Food Chemistry **29** (1988) 189–198

The Role of Noncovalent Forces in Micelle Formation by Vicilin from *Vicia faba*. III. The Effect of Urea, Guanidine Hydrochloride and Sucrose on Protein Interactions

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(Received 26 August 1987; revised version accepted 26 October 1987)

ABSTRACT

The effect of denaturing (urea, guanidine hydrochloride (Gdn HCl)), and stabilizing (sucrose) media on the capacity of vicilin from fababeans to self-associate into a micelle arrangement was examined. The micelle observations were related to protein conformational parameters, specifically thermal stability plus aliphatic (CPA) and aromatic (ANS) surface hydrophobicity (S_o). Both urea and GdnHCl had a negative impact on micelle formation at low concentrations (0·1M urea and 0·2M GdnHCl). At higher denaturant levels, the deteriorating micelle response was correlated with a decrease in thermal stability (T_d, ΔH) and a decrease in aromatic and aliphatic S_o values. Exposure to sucrose was also detrimental to micelle formation; this was attributed to preferential hydration of the protein molecules. In all situations, a delicate balance of hydrophilic–hydrophobic forces seemed to be required for the micelle phenomenon.

INTRODUCTION

The fababean storage protein, vicilin, has the capacity to self-associate into a micelle arrangement under specific environmental conditions. In some circumstances, the micelle is not an end-point, but rather is an intermediate in the formation of extensive networks and amorphous protein sheets (Ismond *et al.*, 1986*a*). The extent of the micelle reaction appears to be controlled by a delicate balance of noncovalent forces, specifically hydrophobic and electrostatic interactions (Ismond *et al.*, 1986*b*). The main

Food Chemistry 0308-8146/88/$03·50

attractive forces seem to be hydrophobic in nature, as the introduction of a controlled volume of water is required for extensive micelle responses. In addition, the exposure of a certain number of hydrophobic residues appears to be critical for intermolecular association. On the other hand, the main repulsive force which inhibits micelle formation and subsequent interaction seems to be electrostatic in nature. The magnitude of electrostatic repulsion appears to be related to the interactions of specific ions with individual protein molecules at the micelle surface (Ismond *et al.*, 1986*b*).

The structures formed by extensive micelle associations are reminiscent of the protein aggregations necessary for protein integrity and functionality in a number of food products. An appreciation of the molecular forces involved in this type of protein–protein association should eventually lead to the controlled development of such types of interactions in a food system. As a result, this study was designed to explore, further, the noncovalent interactions important to micelle formation and interaction. The capacity of vicilin to self-aggregate was assessed in both denaturing (urea, guanidine hydrochloride) and stabilizing (sucrose) media. Several parameters were correlated with observed micelle interaction patterns; these included thermal properties as indicators of the degree of protein denaturation and surface hydrophobicity (both aliphatic and aromatic) as an assessment of the potential for hydrophobic associations.

MATERIALS AND METHODS

Protein isolation

Vicilin was isolated from the seed of the fababean (*Vicia faba* var. *Diana*) using the method described by Ismond *et al.* (1986*a*). Protein samples were dialyzed against distilled water and lyophilized until required.

Environmental influence

To assess the effects of denaturants and sucrose on selected aspects of protein conformation and micelle-forming capacity, solutions of vicilin (approximately 1 mg/ml) were prepared using 0·2M sodium acetate, pH 7·0, plus specific levels of denaturants and sucrose. Urea (Fisher) concentrations ranged from 0·1 to 8·0M; guanidine hydrochloride (Sigma) varied from 0·1 to 4·0M. Levels of sucrose ranged from 10 to 40%. For each concentration of denaturant or sucrose, the capacity of vicilin to form micelles was assessed using the microscopic method described by Ismond *et al.* (1986*a*). The thermal properties of vicilin in each environment were determined using a

DuPont Thermal Analyzer with a 910 Differential Scanning Calorimeter cell base, as described by Arntfield & Murray (1981). Thermal curves 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. The vicilin samples were concentrated to approximately 10 mg/ml using an Amicon Minicom B-15 macrosolute concentrator prior to DSC analyses. The thermal parameters T_d (denaturation temperature) and ΔH (enthalpy of denaturation) were determined from all thermal curves.

In addition, the surface hydrophobicity (S_o) of vicilin exposed to all environments was determined by two methods. Aliphatic hydrophobicity was established using the method of Kato & Nakai (1980) with *cis*-parinaric acid (CPA; Calbiochem–Behring Corp.) as a fluorescence probe. Aromatic hydrophobicity was determined according to the method of Hayakawa & Nakai (1985) using 1-anilino-8-naphthalene sulfonate (ANS; Sigma) as a fluorescence probe. Fluorescence intensities were measured in a Perkin–Elmer fluorescence spectrophotometer (Model No. LS-5) using a slit width of 5·0 nm and a fixed scale of 1·0.

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. All thermal parameters and S_o values were determined using a minimum of four samples; means and standard deviations of the means are given for each.

RESULTS

The response of vicilin to urea varied with the different parameters assessed. From a thermal stability perspective, there was no significant decrease in denaturation temperature (T_d) or enthalpy of denaturation (ΔH) until vicilin was exposed to 1·0M urea (Table 1). Similarly, the aliphatic S_o (CPA) was relatively stable up to a 1·0M urea environment. In contrast, there was a significant decrease in aromatic S_o (ANS) with exposure to 0·5M urea. However, the micelle rating was the most sensitive parameter with respect to denaturant addition. There was a change from extensive coalescence (rating 5; Fig. 1A) to small granular granular networks (rating 2; Fig. 1B) with the inclusion of 0·1M urea. The micelle reaction remained constant in terms of a rating description up to 3·0M urea; however, the actual amount of interaction decreased with increasing urea levels. Above 3·0M urea, the micelle response was totally suppressed. In conjunction with these changes, the thermal stability of vicilin, as reflected by T_d and ΔH values, decreased significantly at urea levels greater than 1·0M. Complete denaturation, as assessed by the absence of an endotherm, occurred at 4·0M urea. Both

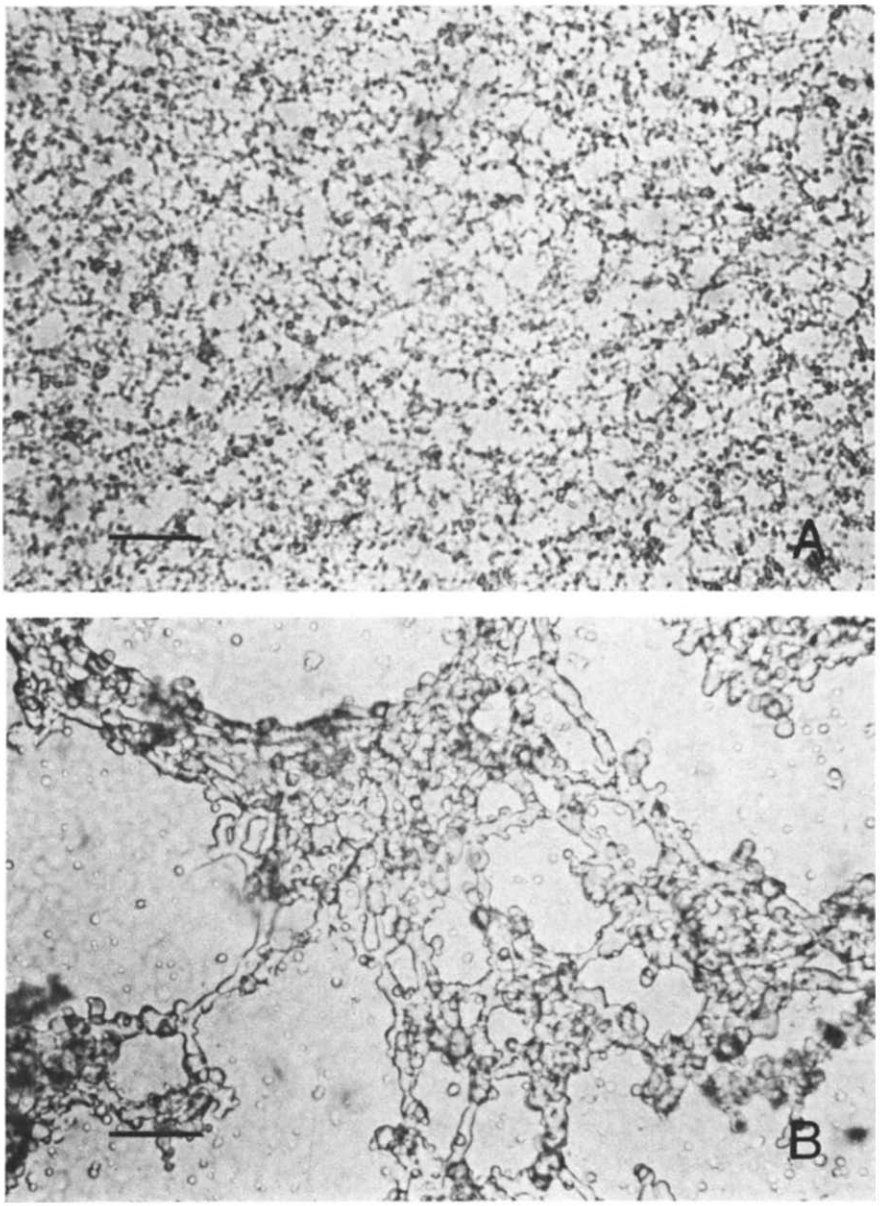

Fig. 1. Photomicrographs of micelle formation by vicilin without urea (A, rating 5) and with 0·1M urea (B, rating 2). Bar represents 25 μm.

TABLE 1

Denaturation Temperatures (T_d), Enthalpies of Denaturation (ΔH), Surface Hydrophobicities (S_o) and Micelle Ratings (MR) for Vicilin Exposed to Different Levels of Urea

Urea concentration	T_d[a] *(0°C)*	ΔH *(Jg^{-1})*	S_o[b] *(ANS)*	S_o[c] *(CPA)*	*Micelle rating*
0·0	89·5 ± 0·0	13·58 ± 2·13	637·7 ± 29·6a	172·0 ± 6.0a	5
0·1	87·6 ± 0·8a	7·56 ± 1·34a	610·7 ± 63·9a,b	168·0 ± 20·0a	2
0·2	88·4 ± 0·7a	6·52 ± 2·13a	560·3 ± 22·7a,b	158·4 ± 30·8a,b	2
0·5	86·2 ± 1·2a	10·41 ± 3·09b,c	528·5 ± 64·8b,c	161·2 ± 7·2a,b,c	2
1·0	83·1 ± 0·7	11·62 ± 1·96b	473·2 ± 2·6c	138·2 ± 2·0b,c	2
1·5	80·3 ± 1·3b	7·48 ± 2·72a,c	330·0 ± 81·0d	129·4 ± 29·6b,c,d	2
2·0	79·3 ± 0·8b	5·23 ± 1·67d	260·4 ± 61·9d	127·2 ± 1·8b,c,d	2
3·0	75·8 ± 0·4	2·97 ± 1·38d	172·8 ± 50·7	122·4 ± 8·4c,d	2
4·0	NA	NA	88·3 ± 7·6	100·6 ± 32·2d	NR
5·0	NA	NA	26·7 ± 1·6	30·2 ± 1·0e	NR
6·0	NA	NA	12·9 ± 7·7e	36·2 ± 7·0e	NR
7·0	NA	NA	2·2 ± 0·4e	23·4 ± 3·8e	NR
8·0	NA	NA	1·4 ± 0·2e	14·6 ± 0·6e	NR

Column values followed by the same letter are not significantly different ($P \leq 0.05$) as determined by a multiple t test.

NA, values not available due to protein denaturation.

NR, no micelle response.

[a] $T_d = 88.0 - 4.3$M. Standard linear regression analysis, $R = -0.9853$; $P < 0.001$.

[b] S_o (ANS) $= 532.8 - 84.1$M. Standard linear regression analysis. $R = 0.9362$; $P < 0.001$.

[c] S_o (CPA) $= 167.2 - 10.3$M. Standard linear regression analysis. $R = 0.9749$; $P < 0.001$.

aliphatic and aromatic S_o values decreased as a direct function of urea concentration (Table 1).

Similar trends were apparent with vicilin exposed to guanidine hydrochloride (GdnHCl). Surface hydrophobicities and T_d values decreased as a linear function of the GdnHCl concentration (Table 2). The ΔH values decreased significantly at 1·0 and 1·5M GdnHCl; complete denaturation occurred with 2·0M GdnHCl. In contrast to the urea results, the micelle response was more stable to denaturant concentration. A strong response (rating 5) was observed at concentrations up to 0·2M GdnHCl; deterioration of the reaction occurred at higher denaturation levels.

The micelle response also decreased with increasing exposure to sucrose, a stabilizing compound. Maximum reaction (rating 5) was observed at 10% and 15% sucrose (Table 3); the response deteriorated to rating 2 at 20% followed by rating 1 (a fine haze) at 40% sucrose. With respect to the molecular parameters, both aromatic and aliphatic surface hydrophobicities decreased in response to an initial exposure to low levels (10%) of sucrose. However, in both cases, S_o values were constant from 10% to 40% sucrose.

TABLE 2

Denaturation Temperatures (T_d), Enthalpies of Denaturation (ΔH), Surface Hydrophobicities (S_o) and Micelle Ratings (MR) for Vicilin Exposed to Different Levels of Guanidine Hydrochloride

GdnHCl concentration (M)	T_d[a] (°C)	ΔH (*joules* g^{-1})	S_o[b] (*ANS*)	S_o[c] (*CPA*)	*Micelle rating*
0·0	89·0 ± 0·0a	13·58 ± 2·13a	637·7 ± 29·6	172 ± 6·0a	5
0·1	87·6 ± 0·5a,b	10·45 ± 3·51a,b	541·6 ± 1·6a	151·6 ± 15·8b	5
0·2	87·2 ± 0·8b,c	11·61 ± 0·17a,b	564·0 ± 9·3a	161·2 ± 18·2a	5
0·5	85·6 ± 1·1c	11·83 ± 1·88a,b	459·2 ± 46·4	144·0 ± 3·0b	2
1·0	80·3 ± 0·3d	8·15 ± 0·38c	359·2 ± 45·6	140·6 ± 9·6b	2
1·5	79·0 ± 1·0d	4·97 ± 2·17c	208·8 ± 9·7	135·6 ± 3·6b	2
2·0	NA	NA	77·4 ± 4·3	81·4 ± 2·4	NR
3·0	NA	NA	10·8 ± 0·4b	32·2 ± 4·4c	NR
4·0	NA	NA	2·5 ± 0·3b	26·2 ± 3·6c	NR

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

NA, values not available due to protein denaturation.

NR, no micelle response.

[a] $T_d = 88{\cdot}6 - 6{\cdot}9$M. Standard linear regression analysis. $R = -0{\cdot}9825$; $P < 0{\cdot}001$.

[b] S_o (ANS) $= 516 - 156{\cdot}1$M. Standard linear regression analysis. $R = -0{\cdot}9475$; $P < 0{\cdot}001$.

[c] S_o (CPA) $= 159{\cdot}6 - 34{\cdot}8$M. Standard linear regression analysis. $R = 0{\cdot}9421$; $P < 0{\cdot}001$.

TABLE 3

Denaturation Temperatures (T_d), Enthalpies of Denaturation (ΔH), Surface Hydrophobicities (S_o) and Micelle Ratings (MR) for Vicilin Exposed to Different Levels of Sucrose

Sucrose concentration (%*w/v*)	T_d[a] (°C)	ΔH (*joules* g^{-1})	S_o (*ANS*)	S_o (*CPA*)	*Micelle rating*
0·0	89·0 ± 0·0a	13·58 ± 2·13a	637·0 ± 29·6	172 ± 6·0	5
10	89·2 ± 0·9a	12·12 ± 2·17a	399·0 ± 70·0a	87·2 ± 25·2a	5
15	90·4 ± 1·7a,b	13·67 ± 1·96a	390·5 ± 23·1a	112·6 ± 11·8a	5
20	91·4 ± 0·7a,b	12·41 ± 1·17a	398·0 ± 33·9a	104·0 ± 12·0a	2
30	92·5 ± 1·4b	15·93 ± 2·26a	319·8 ± 37·8a	109·0 ± 4·8a	2
40	98·9 ± 1·9	14·55 ± 4·35a	384·7 ± 51·8a	101·2 ± 8·4a	1

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

[a] $T_d = 87{\cdot}4 + 0{\cdot}233$°C. Standard linear regression analysis. $R = 0{\cdot}9082$, $P < 0{\cdot}001$.

Evidence of protein stabilization with increasing sucrose levels was reflected by the T_d values; the T_d showed a linear increase with increasing sucrose concentrations (Table 3). There was, however, little variation in the ΔH parameter.

DISCUSSION

Micelle formation by protein molecules has been previously attributed to a delicate balance of noncovalent forces (Ismond *et al.*, 1986*a,b*). This study was designed to pursue this premise by examining the micelle response with solutes having known influences on noncovalent forces. Urea and GdnHCl, two of the solutes chosen, are denaturants which have been the subject of many investigations (Tanford, 1968, 1970; Pace, 1975). In both cases, the end-product of denaturation is a randomly coiled protein; this result is caused primarily by the disruption of noncovalent interactions (Tanford, 1968). The impact of disrupting these noncovalent forces in the protein vicilin was monitored, both in terms of the micelle response and specific conformational parameters. Interestingly, micelle formation by vicilin was affected by 0·1 M urea, a concentration at which only changes in ΔH of the thermal parameters could be detected. These results were similar to a study by Yao *et al.* (1984) on creatine kinase; in this situation, the enzyme was inactivated at urea levels that had no influence on protein conformation as monitored by UV spectroscopy, fluorescence and exposure of sulfhydryl groups.

The micelle response for vicilin remained constant from 0·1 to 3·0 M urea. Minor conformational disturbances were evident at 0·1 M urea in terms of a decreased ΔH value. An interesting response occurred at 1·0 M and 1·5 M urea; at both these concentrations the ΔH increased significantly. This increase in ΔH may be a function of the mode of operation of urea. If urea is interfering with hydrophobic interactions, then the exothermic contribution to ΔH from disruption of the hydrophobic association with heating is reduced; this results in an overall increase in ΔH.

Major conformational disturbances were apparent with vicilin exposed to 1·0 M urea; both the thermal stability and the S_o decreased significantly at this point. This trend continued in a linear progression as the concentration of urea was increased. The decrease in both aromatic and aliphatic S_o values was unexpected; deterioration of conformation to a random coil should be paralleled by an increase in the exposure of hydrophobic residues. The decreased values observed here may reflect intermolecular aggregation as denaturation progressed. Other studies have shown noncovalent associations of various proteins with urea and GdnHCl-induced unfolding

(Prakash & Nandi, 1977; Fish *et al.*, 1985). The decrease in S_o was not attributed to fluorescence quenching; preliminary studies showed that the binding of ANS and CPA was not impacted by the presence of urea or GdnHCl.

Similar results to those with urea were observed for vicilin exposed to various levels of GdnHCl. Although GdnHCl is considered to be a more potent denaturant than urea (Aune *et al.*, 1967; Tanford, 1968, 1970; Salahuddin & Tanford, 1970; Green & Pace, 1974), the micelle response remained unaffected up to a concentration of 0·5M GdnHCl. From that point, an increase in GdnHCl levels resulted in a decrease in the micelle response plus a decrease in both S_o and thermal stability. From the data for both urea and GdnHCl, there was not a definite aromatic or aliphatic S_o value below which the micelle response changed. A decrease in aromatic S_o to 610·7 and aliphatic S_o to 168 was sufficient to reduce the micelle response from the maximum reaction (rating 5) to a lesser reaction (rating 2) in urea. On the other hand, the same micelle change occurred for vicilin in GdnHCl when the aromatic S_o decreased to 459·2 and the aliphatic S_o to 144. In both cases, the overall decrease in surface hydrophobicity may have occurred in conjunction with increased electrostatic repulsion to result in a reduction of the micelle response.

With complete denaturation of vicilin by both urea and GdnHCl, the micelle response was totally suppressed. 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—a reasonable conclusion as micelle formation is considered to be a hydrophobically driven event. Electrostatic repulsion may have also become prohibitive to micelle formation at these denaturant levels.

In contrast to the denaturants, sucrose is a solute that stabilizes proteins in their native conformation (Ball *et al.*, 1943; Frigon & Lee, 1972; Timasheff *et al.*, 1976; Lee & Timasheff, 1981). This stabilizing effect is apparent from the thermal data for vicilin; an increase in sucrose concentration resulted in a direct increase in denaturation temperature. In addition, the presence of even a low level of sucrose impacted the surface properties of vicilin. A decrease in both aliphatic and aromatic S_o values was observed; this may reflect the consequence of preferential hydration of vicilin in the presence of sucrose. That is, the solvation of the protein surface may result in increased intramolecular hydrophobic interactions.

Although the S_o values decreased in response to 10% sucrose, the overall surface hydrophobicity was constant with increasing sucrose levels. However, the capacity for vicilin to self-associate was not constant; the micelle response deteriorated gradually with increasing sucrose concentrations. Rather than attributing the decrease in molecular interaction to

a decrease in the S_0 the observed micelle responses may also be related to the preferential hydration phenomenon. As the level of sucrose increases, there is an increase in the solvent cohesive force (Lee & Timasheff, 1981). As a result, the energy required for cavity formation for the associated structures in the solvent may be prohibitive to the interaction process.

CONCLUSIONS

Both denaturing and stabilizing media had a negative impact on the capacity of vicilin to self-associate into a micelle arrangement. Environmental manipulation affected vicilin conformational parameters; this, in turn, disrupted the critical hydrophobic–hydrophilic balance necessary for the micelle type of interaction. Reduction of the surface hydrophobicity either by molecular aggregation or preferential hydration of the protein surface to minimize hydrophobic residue exposure was detrimental to micelle formation and interaction. These observations reinforce the concept that a delicate balance of noncovalent forces is required for the micelle phenomenon. Further study will be directed toward the controlled development of such protein interactions in a food system.

ACKNOWLEDGEMENT

The support of this project by operating grants from the Natural Sciences and Engineering Council of Canada is gratefully acknowledged.

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