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The Rôle of Non-Covalent Forces in Micelle Formation by Vicilin from *Vicia faba*. The Effect of pH Variations on Protein Interactions

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ABSTRACT

The influence of environmental pH on the capacity of vicilin from fababeans to self-associate into a micelle arrangement was investigated. Various types of micelle-based protein interactions were identified; these responses were related to certain vicilin conformational parameters, specifically, thermal stability and surface hydrophobicity (So). The most extensive micelle responses were observed from pH 6·0 to 6·8; a gradual deterioration in the degree of protein self-association occurred with pH increases from 6·8 to 8·0. These results corresponded with significant decreases in vicilin So, i.e. from 296 at pH 6·5 to 158 at pH 8·0. Thermal parameters over this range reflected minimal conformational disturbances. The observed importance of molecular surface hydrophobicity was supportive of the original premise that micelle structures are products of hydrophobic associative forces.

INTRODUCTION

Certain protein molecules have the capacity to aggregate into a micelle arrangement in response to an aqueous environment without the electrolyte intervention associated with the well recognized casein micelle (Murray *et al.*, 1978; Simons *et al.*, 1978; Evans & Philips, 1979). However, proteins, unlike lipids, have definite structural limitations in attempting to assume this thermodynamically favorable format.

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Hydrophilic and hydrophobic residues occur in scattered patches on the protein surface, often without showing definite polarity of arrangement (Klotz, 1970; Lee & Richards, 1971); in addition, relative amounts of these types of amino acid side-chains are highly variable among different proteins. As a further complication, protein surface properties are not constant; the dynamic flexible nature of a protein may allow considerable conformational variations due to environmental changes. With these considerations, it appears that micelle formation by proteins must be a sensitive function of the structural characteristics of the interacting molecules and of environmental parameters which influence, strongly, protein surface properties.

Despite the sensitivity of this specific response, protein micelle formation has been demonstrated by Murray *et al.* (1978) to be fundamental in the development of a mild technique for the isolation of storage proteins from a variety of seeds. In this method, a protein concentrate (e.g. from fababeans (*Vicia faba*)), suspended in a high salt medium was exposed suddenly to an aqueous environment; the result was a massive precipitation of protein molecules aggregated in a micelle arrangement. Not only were the proteins isolated by this method in a highly native state but, in addition, the isolated material, designated as PMM (protein micellar mass), was approximately 96% protein (N × 5·85; Murray *et al.*, 1981). Furthermore, most of the problematic anti-nutritional factors associated commonly with fababean protein isolates were eliminated (Arntfield *et al.*, 1985).

With the recognition of micelle formation as a valuable method for the isolation of non-denatured protein, 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 association within a micelle are of fundamental concern. As an initial phase of this ultimate consideration, 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. In this respect, the storage protein, vicilin, was isolated from fababean seeds as a study molecule for micelle assessment. Environmental manipulation in the first phase of the investigation was restricted to controlled changes in pH.

In preliminary investigations it was noted that protein micelle formation was not an all-or-none phenomenon; that is, a population of micelles might undergo dynamic interaction to produce highly structured

networks and amorphous protein sheets. This was of interest from an applied viewpoint. Such a progression might be used in a situation requiring massive protein aggregation to give a three-dimensional framework, as is frequently necessary within specific food systems. From an experimental consideration, the possibility of various degrees of micelle-based protein interactions necessitated the establishment of a descriptive assessment scheme that could be used to identify, readily, a particular reaction pattern. With this consideration, the different types of molecular responses observed were grouped into subjective categories identified by a specific set of microscopic characteristics. Criteria for the establishment of these categories were based primarily on vicilin responses to a variety of electrolyte environments. A detailed study of these electrolyte effects is the subject of a subsequent paper (Ismond *et al.*, 1985*a*).

The established reaction categories were used to assess the interactive capacity of vicilin under different pH regimes. The capacity of vicilin to self-associate was, in turn, correlated with specific protein structural parameters in an attempt to understand further the forces involved in micelle-based interactions. The molecular properties of interest included thermal characteristics, as an indication of the degree of protein denaturation, and the relative exposure of hydrophobic surface residues, as an important consideration in the interaction of hydrophobic side-chains for micelle formation.

MATERIALS AND METHODS

Protein isolation

An initial globulin fraction was prepared from seeds of the fababean (*Vicia faba* var. Diana) using the micellization technique of Murray *et al.* (1978). Isolation of vicilin from this starting material involved the method of Ismond *et al.* (1985*b*). Homogeneity of the vicilin fraction was assessed by isoelectric focusing and ultracentrifugation.

Micelle characterization

In preliminary micelle experimentation, various types and degrees of intermolecular association were observed. To establish whether different

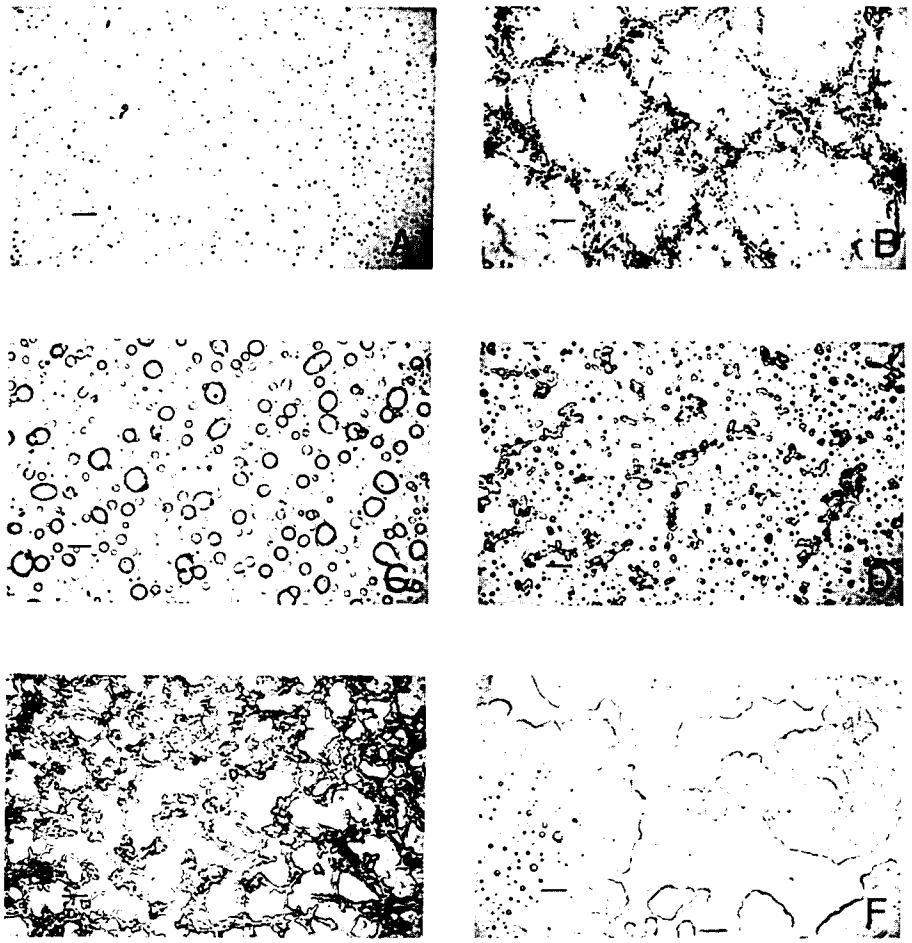

Fig. 1. Photomicrographs of various types of micelle formulation observed with vicilin in different environments. Each micelle response has been assigned a numerical value or micelle rating. Bar represents 25 μm. A. Rating 1 (0·1M $Na_3C_6H_5O_7$)—small single micelles (< 2 μm in diameter); B. Rating 2 (0·2M NaSCN)—small micelles in aggregates, possible granular networks; C. Rating 3 (0·1M NaBr)—small, intermediate and large discrete micelles (2 to 20 μm in diameter); D. Rating 4 (0·1M NaSCN)—all micelle sizes; homogeneous coalescence; E, F. Rating 5 (0·2M NaCl)—extensive coalescence of all micelle sizes to homogeneous networks (E) and protein sheets (F).

reaction end-points could be identified and described, the ability of vicilin to self-associate was assessed in a variety of destabilizing and stabilizing electrolyte media. Exposure of vicilin to the various environments (NaBr, NaCl, $Na_3C_6H_5O_7$, NaSCN; as given in Fig. 1) involved extensive dialysis. Protein samples (10 ml, approximately 1 mg ml^{-1}) were dialyzed against a minimum of six 400 ml buffer changes over a 36 h period with continuous stirring. Prior to micelle assessment, all vicilin samples were concentrated to approximately 25 mg ml^{-1} using an Amicon Minicon B-15 macrosolute concentrator. It should be noted that the experimental salt media reported here were selected from a more extensive electrolyte study (Ismond *et al.*, 1985*a*). These media resulted in an extensive spectrum of vicilin interaction patterns observed under a variety of environmental conditions; their inclusion at this point is designed to illustrate how the various categories for different degrees of vicilin self-association were established. A standard method was established to evaluate micelle formation with all experimental conditions. Initially, a drop (20 μl) of concentrated vicilin solution was examined microscopically for possible structural detail. The vicilin was diluted subsequently with an equivalent volume of distilled water; post-dilution structures were monitored and photographed with a Zeiss Universal Research microscope equipped with a model C35M Zeiss automatic exposure 35 mm camera. All vicilin responses were observed using a minimum of duplicate samples.

The influence of pH on vicilin interaction

To establish the influence of pH on micelle formation, vicilin (approximately 1 mg ml^{-1}) was exposed initially by extensive dialysis to a series of 0·1 M phosphate buffers ranging in pH from 6·0 to 8·0. The buffers differed in pH by 0·1 unit from 6·0 to 6·8 and by 0·5 unit from 7·0 to 8·0. This distribution was selected on the basis of preliminary results; pH values between 6·0 and 7·0 appeared most favorable for extensive micelle interaction. At each pH level, the capacity of vicilin to form micelles was assessed using the standard procedure. In addition, the thermal properties of vicilin in each environment were determined with a Du Pont 990 Thermal Analyzer with a 910 Differential Scanning Calorimeter (DSC) cell base, as described by Arntfield & Murray (1981). Thermal curves were established at a heating rate of 10 °C min^{-1} over a temperature range of 25 to 150 °C with sensitivities of 0·016 or

0·032 mW cm^{-1}. The vicilin samples were concentrated to approximately 10 mg ml^{-1} using an Amicon Minicon B-15 macrosolute concentrator prior to DSC analyses. The thermal parameters *Td* (denaturation temperature), ΔH (enthalpy of denaturation) and 1/2 *bw* (one-half band width) were determined from all thermal curves.

In addition, the surface hydrophobicity of vicilin exposed to each pH regime was assessed by the method of Kato & Nakai (1980) using *cis*-parinaric acid (Calbiochem-Behring Corp.) as a fluorescent probe. Fluorescence intensities were measured with an Amico-Bowman fluorescence spectrophotometer (Model No. 4–8202) using a slit width of 0·5 mm. The method was standardized by initially adjusting the relative fluorescence to 5·0/10 full scale when 10 μl of *cis*-parinaric acid were added to decane (2 ml).

Protein concentrations for both calorimetric and fluorescence analyses were determined by the method of Lowry *et al.* (1951) using bovine serum albumin (Sigma) as a standard. All thermal parameters and *So* values were determined using a minimum of four samples; means and standard deviations of the mean are given for each.

RESULTS

Characterization of the micelle response

The micelle response of fababean storage proteins in 0·5M NaCl originally described by Murray *et al.* (1978) was characterized by discrete spherical structures of variable diameter. However, with further investigation using the single protein, vicilin, it was found that a number of different interaction patterns could be observed if the original electrolyte suspending medium were varied. In addition to single micelles designated as small, intermediate and large (approximately 2, 10 and 20 μm in diameter, respectively), different degrees of inter-micelle association resulted in a spectrum of structures ranging from heterogeneous aggregates of discrete micelles to diverse protein networks and/or amorphous protein masses. On the basis of these observations, different micelle responses were arranged subjectively into categories identified by a defined set of descriptive characteristics. A numerical scheme was devised with each number representing a specific pattern of micelle formation; corresponding photomicrographs showing key aspects of

each interaction level are given in Fig. 1. Examples of electrolyte environments resulting in the different micelle responses are also presented in Fig. 1. As mentioned previously, these specific salt environments are presented only in this paper to demonstrate the basis on which a subjective evaluation scheme for types of vicilin self-association was established. A consideration of the mechanisms involved for the different interactions observed in these electrolyte environments is the subject of a subsequent study (Ismond *et al.*, 1985*a*).

With respect to the characteristics ascribed to individual micelle ratings, several descriptive terms warrant further clarification. For rating 2, small micelles (approximately 2 μm in diameter) were present either in aggregates or in fine granular networks. The term 'aggregate' implies association of individual micelles but limited inter-micelle interaction; the phrase 'granular network' describes a three-dimensional arrangement formed by incomplete coalescence of the micelle structures (Fig. 1B). With rating 3, small micelles coalesced to form discrete micelles of larger sizes, designated as intermediate and large, based on approximate diameters of 10 and 20 μm (Fig. 1C). Despite the interaction observed in rating 3, a significant static population of small micelles was still apparent. Rating 4 was characterized by limited 'homogeneous' coalescence (Fig. 1D). In contrast to the granular phenomenon (rating 2; Fig. 1B), this coalescence involved complete assimilation of multiple-sized structures such that any impression of individual micelles was lost. As described for rating 5, the homogeneous coalescence of rating 4 was followed frequently by the formation of extensive protein networks (Fig. 1E). These, in turn, were inevitably replaced by amorphous patches of aggregated protein (Fig. 1F) or nearly complete protein sheets.

Influence of pH on micelle formation and vicilin structure

The established rating scheme was used subsequently in the assessment of micelle formation by vicilin with limited pH variation. Extremes of pH were not considered. Levels below pH 6·0 resulted in the initiation of a particulate isoelectric type of protein precipitation; values greater than 8·0 were avoided on the basis of potential denaturing conditions. At this point, the possible sensitivity of micelle formation to a mild pH regime was of interest. In this respect, vicilin exhibited a strong micelle reaction from pH 6·0 to 6·8 culminating in protein masses and sheets (rating 5; Fig. 1F). At pH 7·0, the micelle response showed some deterioration with the

formation of small discrete micelles and fine granular networks (rating 2; Fig. 1C). Further degeneration in micelle formation was evident above pH 7·0. At pH 7·5, small discrete micelles were formed (rating 1; Fig. 1A); at pH 8·0, only a limited number of small micelles were observed.

In order to relate the capacity of vicilin to form micelles with molecular properties, several structural aspects of the protein were monitored. The surface hydrophobicity (*So*) of vicilin was identified to be a potentially important parameter on the basis of the suggested significance of hydrophobic interactions to micelle formation (Murray *et al.*, 1981), The *So* values were determined using *cis*-parinaric acid, a probe specific for aliphatic hydrophobic residues. The aliphatic hydrophobicity was considered to be a reasonable assessment of the surface properties of vicilin as the aromatic residue content of this protein is highly reduced (Ismond *et al.*, 1985*b*). As an indication of surface variation, the *So* of

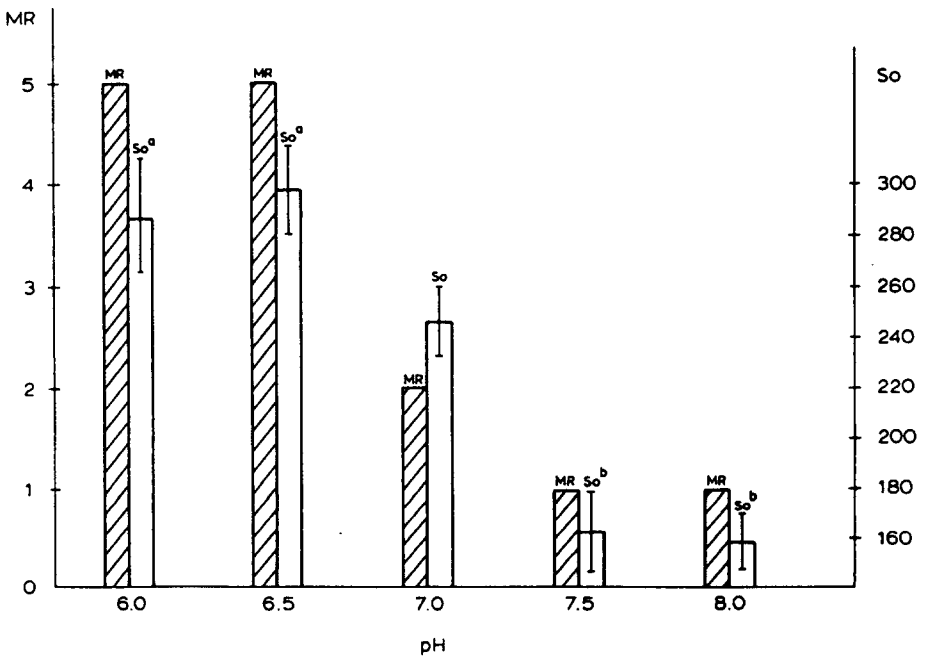

Fig. 2. Micelle ratings (MR) and surface hydrophobicities (*So*) for vicilin exposed to a series of 0·1 M phosphate buffers ranging in pH from 6·0 to 8·0. The *So* values followed by the same letter are not significantly different ($P < 0·05$) as determined by the multiple *t* test.

vicilin decreased with pH from a maximum value of 286 ± 22 at pH 6·0 to a minimum of 158 ± 12 at pH 8·0 (Fig. 2).

In addition to *So* considerations, the thermal properties of vicilin, as assessed by DSC, were used to monitor variations in protein conformation. The application of DSC as a monitor for protein structural variations is based on the premise that both the denaturation temperature (*Td*) and enthalpy of denaturation (ΔH) are characteristic of a specific unfolding process and reflect the conformational stability of the initial state of the protein. As a result, any changes in these thermal parameters should reflect differences in the structure of the original protein (Chlebowski & Williams, 1983). The thermal behaviour of vicilin did not exhibit dramatic changes from pH 6·0 to 8·0; however, some specific trends occurred (Table 1). The *Td* value remained constant from pH 6·0 to 6·4, then decreased progressively to a value of 81·2 °C at pH 8·0, a total change of 5·3 °C. In conjunction with the *Td* change, the 1/2 *bw* values for the thermal curves increased a total of 4·0 °C from pH 6·0 to 8·0. In spite of an apparent gradual increase in this parameter with increasing pH, only the 1/2 *bw* values at the extreme ends of the pH range were different statistically. In contrast to the changes observed in the *Td* and 1/2 *bw*

TABLE 1

Micelle-forming Capacity and Thermal Properties (*Td*, ΔH, 1/2 *bw*) of Vicilin Exposed to a Series of 0·1 M Phosphate Buffers Ranging in pH from 6·0 to 8·0

pH	*Micelle rating*	*Td* (°C)	ΔH (*joules* g^{-1})	*1/2 bw* (°C)
6·0	5	$86{\cdot}9 \pm 1{\cdot}1_{1,2}$[a]	$11{\cdot}75 \pm 1{\cdot}21_{1,2}$	$9{\cdot}9 \pm 2{\cdot}3_{1,2}$
6·1	5	$86{\cdot}8 \pm 1{\cdot}0_{1,2}$	$12{\cdot}87 \pm 0{\cdot}84_{1,2}$	$8{\cdot}9 \pm 0{\cdot}2_{1}$
6·2	5	$86{\cdot}4 \pm 0{\cdot}4_{1}$	$13{\cdot}67 \pm 1{\cdot}59_{1,2}$	$10{\cdot}3 \pm 0{\cdot}1_{2,3}$
6·3	5	$86{\cdot}3 \pm 0{\cdot}5_{1}$	$15{\cdot}80 \pm 4{\cdot}01_{1,3,4,5}$	$10{\cdot}5 \pm 0{\cdot}9_{2,4}$
6·4	5	$86{\cdot}3 \pm 0{\cdot}9_{1,2}$	$12{\cdot}92 \pm 2{\cdot}13_{1,5}$	$10{\cdot}7 \pm 1{\cdot}0_{2,4}$
6·5	5	$85{\cdot}6 \pm 0{\cdot}4_{2,3}$	$17{\cdot}97 \pm 2{\cdot}34_{3}$	$11{\cdot}4 \pm 0{\cdot}8_{2,4,5}$
6·6	5	$84{\cdot}6 \pm 0{\cdot}5_{4}$	$15{\cdot}05 \pm 2{\cdot}59_{2,3,5}$	$11{\cdot}2 \pm 1{\cdot}0_{2,4,5}$
6·7	5	$83{\cdot}8 \pm 0{\cdot}3_{4,5}$	$13{\cdot}80 \pm 0{\cdot}88_{2,4}$	$11{\cdot}8 \pm 0{\cdot}3_{4,5}$
6·8	5	$83{\cdot}8 \pm 0{\cdot}3_{4,5}$	$12{\cdot}54 \pm 0{\cdot}42_{2,5}$	$11{\cdot}3 \pm 0{\cdot}1_{4,6}$
7·0	2	$83{\cdot}9 \pm 1{\cdot}3_{3,4,5}$	$13{\cdot}42 \pm 2{\cdot}26_{2,5}$	$11{\cdot}2 \pm 0{\cdot}9_{3,4,6,7}$
7·5	1	$82{\cdot}0 \pm 0{\cdot}6_{6}$	$12{\cdot}00 \pm 0{\cdot}96_{2,5}$	$12{\cdot}6 \pm 0{\cdot}8_{5,7}$
8·0	1	$81{\cdot}2 \pm 1{\cdot}2_{6}$	$13{\cdot}67 \pm 0{\cdot}84_{2,5}$	$14{\cdot}3 \pm 1{\cdot}6_{5,6,7}$

[a] Column values followed by the same number are not significantly different ($P \leq 0{\cdot}05$) as determined by a multiple *t* test.

values from pH 6·0 to 8·0, the ΔH exhibited little variation. As a single variable, a mean ΔH value of 13·79 joules g^{-1} over this pH range was indicative of a relatively native protein structure.

DISCUSSION

From the results of this study, it is apparent that the protein vicilin has the capacity to self-associate under certain environmental conditions to form spherical structures designated as micelles. In addition, the resulting micelles are often not static end-points, but, rather, highly interactive structures which continue association to form elaborate networks or amorphous protein sheets. This observation is of significance from both a theoretical and an applied viewpoint. In the first instance, vicilin association into a micelle arrangement appears to be the result of molecular orientation with a thermodynamic driving force. As these protein interactions occurred only with the introduction of a controlled volume of water, the thermodynamic impetus is considered to be hydrophobic in origin (Murray *et al.*, 1981). The formulation of a static population of discrete micelles (e.g. ratings 1 and 3) is probably the consequence of the co-operative interaction of non-covalent forces. According to Tanford (1973), attractive forces must be dominant for the formation of a detergent micelle arrangement, whereas surface repulsive forces must be dominant for such micelles to be restricted to a spherical shape of a particular size. Micelles, in general, are not stoichiometric compounds but aggregates capable of existing over a wide range of sizes. There is a limitation to the minimum size, based on the ability to reduce the water–nonpolar interface; similarly, there is a maximum size restriction based on surface repulsive characteristics (Tanford, 1973). For vicilin, the environmental media have a definite impact on the development of these surface repulsive phenomena. Even at salt concentrations where anionic influences are assumed to be primarily electrostatic at a molecular level (i.e. $\mu < 0{\cdot}5$; von Hippel & Schleich, 1969), the degree of protein interaction is influenced by the identity of the anion. Some media resulted in rapid development of inter-micelle surface repulsion (e.g. rating 1 in 0·2M NaSCN) with the observed formation of only small discrete micelles. In other environments, a major surface repulsive situation did not appear to develop (e.g. rating 5 in 0·2M NaCl); inter-micelle coalescence occurred until amorphous protein masses predominated. Although these observations are of interest, the intent at

this point was to recognize the spectrum of interactions possible with different environmental conditions. This was considered to be a preliminary step to understanding the molecular mechanisms operative in producing various types of protein associations.

From a practical viewpoint related to food applications, the extensive networks formed under certain environmental conditions (ratings 4 and 5) are similar to those observed with protein frameworks in a number of food products. Consequently, an appreciation for the interactive forces involved in this type of protein association may result in the eventual controlled manipulation of this reaction phenomenon within a specific food system. In this respect, one general environmental concern is the pH of the system. In considering the influence of pH on micelle formation, some attempt was made to correlate specific molecular parameters with different degrees of protein interaction. From the micelle observations, the optimum structural characteristics of vicilin, for micelle formation and subsequent interaction, existed from pH 6·0 to 6·8. Significant deterioration of the micelle response occurred from pH 7·0 to 8·0, the pH range in which changes in *So*, *Td* and 1/2 *bw* appeared to reflect subtle conformational variations in vicilin. However, the relatively stable ΔH values from pH 6·0 to 8·0 appeared to preclude major structural alterations. An overall decrease in *Td* was indicative of molecular destabilization; the corresponding increase in 1/2 *bw* may have reflected a gradual distortion in subunit association accompanied by a reduction in co-operativity within the multimeric molecule. Due to the low ionic strength environment, both thermal responses can be correlated with changes in the charge profile of the vicilin molecules. As the pH was increased above the isoelectric point (for vicilin, pH 5·0; Ismond, 1984), the overall negativity of the molecule increased. This increase in repulsive negative surface charge may have stressed the structure and induced conformational changes of varying magnitudes within the molecule. Furthermore, Perutz (1978) suggested that progressive ionization of internal residues results in the attraction and incorporation of numerous hydration shells. This leads to a shift in the equilibrium from a native to an altered conformation. In addition, the contribution of all internal residues to overall molecular stability is not equal; the ionization of some residues may exert more deleterious effects than others. Consequently, molecular destabilization is a complex phenomenon not necessarily occurring in a sequential relationship with manipulation of an environmental parameter such as pH.

Although the changes in the thermal parameters *Td* and *1/2 bw* appeared to reflect a gradual destabilization of vicilin, the corresponding decrease in *So* over the same pH range was not characteristic of a progressive unfolding of the molecule. With preliminary studies, exposure of vicilin to a pH extreme of 10·0 resulted in an *So* value of 500, an expected response if the conformation of the molecule was deteriorating. In contrast to this exposure of hydrophobic side-chains with more extreme alkaline pH values, the conformational fluctuations observed from pH 6·0 to 8·0 seemed to be characterized by an increased burial of hydrophobic residues. This observation would appear to reinforce the concept of a gradual distortion in molecular structure with minimal pH changes being related to disturbed subunit associations at the quaternary level, rather than a deterioration of more fundamental secondary structural arrangements.

As pH influences some conformational aspects of vicilin, it is not unexpected that these molecular ramifications would ultimately affect micelle formation. For example, at pH 6·0 and 6·5, the *So* values were high and the micelle response was strong; with an increase in pH, there was a corresponding decrease in both *So* and the degree of vicilin self-association. From these results, it would appear that manipulation of vicilin conformation is not optimal for micelle formation if hydrophobic interactions are suppressed due to a decrease in *So* values resulting from an increase in intramolecular electrostatic contributions. This observed importance of *So* is supportive of the original premise by Murray *et al.* (1978) that these micelle structures are a product of hydrophobic associative forces. In addition, the observed micelle populations from pH 7·0 to 8·0 were characterized by a progressive decrease in inter-micelle interaction. Increasing molecular negativity with increasing pH may result in extensive surface repulsion among established micelles.

CONCLUSIONS

Hydrophobic, electrostatic and steric parameters associated with the conformation of vicilin appear to influence the capacity of the protein molecules to self-associate into a micelle arrangement. Manipulation of vicilin structure is not optimal for micelle formation if surface hydrophobic interactions are suppressed as a result of electrostatic contributions. This observed importance of *So* is supportive of the

original premise that micelle structures are products of hydrophobic associative forces. A strong micelle response by vicilin is exhibited in that pH range (6·0 to 6·8) where conformational fluctuations, as assessed by thermal properties and *So* parameters, are minimal. This represents an important consideration if micelle formation and interaction were desired in a food system.

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