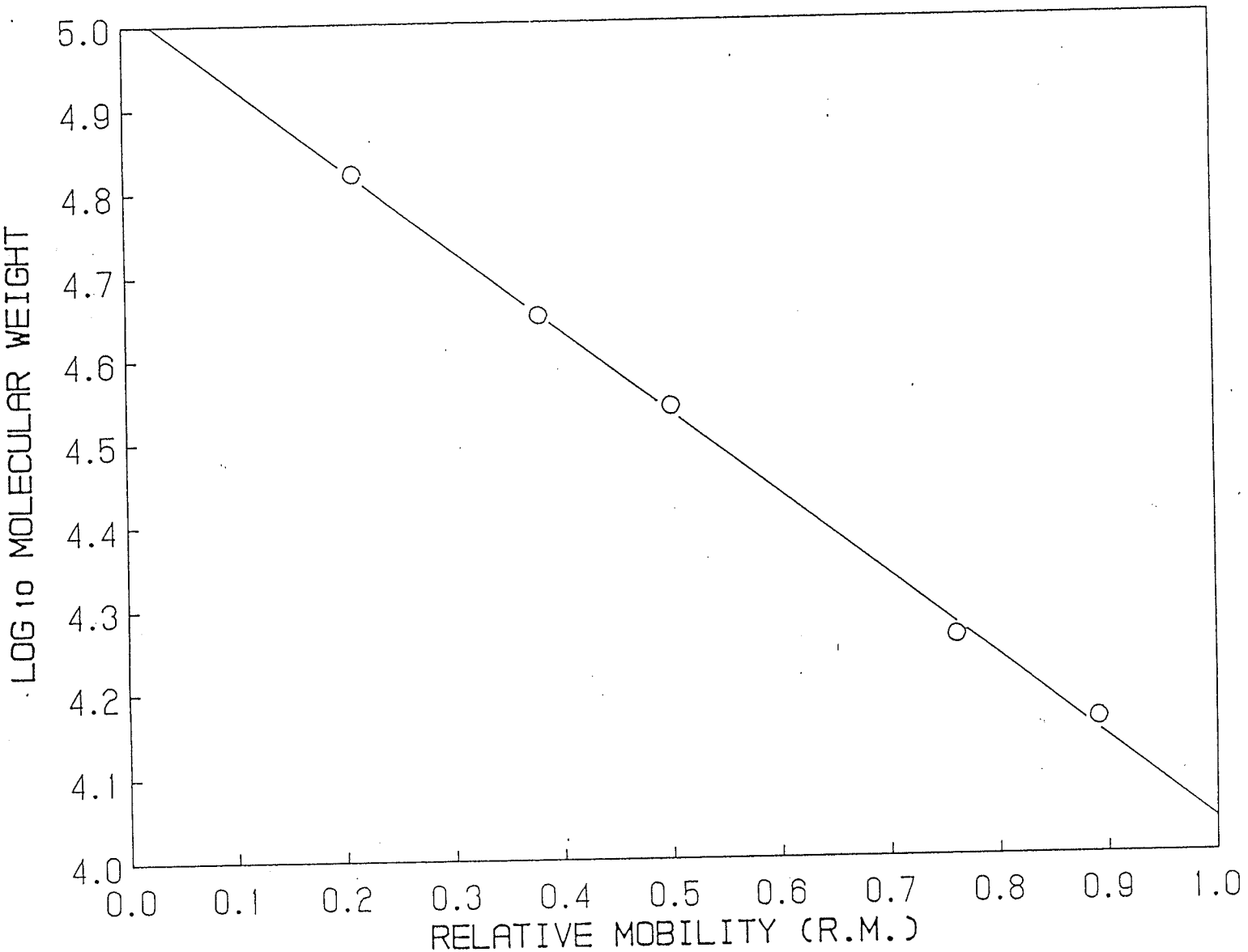
Appendix I. Calibration curve for isoelectric point determination of legumin by isoelectric focusing.


ISOELECTRIC POINT
5
4
3
0
1
2
3
4
5
6
7
DISTANCE FROM THE ANODE (CM)

Appendix II. Calibration curve for molecular weight determination of legumin by gel filtration.


6.0
5.5
5.0
4.5
4.0
3.5
3.0
LOG 10 MOLECULAR WEIGHT
200
250
300
350
400
450
500
ELUTION VOLUME (mL)

Appendix III. Relative mobility (R.M.) of SDS-PAGE protein standards as a function of $\log_{10}$ molecular weight.


LOG 10 MOLECULAR WEIGHT
5.0
4.9
4.8
4.7
4.6
4.5
4.4
4.3
4.2
4.1
4.0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
RELATIVE MOBILITY (R.M.)