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The Rôle of Non-covalent Forces in Micelle Formation by Vicilin from *Vicia faba*. II. The Effect of Stabilizing and Destabilizing Anions on Protein Interactions*

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ABSTRACT

The effect of a variety of stabilizing and destabilizing anions on the capacity of vicilin from fababeans to self-associate into a micelle arrangement was examined; these observations were related to protein conformational parameters, specifically, thermal stability and surface hydrophobicity (So). Extensive vicilin interactions occurred with moderately stabilizing environments (denaturation temperature (Td) values of 87·7 to 92·5 °C) in which the protein So values were not extreme (240 ± 40). In contrast, highly stabilized molecules (Td > 95 °C) with low So values (e.g. 137) or destabilized molecules (Td < 80 °C) with high So values (e.g. 572) did not self-associate into a micelle arrangement. From these relationships, the micelle response appeared to be dependent on a critical balance of non-covalent forces operative at several levels. A specific intramolecular hydrophilic–hydrophobic balance was essential for protein association; similarly, intermolecular attractive hydrophobic forces had to be dominant over electrostatic repulsive forces for micelle formation to occur.

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INTRODUCTION

Under specific environmental conditions, the fababean storage protein, vicilin, has the capacity to self-associate into a stable micelle arrangement. However, in some instances, the micelles derived from vicilin interaction represent reactive intermediates rather than static end-points; inter-micelle association may continue to result in elaborate three-dimensional networks and amorphous protein sheets (Ismond *et al.*, 1985*a*). In order to predictably manipulate the progression of this type of interaction, as may be required in a particular food application, the specific forces promoting protein–protein association must be understood. Two levels of molecular interaction are of concern. First, the conformational characteristics of soluble vicilin molecules must be appropriate for micelle formation. In this respect, the relative exposure of hydrophobic residues on the protein surface appears significant (Ismond *et al.*, 1985*a*), a reinforcement of the original premise that micelle formation represents a precipitation phenomenon resulting from hydrophobic associations (Murray *et al.*, 1981). However, it would seem that hydrophobic interactions should also be influenced by electrostatic parameters resulting from the overall charge of the protein as determined by the degree of amino acid ionization and possible association of solubilizing electrolytes. Secondly, the surface properties of intact micelles must be influential in promoting inter-micelle aggregation for the formation of extensive protein structures. The basis for this interaction may be hydrophobic; however, electrostatic parameters must not be prohibitive in order to allow hydrophobic associations to occur.

With these considerations, this study was designed to explore some of the non-covalent forces that appear to be involved in protein micelle formation and interaction. The capacity of vicilin to self-associate was investigated in a variety of electrolyte media. At low salt concentrations, the influence of protein stabilization, by non-specific electrostatic interactions, on micelle formation was examined. Several molecular parameters were correlated with observed micelle interaction patterns; these included thermal properties as indicators of the degree of protein denaturation and surface hydrophobicity as an assessment of the potential for hydrophobic association. Similar studies were also conducted at higher salt concentrations. The influence of specific stabilizing or non-chaotropic anions was compared with the effects of certain destabilizing or chaotropic anions. Experimental results were used

to assess the relative significance of non-covalent forces with respect to micelle formation and subsequent interaction.

MATERIALS AND METHODS

Protein isolation

Vicilin was isolated from the seeds of the fababean (*Vicia faba* var. *Diana*) using the method described by Ismond *et al.* (1985*b*).

Influence of electrolytes

To assess the effect of various electrolytes on selected aspects of protein conformation and micelle-forming capacity, vicilin (approximately 1 mg ml^{-1}) was initially exposed by extensive dialysis to a series of different salts. Protein samples (10 ml) were dialyzed against a minimum of six 400 ml solution changes over 36 h with continuous stirring. Salt media included $NaC_2H_3O_2$, NaBr and NaCl (0·2, 0·5, 1·0M), plus $Na_3C_6H_5O_7$, Na_2SO_4, NaSCN (0·1, 0·2, 0·5, 1·0M) and NaI (0·2 and 0·5M). Sodium acetate, NaBr, NaCl and NaI were not considered at the 0·1M level due to reduced vicilin solubility in these media. The pH was allowed to remain an uncontrolled parameter for solutions in the acidic and neutral ranges. As a result of the pH effects observed previously (Ismond *et al.*, 1985*a*), the alkaline environments ($NaC_2H_3O_2$ and $Na_3C_6H_5O_7$) were adjusted to pH 6·6 with acetic and citric acids, respectively.

For each electrolyte, the capacity of vicilin to form micelles was assessed using the standard procedure given by Ismond *et al.* (1985*a*). The thermal properties of vicilin in each environment were determined with a DuPont Thermal Analyzer with a 910 Differential Scanning Calorimeter cell base, as described by Arntfield & Murray (1981). Sample preparation and running conditions were given in Ismond *et al.* (1985*a*). In addition, the surface hydrophobicity (*So*) of vicilin exposed to each electrolyte was determined using a modification of the method of Kato & Nakai (1981) involving *cis*-parinaric acid (Calbiochem–Behring Corp.) as a fluorescence probe. Fluorescence intensities were measured in an Aminco–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 *cis*-parinaric acid (10 μl) was added to decane (2 ml).

Protein concentrations for DSC and fluorescence analyses were determined by the method of Lowry *et al.* (1951) using bovine serum albumin (Sigma) as a standard. Levels of vicilin in $NaC_2H_3O_2$ and NaSCN were assessed using a Coomassie Blue R-250 reagent (Pierce Chem. Co.) to avoid possible anionic interference with the Lowry reaction. 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

Effects of specific anions on conformational parameters

For comparative purposes, the influence of most salts on vicilin was assessed at concentrations representing two levels of salt action. At low ionic strengths ($\mu < 0{\cdot}5$), a general salting-in or solubilizing of the protein occurred as a result of reduced inter-protein electrostatic interactions. As the salt concentration increased ($\mu > 0{\cdot}5$), the influence of various anions on vicilin conformation was related, at least partly, to the modification of the solvent environment, or a lyotropic effect. These represent two extreme types of salt behaviour with various intermediate situations possible; consequently, an absolute boundary is difficult to identify (Eagland, 1975). According to von Hipple & Schleich (1969), electrostatic effects become negligible from ionic strengths of 0·5 to 1·0. As a result, the effects of all univalent salts in this study were considered to be predominantly electrostatic at 0·1M and 0·2M and mainly lyotropic at higher concentrations. For both Na_2SO_4 and $Na_3C_6H_5O_7$, electrostatic interactions were assumed to be dominant only at 0·1M levels. The thermal properties of vicilin, reflecting protein structural parameters, were affected by the concentration and identity of the various anions. For most anions ($C_2H_3O_3^-$, Br^-, Cl^-, SO_4^{-2}, $C_6H_5O_7^{-3}$), there was an increase in denaturation temperature (*Td*) with an increase in salt concentration (Table 1); this observation implies an increased stabilization of the vicilin molecule. Relationships defining the significant positive correlations between concentrations of these stabilizing anions and *Td* values are given in Table 2. In contrast, there was a destabilization of vicilin exposed to

TABLE 1

Denaturation Temperature (*Td*) Values for Vicilin in Various Concentrations of Sodium Salts. Salts are Listed in Sequence According to the Molal Surface Tension Increments (σ)[a]

Salt	*Td* (°C)				
	σ[b]	0·1M	0·2M	0·5M	1·0M
NaSCN	0·60[c]	$79{\cdot}0 \pm 3{\cdot}0^{A,B,C}$	$82{\cdot}3 \pm 0{\cdot}8_1^A$	$80{\cdot}8 \pm 1{\cdot}2^B$	$79{\cdot}9 \pm 0{\cdot}7^C$
NaI	1·02[d]	nd[e]	$85{\cdot}4 \pm 1{\cdot}7_1^A$	$86{\cdot}1 \pm 0{\cdot}7^A$	nd[e]
$NaC_2H_3O_2$	1·27[d]	nd[e]	$87{\cdot}7 \pm 0{\cdot}7_1$	$91{\cdot}5 \pm 0{\cdot}7$	$97{\cdot}8 \pm 1{\cdot}9_{1,2}$
NaBr	1·32[d]	nd[e]	$89{\cdot}8 \pm 1{\cdot}2_{2,3}$	$92{\cdot}4 \pm 0{\cdot}4_1$	$95{\cdot}4 \pm 0{\cdot}7_1$
NaCl	1·64[d]	nd[e]	$88{\cdot}0 \pm 0{\cdot}8_{1,2}$	$92{\cdot}5 \pm 0{\cdot}6_1$	$98{\cdot}0 \pm 0{\cdot}0_2$
Na_2SO_4	2·73[d]	$88{\cdot}5 \pm 0{\cdot}8_1$	$90{\cdot}6 \pm 0{\cdot}5_3$	$99{\cdot}1 \pm 0{\cdot}5_2$	$109{\cdot}3 \pm 0{\cdot}4$
$Na_3C_6H_5O_7$	3·27[c]	$86{\cdot}8 \pm 0{\cdot}8_1$	$91{\cdot}3 \pm 1{\cdot}6_3$	$101{\cdot}5 \pm 2{\cdot}3_2$	$114{\cdot}6 \pm 1{\cdot}4$

[a] Horizontal values followed by the same upper case letter and vertical values followed by the same number are not significantly different (multiple *t*-test; $P \leq 0{\cdot}05$).
[b] Units are 10^{-3} dyn g cm^{-1} mol^{-1}.
[c] Melander & Horvath (1977).
[d] International Critical Tables (1929).
[e] Value not determined due to solubility limitations.

TABLE 2

Relationships Between Pairs of Variables as Defined by Standard Linear Regression Analysis ($P < 0{\cdot}001$)

A. *Td* (°C) as a function of salt concentration (M):

$NaC_2H_3O_2$:	$Td = 9{\cdot}62\text{M} + 86{\cdot}6$ ($r = 0{\cdot}9784$)
NaBr:	$Td = 7{\cdot}65\text{M} + 88{\cdot}0$ ($r = 0{\cdot}9440$)
NaCl:	$Td = 9{\cdot}47\text{M} + 87{\cdot}4$ ($r = 0{\cdot}9856$)
Na_2SO_4:	$Td = 23{\cdot}4\text{M} + 86{\cdot}4$ ($r = 0{\cdot}9942$)
$Na_3C_6H_5O_7$:	$Td = 30{\cdot}5\text{M} + 84{\cdot}8$ ($r = 0{\cdot}9847$)

B. *Td* (°C) as a function of σ (dyn g cm^{-1} mol^{-1})

0·2M:	$Td = 2{\cdot}91\sigma + 83{\cdot}3$ ($r = 0{\cdot}8158$)
0·5M:	$Td = 7{\cdot}21\sigma + 80{\cdot}3$ ($r = 0{\cdot}9241$)
1·0M:	$Td = 12{\cdot}5\sigma + 75{\cdot}9$ ($r = 0{\cdot}9371$)

C. *So* as a function of σ (dyn g cm^{-1} mol^{-1})

0·5M:	$So = -132{\cdot}4\sigma + 517$ ($r = -0{\cdot}8463$)
1·0M:	$So = -146{\cdot}2\sigma + 537$ ($r = -0{\cdot}8599$)

D. ΔH (joules g^{-1}) as a function of σ (dyn g cm^{-1} mol^{-1})

1·0M:	$\Delta H = 5{\cdot}88 + 4{\cdot}62$ ($r = 0{\cdot}8438$)

TABLE 3

Denaturation Enthalpy (ΔH) Values for Vicilin in Various Concentrations of Sodium Salts. Salts are Listed in Sequence According to the Molal Surface Tension Increments (σ)[a]

Salt	ΔH (*joules* g^{-1})				
	σ[b]	*0·1*M	*0·2*M	*0·5*M	*1·0*M
NaSCN	0·60[c]	$15{\cdot}68 \pm 0{\cdot}79_1$	$11{\cdot}54 \pm 2{\cdot}55^{A}_{1,2}$	$10{\cdot}78 \pm 1{\cdot}92^{A}_{1,2}$	$4{\cdot}22 \pm 0{\cdot}79$
NaI	1·02[d]	$7{\cdot}65 \pm 0{\cdot}67^{A}$	$12{\cdot}04 \pm 1{\cdot}00^{B}_{1}$	$11{\cdot}08 \pm 3{\cdot}34^{A,B}_{1,2}$	nd[e]
$NaC_2H_3O_2$	1·27[d]	nd[e]	$10{\cdot}74 \pm 3{\cdot}34^{A}_{1,2}$	$10{\cdot}45 \pm 0{\cdot}71^{A}_{1}$	$14{\cdot}59 \pm 0{\cdot}96_1$
NaBr	1·32[d]	nd[e]	$9{\cdot}53 \pm 1{\cdot}13_2$	$17{\cdot}05 \pm 1{\cdot}46^{A}_{3}$	$14{\cdot}33 \pm 0{\cdot}67^{A}_{1}$
NaCl	1·64[d]	nd[e]	$15{\cdot}17 \pm 0{\cdot}54^{A}_{3}$	$14{\cdot}59 \pm 2{\cdot}59^{A}_{2,3}$	$16{\cdot}05 \pm 0{\cdot}04^{A}$
Na_2SO_4	2·73[d]	$11{\cdot}45 \pm 1{\cdot}05^{A}_{2}$	$12{\cdot}79 \pm 3{\cdot}76^{A,B}_{3}$	$14{\cdot}09 \pm 1{\cdot}25^{A,B}_{2,3}$	$18{\cdot}35 \pm 0{\cdot}92^{B}$
$Na_3C_6H_5O_7$	3·27[c]	$12{\cdot}83 \pm 2{\cdot}80^{A}_{1,2}$	$17{\cdot}51 \pm 2{\cdot}30^{A}_{3}$	nd[f]	nd[f]

[a] Horizontal values followed by the same upper case letter and vertical values followed by the same number are not significantly different (multiple *t*-test; $P \leq 0{\cdot}05$).

[b] Units are 10^{-3} dyn $g cm^{-1}$ mol^{-1}.

[c] Melander & Horvath (1977).

[d] International Critical Tables (1929).

[e] Value not determined due to solubility limitations.

[f] Value not determined due to large post-denaturation exotherms.

increasing concentrations of NaSCN, as evidenced by a significant decrease in *Td* values (Table 1). With NaI, the *Td* values remained constant at the two concentration levels examined (Table 1). However, I^- was considered to be a destabilizing anion as a result of the low *Td* (86·1 °C) at 0·5M concentration levels.

In terms of other thermal parameters, denaturation enthalpy (ΔH) values (Table 3) for vicilin in the stabilizing media either increased with increasing salt concentrations ($C_2H_3O_2^-$, Br^-, SO_4^{-2}, $C_6H_5O_7^{-3}$) or remained constant over the salt concentrations used (Cl^-). The ΔH values for vicilin in NaI also showed no significant change; however, with NaSCN, the destabilizing influence was apparent. The ΔH value decreased significantly from 15·68 joules g^{-1} at 0·1M to 4·22 joules g^{-1} at 1·0M NaSCN.

Results from the *So* determinations for vicilin were also indicative of conformational changes with increases in the concentration of specific anions (Table 4). In general, the stabilizing anions caused either a decrease in vicilin *So* with increasing concentration (SO_4^{-2}, $C_6H_5O_7^{-3}$) or had virtually no effect on *So* values ($C_2H_3O_2^-$, Cl^-). The *So* for vicilin in NaSCN showed an opposite trend; there was a significant increase with increasing salt concentration, especially from 0·1 to 0·2M.

To this point, the results have been presented mainly with a view to

TABLE 4

Surface Hydrophobicity (*So*) Values for Vicilin in Various Concentrations of Sodium Salts. Salts are Listed in Sequence According to the Molal Surface Tension Increments (σ)

Salt	*So*				
	σ^b	*0·1M*	*0·2M*	*0·5M*	*1·0M*
NaSCN	0·60[c]	$267 \pm 55_1$	474 ± 37^A	556 ± 83^A	572 ± 78^A
$NaC_2H_3O_2$	1·27[d]	nd[e]	$279 \pm 32_1^A$	270 ± 20^A	268 ± 37^A
NaCl	1·64[d]	nd[e]	$248 \pm 18_1$	202 ± 16^A	201 ± 9^A
Na_2SO_4	2·73[d]	$220 \pm 4_1^A$	$273 \pm 39_1^A$	164 ± 11	$129 \pm 15_1$
$Na_3C_6H_5O_7$	3·27[c]	$191 \pm 23_1^A$	183 ± 18^A	137 ± 8^B	$124 \pm 11_1^B$

[a] Horizontal values followed by the same upper case letter and vertical values followed by the same number are not significantly different (multiple *t*-test; $P \leq 0.05$).

[b] Units are 10^{-3} dyn g cm^{-1} mol^{-1}.

[c] Melander & Horvath (1977).

[d] International Critical Tables (1929).

[e] Value not determined due to solubility limitations.

examining the effects of increasing the concentration of a specific anion on the conformational properties of vicilin. In order to assess relative electrostatic and lyotropic anionic contributions to vicilin conformational parameters, the structural effects of different anions at different concentration levels can be compared. As anionic effects become more lyotropic, the capacity of an anion to exert a stabilizing influence becomes related to its position in the Hofmeister series. For example, the relative stabilizing effectiveness of the anions examined in this study can be described by the following hierarchy, with reference to *Td* values at high salt levels:

STABILIZING		DESTABILIZING
$C_6H_5O_7^{-3} > SO_4^{-2} > Cl^-$,	$C_2H_3O_2^-$,	$Br^- > I^- > SCN^-$

This arrangement closely parallels the order of anions in the original Hofmeister series. However, the Hofmeister series is a subjective ranking scheme; consequently, correlations of conformational parameters with the position of various anions in this hierarchy is difficult. To overcome this general problem, Melander & Horvath (1977) related the position of an anion in the Hofmeister series to σ, the molal surface tension increment, a numerical assessment of the capacity of an anion to increase the surface tension of water. This relationship is not expected to exist at low ionic strength values where anion effects are generally electrostatic with respect to protein structure. At higher salt levels, as the predominant salt effect is more lyotropic, relationships between conformational parameters and σ may become more obvious. With this viewpoint, data for all salts have been tabulated according to increasing σ values (Tables 1, 3, 4). In addition, relationships between σ and the different structural parameters (*Td*, ΔH, *So*) have been examined at each concentration level; significant correlations are listed in Table 2. Analysis of these relationships indicated an increase in the degree of correlation between σ and the various conformational parameters at higher salt concentrations. For example, there was a significant positive correlation between *Td* and σ at all salt levels from 0·2 to 1·0M as defined by the equations given in Table 2. The degree of correlation between the two variables, however, increased with increasing salt levels. For ΔH values, there was no correlation between the two parameters at the 0·2 and 0·5M levels; however, a significant positive relationship existed at 1·0M (Table 2). With the *So* parameter, there was a significant negative correlation between *So* and σ at 0·5 and 1·0M salt

concentrations (Table 2). A similar relationship did not exist at the 0·2M level.

Effects of specific anions on micelle formation

The ability of vicilin to self-associate in a micellar arrangement was assessed using the rating scheme given by Ismond *et al.* (1985*a*); an increase in the numerical value assigned reflects an increase in the degree of protein interaction. Figures 1 to 4 were established in order to investigate the relationships between vicilin molecular parameters, specifically, thermal stability and surface hydrophobicity, and the capacity for vicilin to self-associate. These results were, in turn, related to the molal surface tension values for the individual anions. At 0·1M concentration levels, vicilin exposed to NaSCN and Na_2SO_4 showed a strong micelle reaction (rating 4, Fig. 1); these responses were correlated with relatively high *So* values of 267 and 220, respectively. However, vicilin in 0·1M NaSCN appeared somewhat destabilized with a *Td* value of 79 °C. For vicilin in 0·1M $Na_3C_6H_5O_7$, only a small population of discrete micelles was observed (rating 1). As the degree of molecular stabilization

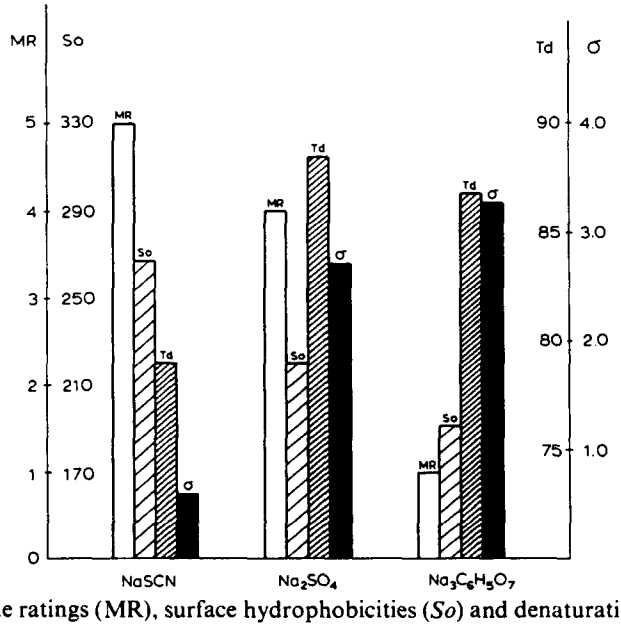

Fig. 1. Micelle ratings (MR), surface hydrophobicities (*So*) and denaturation temperatures (*Td*, °C) for vicilin exposed to a series of 0·1M sodium salts with different molal surface tension increments (σ, 10^{-3} dyn g cm^{-1} mol^{-1}).

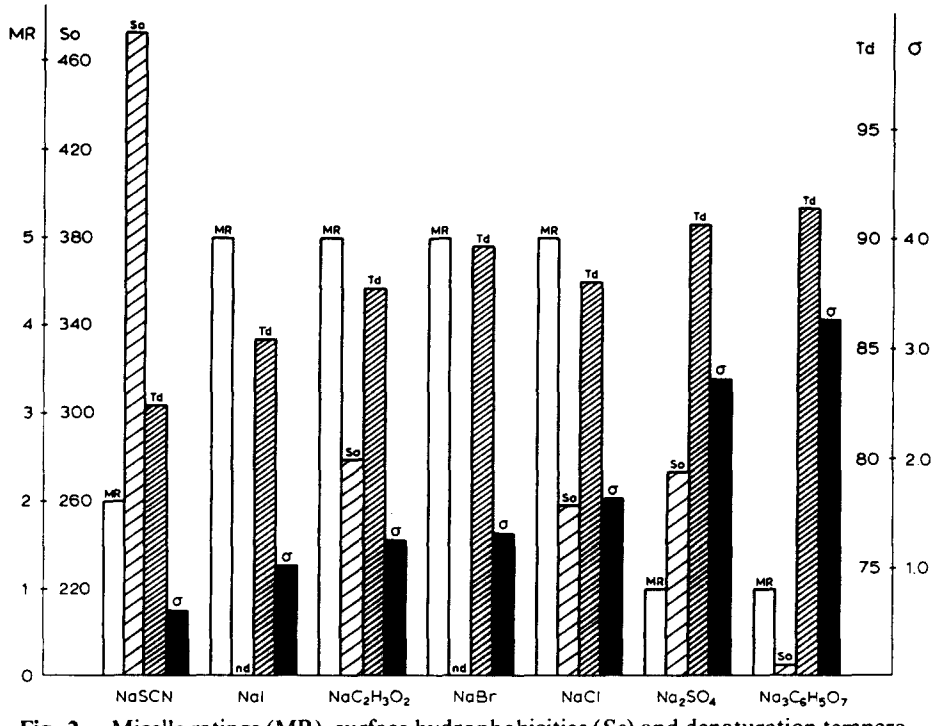

Fig. 2. Micelle ratings (MR), surface hydrophobicities (*So*) and denaturation temperatures (*Td*, °C) for vicilin exposed to a series of 0·2M sodium salts with different molal surface tension increments (σ, 10^{-3} dyn g cm^{-1} mol^{-1}). *So* values designated as nd—not determined due to quenching effect of anion.

appeared to be similar in the sulfate and citrate media, the lower *So* value (191) for vicilin in citrate may contribute to the different micelle responses.

A general trend in micelle response with respect to the Hofmeister or lyotropic series of anions became apparent at 0·2M salt concentrations; groups of anions with similar σ values tended to exhibit comparable reactions (Fig. 2). A strong micelle response (rating 5) was observed with vicilin exposed to the stabilizing univalent anions ($C_2H_3O_2^-$, Br^-, Cl^-) and the more destabilizing iodide. These observations were associated with relatively high *So* values (279 and 248 for $C_2H_3O_2^-$ and Cl^-) and similar thermal properties for all four environments. Significant deterioration in the micelle response was observed for vicilin in 0·2M levels of NaSCN and Na_2SO_4 (rating 1) in comparison with the 0·1M results. The low rating in the NaSCN medium seemed to be associated with molecular destabilization, as evidenced by a significantly decreased *Td*

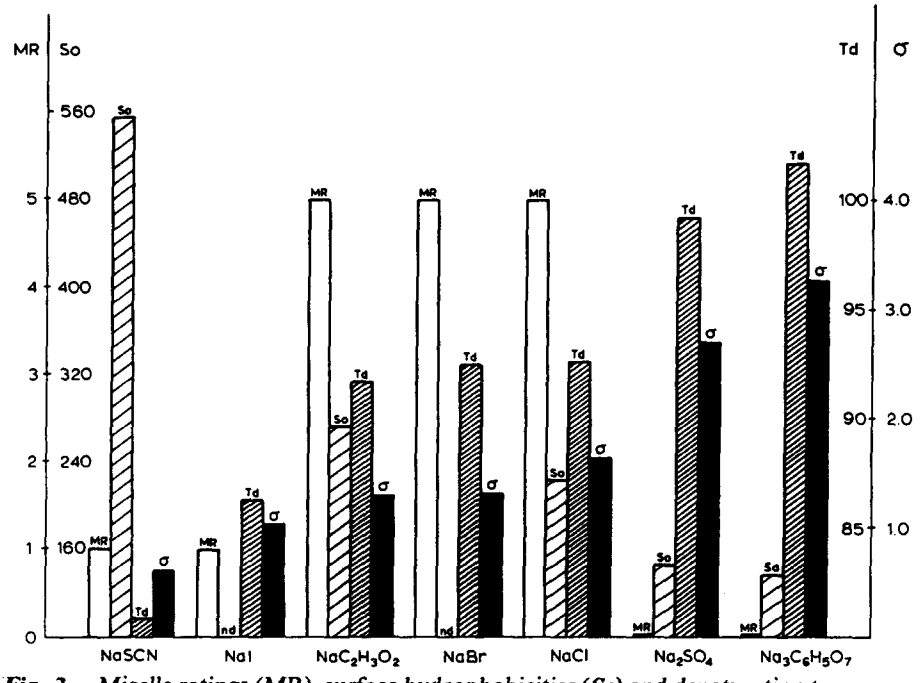

Fig. 3. Micelle ratings (MR), surface hydrophobicities (*So*) and denaturation temperatures (*Td*, °C) for vicilin exposed to a series of 0·5M sodium salts with different molal surface tension increments (σ, 10^{-3} dyn g cm^{-1} mol^{-1}). *So* values designated as nd—not determined due to quenching effect of anion.

value (82·3 °C) and an elevated *So* (474). With Na_2SO_4, however, thermal properties and *So* values were similar to those of the stabilizing univalent anions. A reduced micelle reaction (rating 1) was also observed for vicilin in 0·2M $Na_3C_6H_5O_7$; a similar result to that observed at 0·1M levels. This response was associated with a reduced *So* value (183) and a high degree of thermal stability.

At 0·5M, the micelle-forming capacity of vicilin appeared to be related to the lyotropic influence of the individual salts, as assessed by the molal surface tension increment (Fig. 3). Differences among the various groups of anions were even more exaggerated than at the 0·2M levels. Strong micelle responses with the univalent stabilizing anions were correlated with *So* values of 270 and 201 for $NaC_2H_3O_3$ and NaCl, respectively. The low micelle rating for vicilin in NaSCN, the medium with the lowest σ value, corresponded to an exaggerated *So* value of 556 and a reduced *Td* value of 80·8 °C. A weak micelle response in conjunction with a low *Td* value was also observed for vicilin in 0·5M NaI. Vicilin in the highly

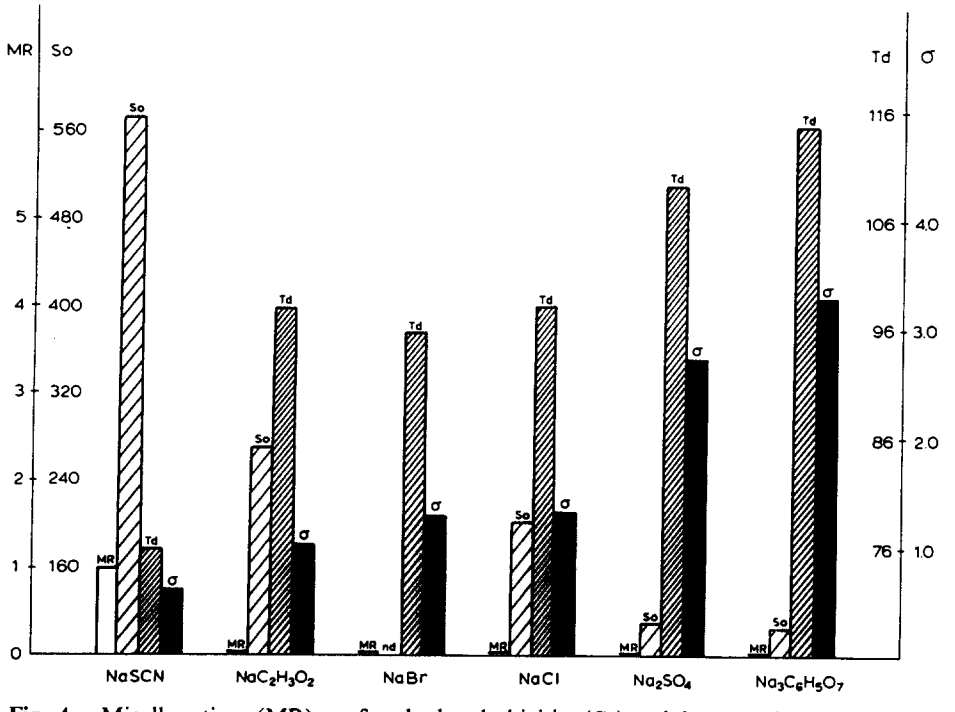

Fig. 4. Micelle ratings (MR), surface hydrophobicities (*So*) and denaturation temperatures (*Td*, °C) for vicilin exposed to a series of 1·0M sodium salts with different molal surface tension increments (σ, 10^{-3} dyn g cm^{-1} mol^{-1}). *So* values designated as nd—not determined due to quenching effect of anion.

stabilizing Na_2SO_4 and $Na_3C_6H_5O_7$ did not exhibit any micelle response; this was correlated with reduced *So* values in both media (164 for sulfate and 137 for citrate). At 1·0M, there was no salt environment that was suitable for the establishment of any stable major micelle population. In 1·0M NaSCN, a few single micelles were formed (rating 1). Vicilin in this medium was characterized by an extreme *So* value of 572 and a low *Td* value of 75·9 °C, both evidence of a highly destabilized molecule. With the moderately stabilizing anions ($C_2H_3O_2^-$, Cl^- and Br^-), the micelle response was initially strong but transitory, disappearing rapidly after formation. Based on previous observations, the *So* values for vicilin in these media, 268 for $NaC_2H_3O_2$ and 201 for NaCl, appeared adequate for micelle formation. There was no evidence of any micelle formation with vicilin in Na_2SO_4 or $Na_3C_6H_5O_7$. Similar to the 0·5M concentration levels, the *So* values were low (129 for sulfate and 124

for citrate) and the elevated *Td* values reflected highly stabilized molecules.

DISCUSSION

Effects of specific anions on vicilin conformation

Protein conformational responses to neutral salts may be affected by both the concentration and identity of the anion. At low electrolyte concentrations ($\mu < 0{\cdot}5$), the anions may be treated more as a collective group as their effects can be attributed to electrostatic interactions related to the polar polyionic nature of the protein (von Hippel & Schleich, 1969). The magnitude of the electrostatic influence is related primarily to the ionic strength of the salt, as well as the density and distribution of charged residues on the protein surface. In this study, the general electrostatic influences of various anions at low concentrations ($\mu < 0{\cdot}5$) on the thermal stability and surface hydrophobicity of vicilin were evidenced by the minimal differences among most of the experimental parameters. However, SCN^- exerted a destabilizing influence even at low ionic strengths, as evidenced by low *Td* values and elevated *So* values. These conformational changes with increased exposure of hydrophobic residues may be related to the capacity of SCN^- to exhibit a high degree of non-specific binding to a number of exposed protein polar sites (Arakawa & Timasheff, 1982).

The ionic strength differential among the multivalent and univalent anions appeared to influence the degree of protein stabilization at 0·2M salt levels. Tatham *et al.* (1983) observed a similar effect with the increased ability of the divalent anions, phosphate and sulfate, to promote α-helix formation in the protein, melittin, as compared with that of the univalent chloride anion for a 0·1 to 1·0M concentration range. Stabilization was attributed to the increased capacity of multivalent negative ions to suppress electrostatic repulsion among basic residues. At 0·2M levels, however, the effective electrostatic shielding by the multivalent anions may be supplemented by a stabilizing lyotropic effect. In fact, the effects of both citrate ($\mu = 1{\cdot}2$) and sulfate ($\mu = 0{\cdot}6$) on vicilin should be predominantly lyotropic at this salt concentration.

At higher salt concentrations ($\mu > 0{\cdot}5$), lyotropic considerations become generally important. The capacity of an anion to exert a

stabilizing influence on protein structure appears to be related primarily to its ability to increase the surface tension of the protein aqueous environment (Melander & Horvath, 1977) in conjunction with its ability to cause preferential hydration of the protein molecule (Arakawa & Timasheff, 1982). This premise seems to be supported by the response of vicilin to increasing concentrations of the various anions. The degree of correlation between specific conformational parameters and the molal surface tension increment increased with increasing molar concentrations. That is, as there was a transition from electrostatic to lyotropic effects with elevated salt concentrations, the ability of an anion to stabilize vicilin was related to its capacity to interact with the aqueous environment. As the response of vicilin to the various anions appears to parallel the original Hofmeister series, this may be indicative of the significance of hydrophobic interactions to the integrity of the vicilin molecule. Theoretical protein studies have shown that at ionic strengths sufficient to minimize electrostatic interactions, changes in protein properties can be attributed to hydrophobic interactions, if the changes can be correlated with the position of these anions in the Hofmeister series (Melander & Horvath, 1977; Franks, 1978). It is reasonable to assume that hydrophobic interactions are important to the quaternary structure of vicilin, a multi-subunit protein lacking in disulfide linkages (Ismond, 1984).

A basic consideration concerning protein stabilization is the type of conformational changes that may occur in conjunction with the stabilization phenomenon. It seems logical to anticipate that the response of a protein to the lyotropic effect of preferential hydration might also involve a change in protein surface properties. For vicilin, there was a significant decrease in *So* with increasing concentrations of the highly stabilizing anions, citrate and sulfate. This would imply dynamic changes in surface features to increase the burial of hydrophobic residues, a thermodynamically favourable event in an aqueous environment. A similar trend was not observed for the moderately stabilizing anions. The relatively constant *So* values for vicilin exposed to $NaC_2H_3O_2$ and NaCl may reflect the tendency of these anions to bind to the protein, especially at high concentrations. Arakawa & Timasheff (1982) demonstrated that acetate, unlike the more stabilizing anions, is not completely excluded from the surface of the protein at high concentrations. Similarly, others (Scatchard *et al.*, 1957) have shown that chloride ions may exhibit binding to proteins. Therefore, the attractive forces between these anions and

vicilin may overcome somewhat the highly stabilizing environment imposed by preferential hydration when salt molecules are excluded from the surface of the protein.

In this study, the inclusion of Br^- as a stabilizing ion with respect to its influence on vicilin is contradictory to a number of other protein observations. For example, ribonuclease (von Hippel & Wong, 1964), sesame α-globulin (Prakish & Nandi, 1977) and a variety of biomembranes and multi-protein complexes (Hanstein *et al.*, 1971) have been destabilized with exposure to even low concentrations of the bromide anion. With vicilin, although the overall increase in *Td* values with increasing bromide concentrations was among the lowest of the values for the stabilizing anions, the bromide anion did not result in protein destabilization as assessed by calorimetry. It would appear that bromide may exert a mildly stabilizing or destabilizing influence, depending on the conformational properties of the protein in question and on the analysis method used.

In contrast to the other anions examined, SCN^- had a destabilizing influence on vicilin, as evidenced by the thermal properties observed. Destabilizing anions in general appear to be characterized by a low capacity to increase the surface tension of water (Melander & Horvath, 1977) and, consequently, by a reduced capacity to induce protein preferential hydration (Arakawa & Timasheff, 1982). In addition, these anions actually appear to remain preferentially bound to the protein structure, creating conformational disturbances (Bull & Breese, 1970; Arakawa & Timasheff, 1982). In fact, Arakawa & Timasheff (1982) demonstrated that a significant amount of SCN^- would bind preferentially to bovine serum albumin at high concentrations of the salt. Binding of the SCN^- should increase the electrostatic free energy of the protein with the resulting repulsive forces causing a decrease in protein stability. This premise appears to be substantiated by the decrease in thermal stability and the increase in *So* values for vicilin with increasing NaSCN concentrations. If protein destabilization involves a progressive unfolding of the molecule, it is expected that *So* values would reflect the increased exposure of hydrophobic residues.

Effects of specific anions on micelle formation

As observed, vicilin is a dynamic protein molecule, which exhibits conformational responses to the composition of the surrounding

medium. Correlation of these conformational parameters with the capacity of vicilin to self-associate under specific environmental conditions should be informative as to the molecular requirements for micelle formation to occur.

At low ionic strengths of environmental media, sudden dilution of the concentrated vicilin solutions may have resulted in a physical disturbance of the electrical double layer associated with the protein molecules. Therefore, intermolecular association of vicilin by hydrophobic association would be thermodynamically feasible in response to the aqueous environment if the protein *So* values were adequate for this interaction. As a further consequence of the dilution effect, electrostatic influences on the micelle surface may be minimized such that inter-micelle repulsive forces are reduced and further associations are favored. This explanation may apply to the extensive protein networks observed for vicilin in NaSCN and Na_2SO_4 (0·1M), plus NaI, $NaC_2H_3O_2$, NaBr, NaCl (0·2M).

Massive protein interactions persisted with 0·5M levels of the moderately stabilizing anions ($C_2H_3O_2^-$, Br^-, Cl^-). Although pre-dilution salt concentrations were at levels where lyotropic effects are assumed to be predominant, the preferential hydration of the protein molecules did not result in *So* values that were detrimental to micelle formation. Post-dilution electrostatic interactions with the micelle surface were also not prohibitive for inter-micelle associations; in fact, extensive coalescence and aggregation occurred in all three media. Deterioration of the micelle response at 1·0M levels of these salts occurred despite little change in the molecular *So* values. As the initial post-dilution response was massive micelle formation and interaction, it was assumed that immediate post-dilution conditions were favorable for protein association. However, following dilution, anions may have rapidly diffused into the dilution area, establishing anion–protein surface interactions; resulting electrostatic repulsion among surface residues of the aggregated mass may have had a destabilizing influence, causing progressive deterioration of protein association with continued anion binding.

In general, the highly stabilizing salts (Na_2SO_4 and $Na_3C_6H_5O_7$) were relatively unsuitable media for extensive micelle formation and subsequent interaction. For all concentrations of $Na_3C_6H_5O_7$ examined, the ionic strength may be such that lyotropic influences predominate in pre-dilution conditions. As a possible consequence of preferential hydration, the reduced *So* values (ranging from 191 to 124) may have been

inadequate to promote extensive hydrophobic associations. For concentration levels where limited micelle formation did occur, the post-dilution environment did not allow inter-micelle association. At low levels of $Na_3C_6H_5O_7$ (i.e. 0·1M), the reduction of salt levels by sudden dilution may have invoked an electrostatic effect by the trivalent cations resulting in protein solubilization as a consequence of an established electrical double layer. With higher $Na_3C_6H_5O_7$ levels (0·2 and 0·5M), continued surface hydration of the micelles may have discouraged hydrophobic residue exposure and subsequent inter-micelle association.

The observed micelle responses with Na_2SO_4 are assumed to result from similar phenomena suggested for the citrate media. One exception exists—with 0·2M Na_2SO_4 the micelle response was reduced despite a high *So* value (273) for vicilin. This may be attributed to extensive electrostatic influences, rather than reduced exposure of hydrophobic residues.

Interesting micelle responses were observed with vicilin in the destabilizing media. As the concentrations of both NaSCN and NaI were increased, the capacity for vicilin to self-associate decreased; this paralleled a destabilization in vicilin conformation, as evidenced by elevated *So* values (SCN^-) and decreased *Td* values (both I^- and SCN^-). Two forces may be operative in this situation. With specific reference to NaSCN, it is known that the anion binds specifically to the protein, resulting in solubilization and destabilization. This binding may persist with dilution causing intermolecular electrostatic repulsion, or may resume after dilution to create a charge repulsion among established micelles. Secondly, the high *So* values for vicilin in NaSCN (474 to 572) would initially seem ideal for hydrophobic association and micelle formation. However, the extensive exposure of originally internal hydrophobic sidechains may disturb the orientation of the hydrophilic–hydrophobic residues such that micelle formation is more difficult. The establishment of a detergent micelle is known to depend on a distinct intramolecular hydrophilic–hydrophobic balance (Tanford, 1973). This may also be applicable to protein micelle structures. For example, Simons *et al.* (1978) suggested that bacteriorhodopsin, an integral membrane protein, would not self-associate into a micelle structure as a result of its high molecular hydrophobicity.

In general, the self-association of vicilin into a micelle arrangement is influenced critically by environmental impact on the protein surface properties. Vicilin was most interactive if the initial molecule was

moderately stabilized, as assessed by thermal properties, with an 'adequate' *So* value, usually in the vicinity of 240 ± 40. Highly stabilized vicilin molecules were frequently characterized by low *So* values; as such, the relative exposure of hydrophobic residues appeared to be insufficient for extensive molecular interaction. Similarly, destabilized vicilin molecules did not promote extensive micelle responses although *So* values were high (>400); this was an observation partially attributed to an imbalance in hydrophobic–hydrophilic intramolecular relationships. The most appropriate media for micelle formation and subsequent interaction were low concentrations (0·2 and 0·5M) of moderately stabilizing univalent anionic electrolytes, such as NaCl or $NaC_2H_3O_2$. In these environments, the physical impact of the aqueous medium may have been sufficient to disturb the electrical double layer associated with vicilin, allowing the thermodynamically favorable aggregation of hydrophobic residues. The post-dilution microenvironment established by the new electrolyte–protein–water mixture favored attractive protein associative interactions rather than intermolecular repulsion.

CONCLUSIONS

The conformational properties of vicilin are influenced greatly by the environmental media; these characteristics, in turn, are related to the capacity of vicilin to self-associate into a micelle arrangement. From these relationships, the micelle response appeared to be ultimately dependent on a balance of non-covalent forces operative at several levels. For example, vicilin appeared to be an appropriate protein for micelle formation on the basis of the molecular surface properties, with specific reference to the distribution of hydrophobic and hydrophilic residues. Distortion of this hydrophilic–hydrophobic balance, either by the destabilizing action of certain electrolytes to exaggerate the surface hydrophobicity or by preferential hydration of the protein surface to minimize hydrophobic residue exposure, was detrimental to micelle formation. Extension of this concept of a balance of non-covalent forces to an intermolecular level was inherent in the realization that non-covalent intermolecular attractive forces had to predominate over repulsive interactions if micelle formation and subsequent association were to occur. The main attractive forces appeared to be hydrophobic in nature; the sudden introduction of a controlled aqueous environment was

essential for massive micelle responses. In addition, the exposure of a certain number of hydrophobic residues appeared to be critical for intermolecular interaction. On the other hand, the main repulsive force appeared to be electrostatic, with the magnitude of the repulsion related to the interactions of specific ions with individual protein molecules at the micelle surface. An appreciation of the molecular forces involved in this type of protein–protein association may eventually lead to the controlled development of such interactions in a food system. The protein networks that form spontaneously from a basic micelle response under certain conditions are reminiscent of the protein aggregations necessary for the integrity of a number of food products. Further study will be directed toward the exploration of the non-covalent forces involved in these types of interaction.

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