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Evidence for a molten globule state in an oligomeric plant protein

Massimo F. Marcone,* Rickey Y. Yada & Yukio Kakuda

Department of Food Science, Ontario Agricultural College University of Guelph, Guelph, Ontario, Canada N1G 2W1

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Three different mustard globulin (isoforms), purified and isolated, using three extraction solutions (differing in ionic strength) were studied for structural integrity as a function of pH. Evidence of a molten globule state (a reversible intermediary state between the native and fully denatured forms) was obtained. This phenomenon may ultimately prove to be important in the translocation of these proteins across biological membranes at the time of their biosynthesis. Circular dichroism, hydrophobic probe, fluorescence spectral scans and differential scanning calorimetry were used to study this phenomenon. Secondary and tertiary structures (circular dichroism (CD) data) were found to be similar for globulins from higher ionic strength extractions, but different from the globulin from distilled water extraction; however, for all three isoforms, little change in secondary structure fractions as a function of pH was observed. Changes in tertiary structure (near-UV CD and intrinsic fluorescence data) as a function of pH were observed for all three globulin isoforms with greatest changes in tertiary structure being seen in the acidic pH range, i.e. 3–5. In contrast, all globulins were shown to undergo the least conformational change in the pH range of 6–9.

INTRODUCTION

The term 'molten globule state' in protein chemistry has been coined to describe a protein's conformational state which is intermediate between that of the native and fully unfolded state (Kuwajima, 1989; Baldwin, 1991; Christensen & Pain, 1991; Hirose, 1993). The molten globule state is characterized by a substantial loss of tertiary structure while maintaining secondary structure and overall compactness and is often associated with low pH (Christensen & Pain, 1991; Dobson, 1992).

From a food science perspective, several food proteins from animal sources such as ovalbumin (Koseki *et al.*, 1988), lysozyme (Kato *et al.*, 1981; Miranker *et al.*, 1991; Radford *et al.*, 1992; Van Dael *et al.*, 1993), α-lactalbumin (Baum *et al.*, 1989) and β-lactoglobulin (Kuwajima *et al.*, 1987; Ptitsyn *et al.*, 1990; Bychkova *et al.*, 1992) have been shown to take on a molten globule state upon exhibiting such functional properties as gelation, foaming and emulsification. The molten globule state may help to explain these functional properties (Hirose, 1993).

In proteins, the exposure of hydrophobic amino acids and hydrophilic amino acid residues to water in the molten globule state can confer an amphiphilic nature to these proteins (Hirose, 1993). It is, therefore, believed that food proteins in the molten globular state would effectively interact with both water and lipid layers at the water–oil interface (such as in emulsions) (Hirose, 1993). The amphiphilic nature of proteins in the molten globular state may also be effective in stabilizing foams (Hirose, 1993) since the involvement of some conformational rearrangement in proteins during foaming has been shown (Kitabatake & Doi, 1987).

Research into the molten globule state in food proteins has been confined, in general, to small monomeric globular proteins that are known to have single domain characteristics. To date, no evidence in the literature exists for the occurrence of the molten globule state in oligomeric and multi-domain seed storage proteins. A study of the structural integrity of three mustard globulin isoforms as a function of pH was undertaken to determine if the molten globule state existed. Mustard globulin

*To whom correspondence should be addressed.
Tel: (519) 824-4120 ext. 6313; fax: (519) 824-6631;
E-mail: mmassimo@voguelph.ca

isoforms have been previously shown to exist as 304 kDa hetero-oligomeric type proteins composed of seven subunits (38.7–11.6 kDa) with pI ~ 7.10 (Marcone *et al.*, 1997).

MATERIALS AND METHODS

Sample preparation

The mustard seed meal of *Brassica alba* passing through a 60 mesh sieve was defatted with hexane in the ratio of 1:10 (w/v) (flour/acetone) as described in Marcone *et al.* (1997).

Protein fractionation

The salt-soluble protein (globulin) was extracted from the defatted meal with three different extraction solutions, i.e. Extraction 1: 1.0 M NaCl, pH 7.5 ($\mu = 1.0$); Extraction 2: 32.6/2.6 mM K_2HPO_4/KH_2PO_4 buffer, pH = 7.5 ($\mu = 0.5$) containing 0.40 M NaCl; and Extraction 3: distilled water, pH 7.5 ($\mu = 0$) as described in Marcone *et al.* (1997).

Determination of protein concentration

Protein was measured using the Bio-Rad protein dye-binding assay (modified Bradford) with bovine serum albumin (BSA) as the standard (Bio-Rad, 1989). In addition, a spectrophotometric method was used based on the difference in absorbance of proteins at 235 and 280 nm (Whitaker & Granum, 1980). Crude protein was also measured by a modified Büchi Kjeldahl procedure (AACC, 1983). Tests were performed in triplicate in the case of the modified Bradford and spectrophotometric methods and in duplicate in the case of the Kjeldahl method.

Gel filtration chromatography (GFC)

A Sephacryl™ S-300 Superfine (2.5×95 cm) gel filtration column with a molecular weight fractionation range of 10 000-1 500 000 Da was packed and equilibrated with three bed volumes of the above extraction buffer, i.e. 32.6/2.6 mM K_2HPO_4/KH_2PO_4 buffer, pH = 7.5, containing 0.40 M NaCl ($\mu = 0.5$). Blue dextran (2 000 000 Da) was used to measure the void volume. A standard curve was prepared using the following proteins: ferritin (440 000 Da), catalase (232 000 Da), aldolase (158 000 Da), and cytochrome c (12 400 Da). Purification of the globulins from each of the three extraction methods was performed as described in Marcone *et al.* (1997).

Anion-exchange chromatography

Anion-exchange chromatography was performed using a Fast Protein Liquid Chromatograph (FPLC) equipped with two P-500 pumps, LCC-500 PLUS/CI controller and UV-280 detector (Pharmacia LKB, Montreal, PQ) as described in Marcone *et al.* (1997). A Mono-Q™ 5/5 anion-exchange column (Pharmacia LKB, Montreal, PQ) was used for protein separation

Near- and far-UV circular dichroism (CD) spectroscopy

Circular dichroic measurements were carried out in the near-UV (240–320 nm) and far-UV (190–250 nm) at 20°C under constant nitrogen purge using a Jasco J-600 spectropolarimeter (Japan Spectroscopic Co. Ltd., Tokyo, Japan) with a cell pathlength of 10 and 1 mm, respectively. A protein concentration of approximately 0.1 mg ml^{-1} in 50 mM citrate/phosphate buffers of pH 3.0, 4.0, 5.0, 6.0, 7.5 and 9.0, containing 0.40 M NaCl was used in the determinations. Secondary structure fractions were determined from the far-UV spectra using the Jasco SSE program, which is based on the algorithm of Chang *et al.* (1978). Analyses were performed in triplicate with six scans per replicate.

Surface hydrophobicity

Aromatic surface hydrophobicities of the globulins were determined using 1-anilino-8-naphthalenesulfonate (ANS) as a function of pH according to the methods of Hayakawa and Nakai (1985) and Akita and Nakai (1990), using protein concentrations of between 0.001 and 0.30% (w/v) in the buffers described earlier. An excitation wavelength of 380 nm and an emission wavelength of 475 nm were used.

Fluorescence spectra

A Shimadzu RF-540 spectrofluorophotometer was used to measure emission spectra in the range 290–500 nm after excitation at 280 nm. Globulin solutions of 0.02% (w/v) in 50 mM citrate/phosphate buffer containing 0.40 M NaCl at pH 3.0, 4.0, 5.0, 6.0, 7.5 and 9.0 were used. Measurements were made in triplicate.

Micro-differential scanning calorimetry

DSC measurements were performed on an MC-2D Instrument (Upscan Ultrasensitive Differential Scanning Calorimeter) with twin 1.2 ml total-fill tantalum cells. Data were collected using the Origin DSCITC Data Collection Software V.1.1. and analyzed using the Origin Version 2.9 DSC Program (MicroCal, Inc., Northampton, MA, USA). Thermograms were obtained employing a heating rate of 1.37°C/min from 30 to 110°C (under 20 psi pressure) and performed in duplicate. Proteins were diluted to a concentration of 0.5 mg ml^{-1} and dialysed against a 35 mM citrate/phosphate buffer pH 3.0 at 4°C overnight prior to analysis. An identical buffer served as the reference. An identical sample was prepared in the same buffer of pH 3.0 and was dialysed against a 32.6/2.6 mM K_2HPO_4/KH_2PO_4

buffer, pH 7.5, containing 0.40 M NaCl overnight and DSC analysis was performed in a similar way. Analyses were performed in duplicate.

Statistical analysis

All data were analyzed using Analysis of Variance (ANOVA) and Duncan's new multiple range tests when appropriate (SAS Institute, 1990).

RESULTS AND DISCUSSION

Secondary structure of globulins as a function of pH

Of the secondary structure fractions measured, the β-sheet secondary structure fraction was the least affected by changes in pH in globulins from Extractions 1 and 2 (high ionic strength), whereas some conformational change was seen in the basic pH range, i.e. pH 9, for the globulin from Extraction 3 (distilled water) (Table 1). β-sheets are usually considered to be buried in the interior of proteins (Hopp, 1986) and, therefore, would indicate that the interior conformations of isoforms 'B' extracted at higher ionic strength (Extractions 1 and 2) are not greatly affected by pH. In contrast, the interior conformation, the β-sheet of the globulin from Extraction 3, showed small but notable changes at basic pH extremes.

Examination of the β-turn secondary structure fraction of the globulins from Extractions 1, 2, and 3 showed slight conformational changes with change in pH. Although the isoforms from Extraction 2 showed the least change, those from Extractions 1 and 3 showed more change at the neutral to alkaline pH range. β-turns have a propensity of being located at the surfaces of protein owing to their hydrophilic nature (Hopp, 1986; Scheidtmann, 1990). The small conformational changes seen could be due to the ionization of surface charged groups causing electrostatic repulsions between acidic amino acids at alkaline pH.

In the case of random coil fraction, globulin from Extraction 2 showed little conformational change whereas isoforms from Extractions 1 and 3 showed significant changes ($P \leq 0.05$) in going from acidic to basic pH values (Table 1).

Tertiary structure of globulins as a function of pH

Changes in near-UV CD spectra were seen for all globulins as a function of pH, as indicated by the general changes in the intensity or amplitude of the spectra (Figs 1–3). Globulins isolated from Extractions 1 ($\mu = 1.0$) and 2 ($\mu = 0.5$) had similar fine structure (bands) changes at all pH values 3–9. It is interesting to note that the spectra between pH 6.0–9.0 for the globulin from Extraction 2 had almost identical fine structures and spectral intensities (amplitudes) indicative of little conformation change. Similarly, the globulin from Extraction 1 had the same spectra order between pH 6.0 and 9.0 as Extraction 2 and had similar tertiary structures, but slightly greater differences among spectral intensities at the individual pHs were observed. On the other hand, the globulin from Extraction 3 had more fine structure than globulins from Extractions 1 and 2, and had different spectral intensities and order from Extractions 1 and 2 globulins between the pH values 6.0 and 9.0.

A general and similar increase in the intensity or amplitude of the spectra for globulins from Extractions 1 and 2 at pH 5.0, as compared to their respective spectra at pH values between 6 and 9, are indicative of a substantial change in tertiary structure at this pH.

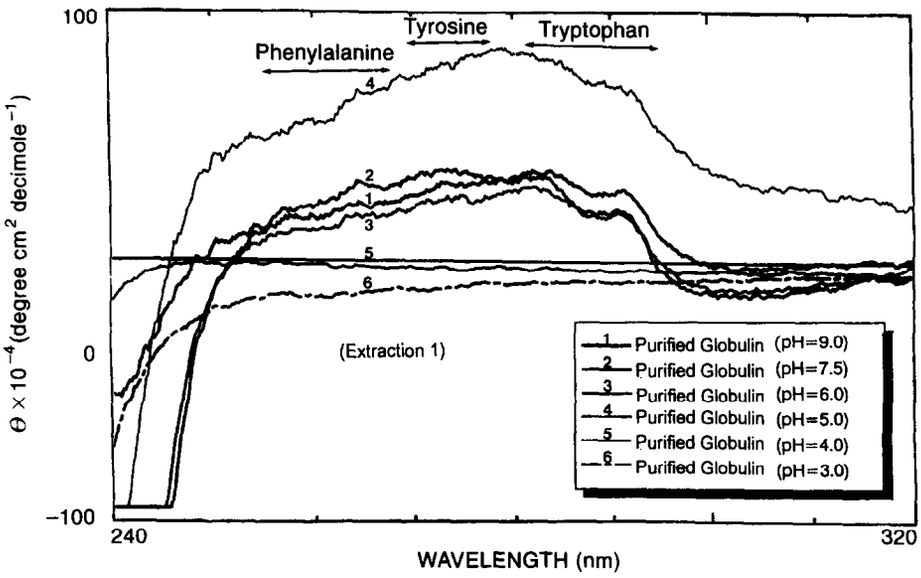

Fig. 1. Circular dichroic tertiary structure spectra of peak B, Extraction 1. Samples were run between 320 and 240 nm at pH 9.0, 7.5, 6.0, 5.0, 4.0 and 3.0. Analyses were performed in triplicate with six scans per replicate.

Table 1. Circular dichroic secondary structure fractions of purified mustard globulin from Extractions 1, 2 and 3 at various pH values

Fraction	Extraction 1						Extraction 2						Extraction 3					
	pH 3.0	4.0	5.0	6.0	7.5	9.0	3.0	4.0	5.0	6.0	7.5	9.0	3.0	4.0	5.0	6.0	7.5	9.0
α-helix	$23.4^{d,e,f}$	$28.1^{c,d,e}$	$38.4^{a,b,c}$	$33.8^{b,c,d}$	$36.0^{a,b,c}$	45.9^{a}	$36.0^{a,b,c}$	$38.7^{a,b,c}$	$34.8^{b,c}$	$41.4^{a,b}$	$35.6^{b,c,d}$	$29.5^{c,d,e}$	$30.1^{c,d,e}$	$29.3^{c,d,e}$	$34.9^{b,c}$	$37.9^{a,b,c}$	$20.7^{e,f}$	17.2^{f}
β-sheet	$26.6^{a,b}$	$17.2^{b,c}$	$12.1^{b,c}$	$17.0^{b,c}$	$9.5^{b,c}$	$19.1^{a,b,c}$	$9.6^{b,c}$	$10.2^{b,c}$	$16.2^{b,c}$	$16.7^{b,c}$	$11.7^{b,c}$	$26.5^{a,b}$	0.8^{c}	$18.4^{a,b,c}$	$12.1^{b,c}$	$11.3^{b,c}$	$30.3^{a,b}$	39.5^{a}
β-turn	$17.3^{b,c}$	$23.7^{b,c,d}$	$15.1^{c,d}$	$20.1^{b,c,d}$	$26.9^{b,c,d}$	16.9^{e}	21.1^{b}	$20.0^{b,c,d}$	$19.6^{b,c,d}$	$16.4^{c,d}$	$24.1^{b,c,d}$	$13.2^{b,c,d}$	24.1^{a}	17.7^{b}	18.4^{b}	$25.0^{b,c,d}$	$19.9^{b,c,d}$	$12.9^{b,c,d}$
Random	$31.8^{b,c}$	$31.0^{b,c,d}$	$24.4^{d,e}$	$29.1^{b,c,d}$	$27.6^{b,c,d}$	18.0^{e}	33.4^{b}	$31.1^{b,c,d}$	$29.4^{b,c,d}$	$25.5^{c,d}$	$28.7^{b,c,d}$	$30.9^{b,c,d}$	44.9^{a}	34.6^{b}	34.5^{b}	$29.2^{b,c,d}$	$27.7^{b,c,d}$	$30.3^{b,c,d}$

Extraction 1: 1.0 M NaCl, pH 7.5; Extraction 2: 32.6 mM/2.6 mM K_2HPO_4/KH_2PO_4 + 0.4 M NaCl; Extraction 3: distilled water, pH 7.5.
Values in the same row with the same letter are not significantly different ($P > 0.05$), pH 7.5. Values represent the mean of three replicates.

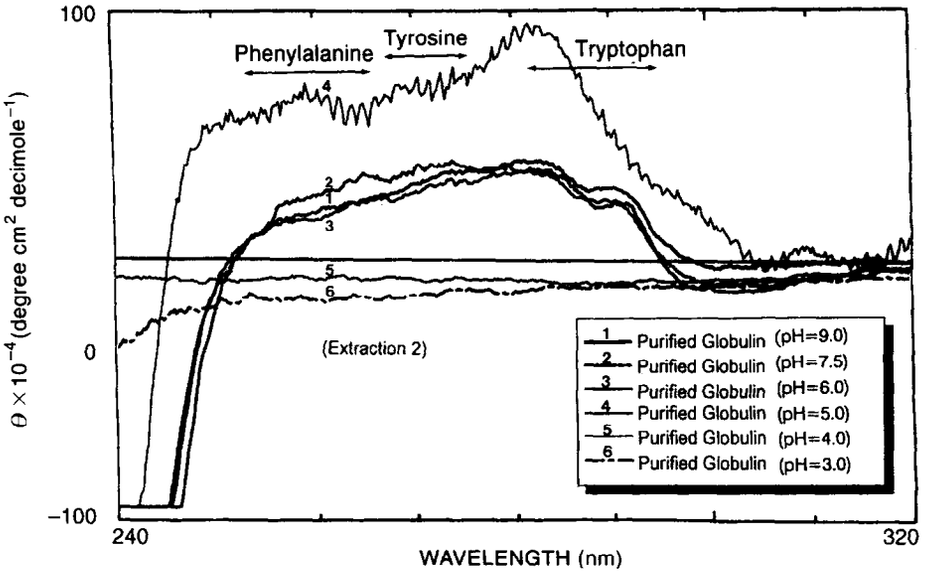

Fig. 2. Circular dichroic tertiary structure spectra of peak B, Extraction 2. Samples were run between 320 and 240 nm at pH 9.0, 7.5, 6.0, 5.0, 4.0 and 3.0. Analyses were performed in triplicate with six scans per replicate.

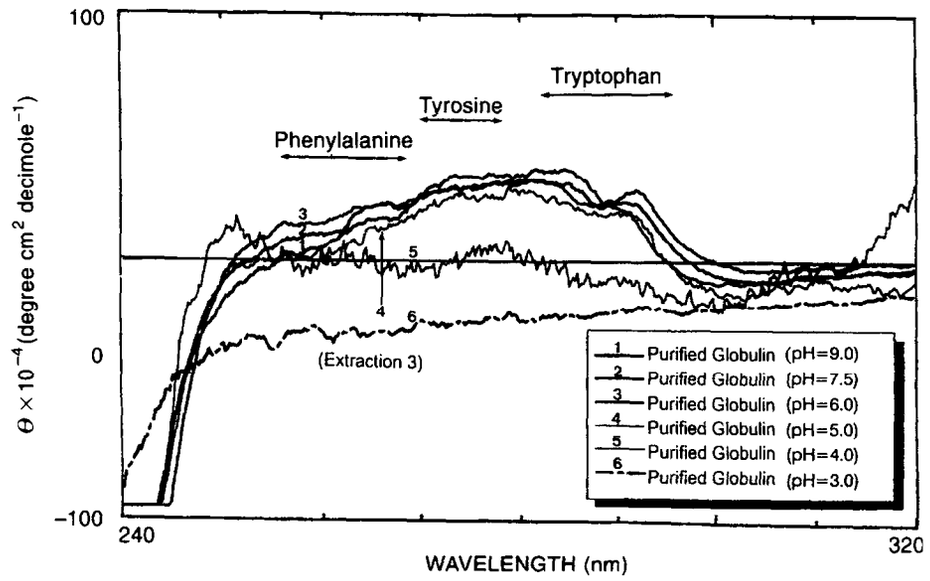

Fig. 3. Circular dichroic tertiary structure spectra of peak B, Extraction 3. Samples were run between 320 and 240 nm at pH 9.0, 7.5, 6.0, 5.0, 4.0 and 3.0. Analyses were performed in triplicate with six scans per replicate.

In contrast, the globulin from Extraction 3 showed a slight decrease in spectral intensity or amplitude at pH 5, as compared with its respective spectra at pH values between 6 and 9.

Evidence of a molten globule state for globulins

In all cases, globulins from Extractions 1, 2 and 3 at acidic pH values, i.e. pH 3.0 and 4.0, showed a dramatic loss in intensity, indicating again a substantial change in surface conformation and loss of tertiary structure. The loss of fine structure may indicate a loss in reinforcement (close proximity) of aromatic amino acids (Strickland, 1974). It was interesting to note that when the globulins were dialysed against buffers of pH 6.0–9.0, tertiary structure was restored indicating that the globulins were not irreversibly denatured at low pH (data not shown). Therefore, the previously observed lack of secondary structure alteration combined with the above observed loss of tertiary structure would support the notion that a molten globule type of structure might be formed for these globulins at acidic pH values.

ANS binding as a function of pH

An additional, very sensitive and specific test for the molten globule state of protein molecules is the binding of the hydrophobic fluorescent probe 8-anilino-1-naph-

thalene-sulfonate (ANS) to exposed aromatic hydrophobic groups (Bychkova *et al.*, 1992). It has been shown that ANS affinity to the molten globule state is much stronger than to native and unfolded proteins (Ptitsyn *et al.*, 1990; Bychkova *et al.*, 1992). Aromatic hydrophobicity (ANS) (Fig. 4) measurements for all three purified globulins were fairly constant in the pH range of 6.0–9.0 and correlated well with the little change in tertiary structure, as noted in near-UV studies in the same range. At acidic pH values (3.0–4.0), all three globulins showed an increase in aromatic hydrophobicity indicative of tertiary structural changes. The latter results may again confirm the existence of a molten globule state at acidic pH values.

Intrinsic fluorescence as a function of pH

Intrinsic fluorescence is recognized as a sensitive monitor of the immediate environment of aromatic amino acid residues in proteins (Farahbakhsh *et al.*, 1987). It has also been well established that the degree of fluorescence of the indole ring of tryptophan reflects the polarity of the surrounding environment (Teale, 1960; Farahbakhsh *et al.*, 1987) and, thus, can be responsive to changes in protein conformation that give rise to the exposure of buried tryptophan residues (Farahbakhsh *et al.*, 1987; Sanyal *et al.*, 1993).

The intrinsic fluorescence intensity maxima for the various globulins with various pH buffers are shown in Table 2. The fluorescence emission spectrum for all three globulins showed a maximum at 332.0 nm in the neutral to alkaline pH range (6–9). The lack of any red or blue shift in this pH range would indicate that little conformational change was occurring due to change in pH. This would agree with near-UV CD spectral data which showed little change.

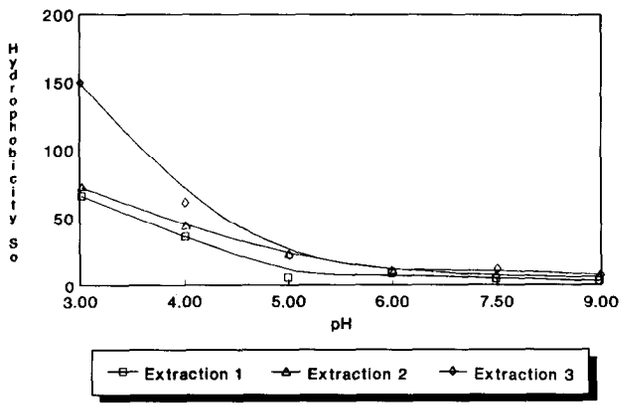

Fig. 4. ANS (aromatic hydrophobicity) of globulins from Extractions 1, 2, and 3.

At pH 5.0, the maximum emission moved from 332.0 to 335.0 for Extractions 1 and 2, which corresponded to near-UV CD data which showed a dramatic increase in intensity. With further decrease in pH, the fluorescence maximum increased for all globulins. The magnitude of the fluorescence maximum change was the greatest for the globulin from Extraction 1 at pH values 4.0 and 5.0 followed by the globulin from Extraction 2 at the same pH values. The globulin from Extraction 3, on the other hand, demonstrated a more moderate increase with decreasing pH (Table 2).

In the case of all three globulins isolated by Extractions 1, 2 and 3, the fluorescence intensity decreased substantially when the pH was lowered below 6.0 (data not shown). This may indicate that the micro-environments of tryptophan residues in each globulin were becoming less hydrophobic and probably increasing in dielectric constant. It has also been shown by other researchers that a progressive red shift of tryptophan fluorescence with decreasing pH may suggest that acidic pH induces exposure of hydrophobic regions, making them accessible to the solvent (Farahbakhsh *et al.*, 1987; Sanyal *et al.*, 1993). The observed red shift seen for all three globulins, in addition to the decrease in fluorescence intensity, indicates that some increased solvent exposure of one or more tryptophan residues was occurring below pH 6.0. Thus, the low pH-induced structural change of globulins appears to involve a subtle exposure of one or more hydrophobic surfaces without an extensive unfolding of the proteins' secondary structure.

Strong evidence now exists that the translocation of proteins across biological membranes involves a non-native or denatured conformational state and it has also been proposed that the molten globule state may serve as a suitable candidate for such translocations (Bychkova *et al.*, 1988). It is worth noting that seed storage globulins belong to the category of secretory proteins which are translocated across the membrane of the endoplasmic reticulum and/or the protein body by a post-translational process (Pernollet & Mosse, 1983).

Table 2. Maxima of fluorescence intensity[a] for the purified mustard globulin as a function of pH

	Globulin Extraction 1	Globulin Extraction 2	Globulin Extraction 3
pH 9.0	332.0 nm	332.0 nm	332.0 nm
pH 7.5	332.0 nm	332.0 nm	332.0 nm
pH 6.0	332.0 nm	332.0 nm	332.0 nm
pH 5.0	335.0 nm	335.0 nm	340.0 nm
pH 4.0	360.0 nm	352.0 nm	352.0 nm
pH 3.0	364.0 nm	358.0 nm	353.0 nm

Extraction 1: 1.0 M NaCl, pH 7.5; Extraction 2: 32.6/2.6 mM K_2HPO_4/KH_2PO_4, pH 7.5, +0.4 M NaCl; Extraction 3: distilled water, pH 7.5.

[a]Excitation: 280 nm.

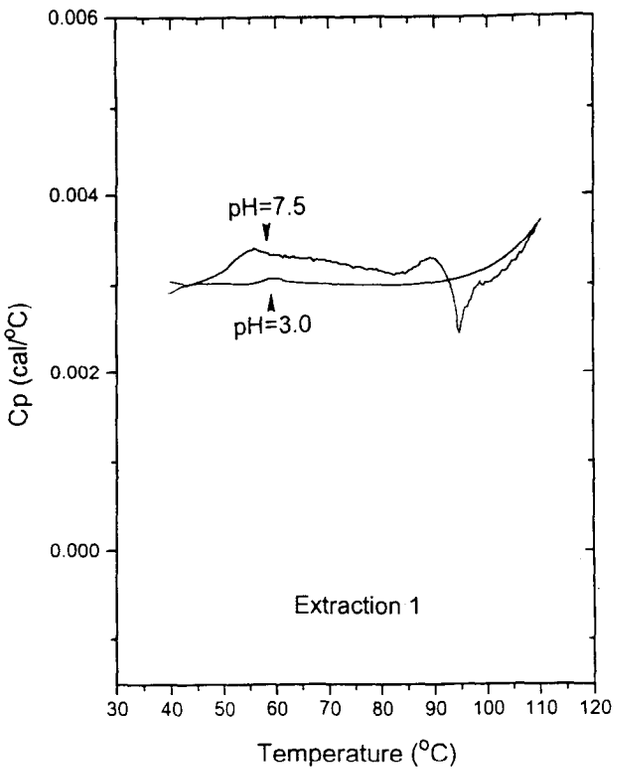

Fig. 5. Differential scanning calorimetric scans of the purified globulin from Extraction 1 at pH 3.0 and at pH 7.5 after the extensive dialysis.

According to Bychkova *et al.* (1988) a protein in the molten globule state would be able to accommodate both polar and non-polar environments due to its amphiphilic nature and would allow for the protein to be translocated through the membrane with a small free energy of activation. This would agree with the ANS data which showed that all globulins had high levels of aromatic hydrophobicity at acidic pH, and the results of Marcone *et al.* (1997) which showed that the globulins were also highly positively charged at these pHs. These two properties would endow the globulins with an amphiphilic nature which would allow for an accommodation of both polar and non-polar environments as described by Bychkova *et al.* (1988). It is, therefore, conceivable that the existence of the molten globule state in globulins or its constituent subunits may serve necessary biological functions, first in their transportation and finally in their proper folding after their biosynthesis.

The noted aggregation as described in Marcone *et al.* (1997), observed for all three globulins below pH 4.0, is also of importance since aggregation via intermolecular non-polar interactions is also a commonly observed characteristic of molten globule states (Christensen & Pain, 1991).

When micro-differential scanning calorimetric analysis was performed on all three globulins at pH 3.0, it was observed that the characteristic transition at 90°C (as observed in Marcone *et al.*, 1997) was lost (Figs 5–7). This would agree with the overall unfolded nature of

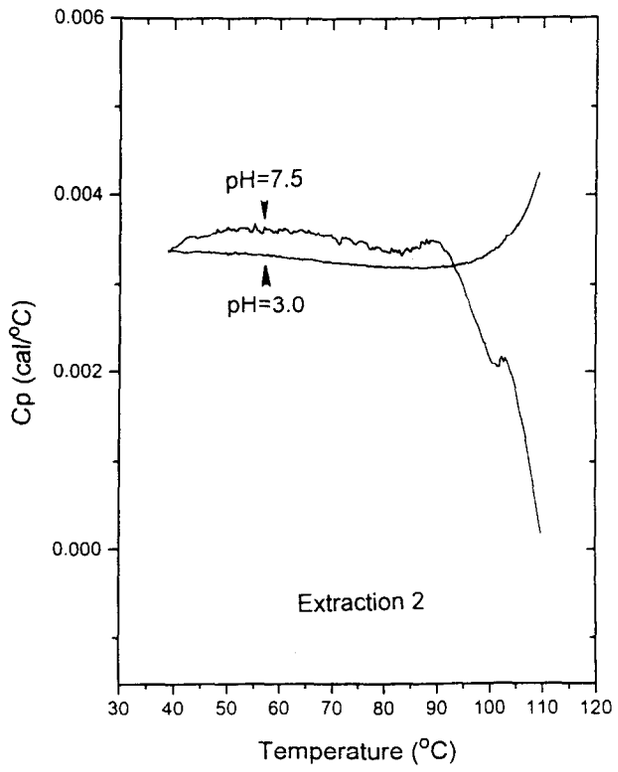

Fig. 6. Differential scanning calorimetric scans of the purified globulin from Extraction 2 at pH 3.0 and at pH 7.5 after extensive dialysis.

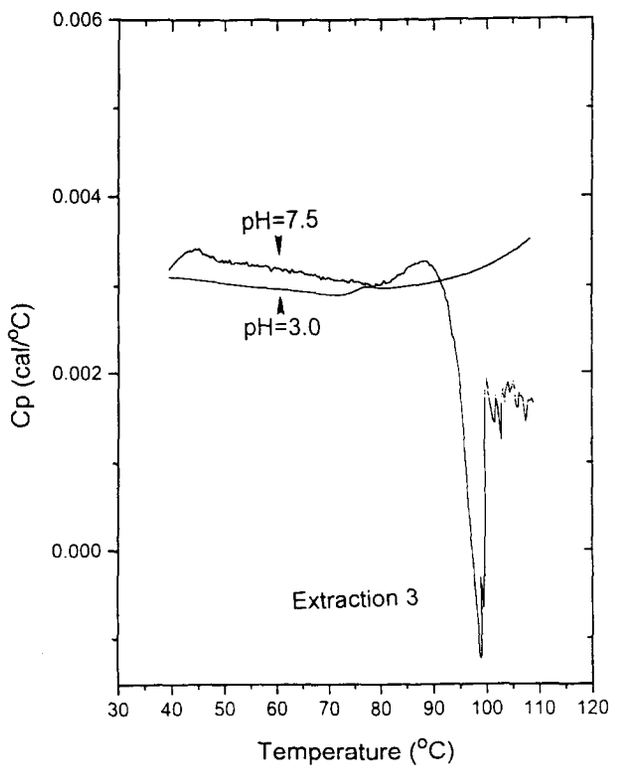

Fig. 7. Differential scanning calorimetric scans of the purified globulin from Extraction 3 at pH 3.0 and at pH 7.5 after extensive dialysis.

these globulins at this pH, as evidenced by tertiary structure, intrinsic fluorescence and hydrophobic probe data.

In contrast, when the globulins at pH 3.0 were dialysed extensively against a buffer at pH 7.5, the characteristic transition occurring at 90°C loss at the former pH was re-established at the latter pH (Figs 5–7). This would indicate that the globulin was not denatured at pH 3.0 but was in a molten globulin state, i.e. the state commonly referred to as state A (Hirose, 1993).

$$N \rightleftharpoons A \leftrightharpoons D$$

where N = native protein state, A = molten globule state and D = denatured state.

CONCLUSIONS

The structural integrity of all three mustard globulin isoforms appears to be affected by pH change. Of the physico-chemical aspects studied, tertiary structure was affected to the greatest extent. All globulins appear to undergo the least conformational change in the pH range of 6–9, whereas the greatest change in structure was found to occur at pH 5.0 in the case of globulins from Extractions 1 and 2. Below pH 5.0, a loss of discernible tertiary structure was found for all globulins studied. Secondary structures for all three isoforms were found to remain relatively unchanged with change in pH. These data confirm previous published data (Marcone *et al.*, 1997) which show that the maximum stability of globulins lies in the pH range 6–9. Fluorescence maxima data agreed well with tertiary structure data which showed little structural change in the pH range of 6–9 for all three globulins. The above data would indicate that a molten globule state exists in mustard seed globulins, and may help to explain how these secretory proteins are translocated across the membrane of protein body during their biosynthesis and storage (Bychokova *et al.*, 1988).

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