Int. J. Peptide Protein Res. 45, 1995, 248–256
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INTERNATIONAL JOURNAL OF
PEPTIDE & PROTEIN RESEARCH
ISSN 0367-8377

Simultaneous differential scanning calorimetry, X-ray diffraction and FTIR spectrometry in studies of ovalbumin denaturation

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Received 3 January, accepted for publication 13 August 1994

The new application of differential scanning calorimetry (DSC) and the susceptibility of ovalbumin to α-chymotrypsin gave a quantitative estimation of protein denaturation in solid ovalbumin. Solid ovalbumin in granules with 11% of water was heated at 100 °C in closed and nonclosed ampules. In order to compare effects of size and crystal structure, two proteins (bovine albumin and γ-globulin) were examined at similar conditions for the extent of denaturation. Ovalbumin and bovine albumin showed similar extents of denaturation, but γ-globulin, with a very different molecular mass, showed the maximal conformational changes. The enthalpy of denaturation was measured to elucidate the conformational changes in solid proteins. Its value was used for calculation of the degree of denaturation. The thermodynamic data associated with transition were calculated and the number of bonds broken during denaturation was determined. Intrinsic fluorescence was utilized in order to compare these two methods. Moreover, X-ray diffraction and FTIR spectrometry were applied to native and denatured proteins. © Munksgaard 1995.

Key words: denaturation; ovalbumin; calorimetry; solid state

Ovalbumin is a widespread matrix for sustained drug release, as well as a food product, rich in amino acids (1–3). Dry heating is a commonly used method for the pharmaceutical production of pellets. This step can induce changes in the matrix–drug mixture connected with protein denaturation; however, there are very few data on protein denaturation in the dry state (4–6). The quantification of these changes is important for understanding the mechanisms which affect sustained drug release.

Ovalbumin, as a protein molecule, represents a well ordered macroscopic system. Because the stability of any structure may be evaluated by its disruption, the stability of a protein can be determined by studying its denaturation. Scanning microcalorimetry, which permits direct measurements of the enthalpy dependence on temperature, became a popular method in thermodynamic studies of the temperature-induced processes in protein molecules. So far all investigations of protein thermal denaturation with DSC techniques were carried out in protein solutions (7). Other known instrumental methods for studying protein denaturation (8–10) also require solutions. A widely used method for examining protein denaturation is intrinsic fluorescence. This method reflects unfolding of proteins by decreased fluorescence intensity and a shift in the maximum of emission (11). It permits quantitative estimation of denaturation using fluorescence parameters which are a linear measure of denaturation in aqueous solutions. This work reports a new application of DSC to determine the conformational changes (percentage of denaturation) in dry-heated solid ovalbumin. To show the effect of proteins with different molecular masses bovine albumin and bovine globulin were examined by the same method. Intrinsic fluorescence, FTIR spectrometry and X-ray diffraction confirmed the results obtained by DSC.

MATERIAL AND METHODS

Ovalbumin (OVA) was received from the Trima Pharmaceutical Industry. All solutions were prepared in 0.01 M phosphate buffer, pH 7.2. Deionized distilled water was used throughout. Other chemicals of reagent grade, including bovine albumin (BA) and γ-globulin (γ-G) were purchased from Sigma Chemical Co. and were used without further purification.

Solid proteins (OVA, BA and γ-G) were denatured in hermetically closed ampules and in non-closed con-

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ditions at 100 °C for different periods of time. Protein samples in closed conditions contained water which was added for preparation of granules (approximately 11%). Some protein samples were mixed with 7.5% of glycerol. Protein samples in non-closed conditions were dried using speed vacuum. After heat treatment, the ampule was removed from the incubator, cooled to room temperature, and DSC, X-ray, fluorescence and FTIR analyses performed.

The extent of denaturation in solid proteins was estimated on a Mettler differential scanning calorimeter using GraphWare TA-72. Portions (4–6 mg) of solid proteins were transferred into preweighed aluminium pans and sealed. An empty pan was used as the reference. The pans were heated in the calorimeter at 5 °C/min over the range 30–120 °C. The denaturation temperature (T_d) and changes in the enthalpy of denaturation (ΔH) were computed from the thermograms.

Fluorescence measurements were carried out in the liquid state. For this purpose the protein solutions were prepared and filtered to remove insoluble materials, and then the protein concentration was adjusted spectrophotometrically in accordance with the absorbance at 280 nm for fluorescence analysis. Absorbance values were measured using a Uvikon 930 spectrophotometer. Fluorescence was measured using a model FP-770 Jasco spectrofluorometer. The temperatures of the samples were maintained at 30 °C using a thermostatically controlled cell holder. Fluorescence emission spectra of native and denatured OVA, BA and γ-G were measured at excitation wavelengths of 295 nm and recorded from 295 to 450 nm. The magnitude of protein denaturation based on the data, which were obtained from fluorometry measurements, was calculated using the following equation:

$$\text{Percent denaturation } (\%D) = [(I_0 - I_t)/I_0] \times 100$$

where I_0 and I_t are the fluorescence intensities of native and denatured protein, respectively (the height of emission peaks).

A Perkin Elmer 2000 FTIR spectrometer was used to record IR spectra. The samples for measurements were prepared from granulated protein mixed with and without guanidinium chloride (GdnHCl) and with KBr, and the pellets were pressed applying 10^4 kg/cm^2 for 15 s.

X-Ray diffractograms of native and denatured OVA and BA were recorded by a Rigaku (MAX-IIIA, Rigaku Keisoku Co.) powder X-ray diffractometer according to Hizukuri's method (12). The X-ray, Cu K_α irradiation was performed with a monochromator. The operating conditions were the following: voltage 35 kV; current 25 mA; scanning speed 1°/min; chart speed 5 mm/min; time constant 1 s. Samples were densely packed on a glass plate using an aluminium frame.

Values of intensities were read from the curves over the angular range 4–30° which includes most of the crystalline peaks. Percent crystallinity was determined by an integral method. d-Spacings were computed by Bragg's law using $\lambda = 2d \sin \theta$, where λ is the wavelength of the X-ray beam (1.5405 Å), d is the spacing between unit-cell edges of the specific crystal to be studied, and θ is the angle of diffraction. The quantitative measurement of crystallinity was undertaken according to Nara *et al.* (13). Each point of minimum intensity on the X-ray diffractograms of OVA and BA was joined by a smooth curve. The upper region under the most prominent peaks in native OVA and BA was the area of 100% crystalline fraction.

Protease digestibility was determined using chymotrypsin. The enzymatic reaction was carried out at 38 °C for 20 min according to the procedure of Kato *et al.* (6). The extent of digestion was calculated as the percentage of absorbance of 0.05% untreated sample.

RESULTS AND DISCUSSION

DSC measurements of OVA denatured in closed conditions

DSC methods have been extensively used to study the protein unfolding in liquid state (7). This method is

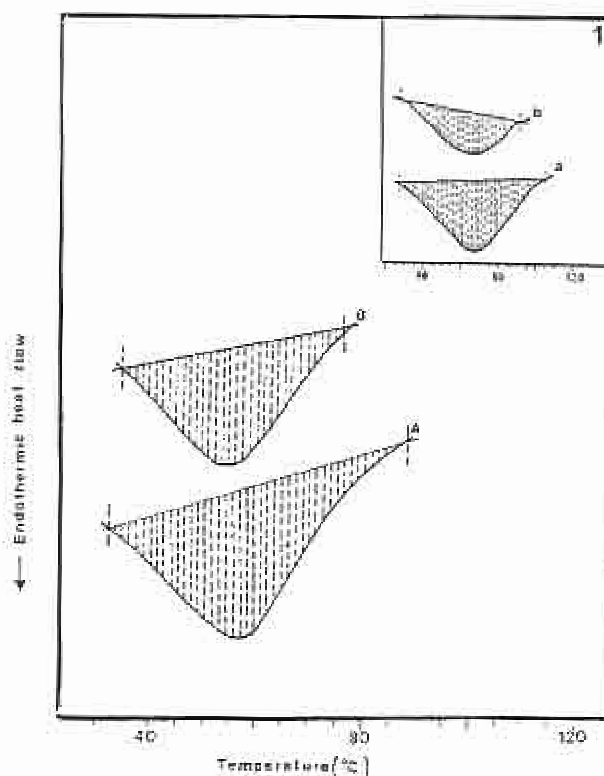

FIGURE 1

DSC thermograms of granulated OVA with 11% of water: native (A) and denatured overnight at 100 °C in a hermetically closed ampule (B). Insert 1: DSC thermograms of granulated OVA with 7.5% of glycerol: native (a) and denatured 1 h at 100 °C in non-closed conditions (b)

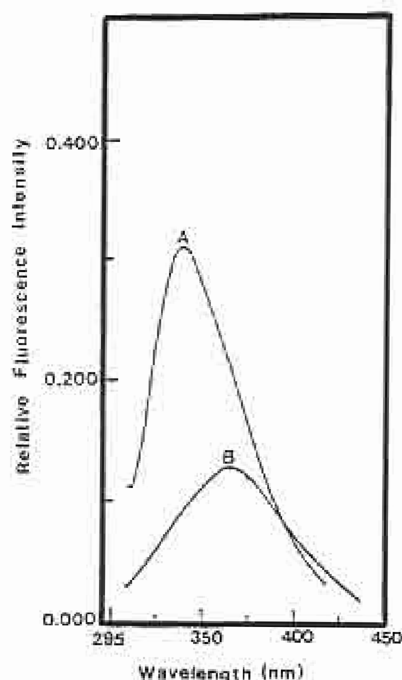

FIGURE 2
Effect of temperature treatment (100 °C overnight in ampules) on the fluorescence emission spectrum of granulated OVA. Excitation at 295 nm. A and B correspond to the intensity of native and denatured OVA, respectively.

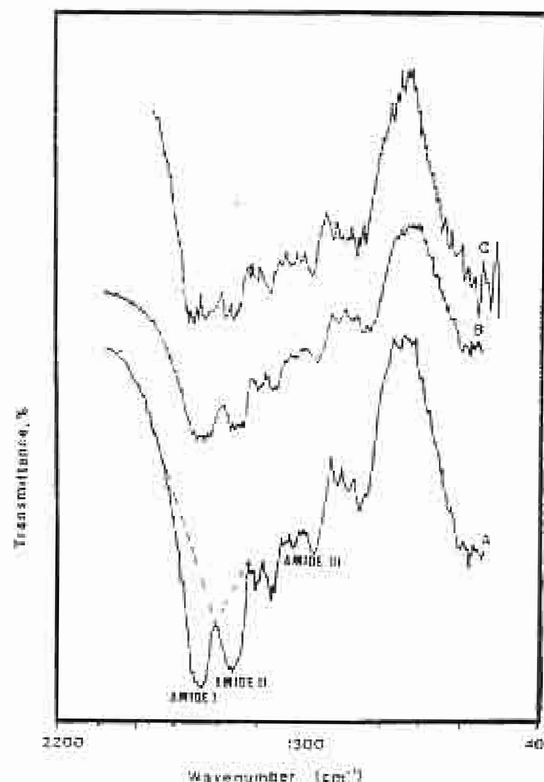

FIGURE 3
FTIR spectra of ovalbumin: (A) native granulated; (B) granulated heated at 100 °C in ampules overnight; (C) powdered mixed with guanidinium chloride (GdnHCl) (1:1).

highly sensitive to conformational changes. The DSC scans for native OVA and a sample denatured in an ampule are shown in Fig. 1. The native structure of moisture-treated ovalbumin was stable up to a critical temperature, and then disrupted with intense heat absorption (Fig. 1, curve A). Heat pretreatment destabilized the protein conformation as reflected by the marked decrease in ΔH and T_d values (Fig. 1, curve B). This result suggests changes in molecular conformation of ovalbumin and is consistent with those of other authors (14).

Disordering of the system takes place upon heating. A considerable number of ovalbumin molecules shifts to a state that contributes much less to the unfolding transition, thus causing a significant decrease in the calorimetric enthalpy. The enthalpy changes of the initial and remaining DSC endotherm were measured and used for calculation of the percent of denatured ovalbumin (Table 1). The entropy (S) values which are associated with state transition and affirmed disordering

TABLE 1
Thermodynamic data of native and denatured proteins in closed conditions[a]

Samples	T_d (°C)	ΔH (J/g)	ΔH (kcal/mol)	ΔS (kcal mol K)	I	%D	%D^*
Native granulated OVA with 11% of water	58.7	228.3	2455.4	7.40	0.312		
Granulated OVA heated at 100 °C in ampule overnight	54.8	97.9	1052.9	3.21	0.124	57	60
Native granulated BA with 11% of water	66.6	203.2	3205.4	9.41	0.423		
Granulated BA heated at 100 °C in ampule overnight	64.2	102.1	1610.6	4.82	0.189	50	55
Native granulated γ-G with 11% of water	58.2	257.3	9224.4	27.90	0.382		
Granulated γ-G heated at 100 °C in ampule overnight	55.6	134.9	4836.3	14.72	0.167	48	56

[a] T_d, Denaturation temperature; I, fluorescence intensity; %D, percent of denaturation calculated from DSC-experiment; %D^*, percent of denaturation calculated from intrinsic fluorescence method.

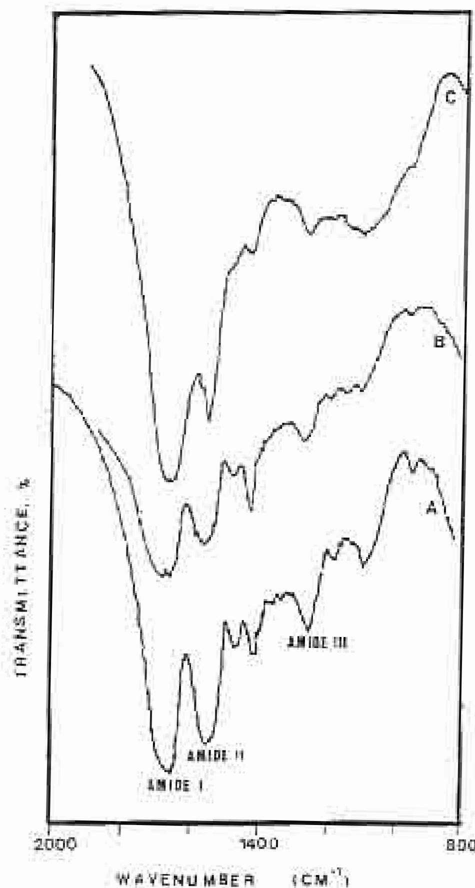

FIGURE 4
FTIR spectra of γ-globulin: (A) native granulated; (B) granulated heated at 100 °C in ampules overnight; (C) powdered mixed with guanidinium chloride (GdnHCl) (1:1).

of protein structure were also calculated (Table 1). Results for bovine albumin and γ-globulin were obtained and interpretated to compare proteins with different molecular weights (OVA, 45 000; BA, 66 000; γ-G, 150 000). Table 1 demonstrates the relation between enthalpy values and molecular weights of proteins. The largest enthalpy value was registered for γ-globulin. Denaturation of around 50% was observed in all cases.

Intrinsic fluorescence method
In our previous report (15) intrinsic fluorescence was used to study the unfolding of ovalbumin induced by different denaturing agents and high temperature. At the excitation of 295 nm, tryptophan is the only aromatic amino acid to absorb light. Effects of denaturants and heating cause the displacement of the tryptophan residues to a more polar environment upon unfolding. Such a displacement gives a shift to lower energy in the maximum of emission and quenching of fluorescence intensity (11). The present work employed fluorescence measurements to examine changes in dry-heated ovalbumin with and without 11% water and for comparison with the data of calorimetric analysis. Figure 2 shows fluorescence spectra of ovalbumin in the native state and after 100 °C treatment, and demonstrates a shift in the wavelength of emission from 339.5 to 363.5 nm and a decrease in the fluorescence intensity of the denatured sample. The percent of denaturation was calculated from both methods (DSC and intrinsic fluorescence) and compared satisfactorily (Table 1). The fluorescence intensity was measured for all protein samples, but in the dry state it did not indicate denaturation. Therefore these results were omitted in Tables 4–6.

FTIR spectrometry
Kaiden et al. (16) applied infrared spectroscopy for the study of the secondary structure of a living protein. On the basis of their investigation, infrared spectra of ovalbumin were obtained to analyze the protein conformation (Fig. 3). These changes induced by heat treatment resulted in the alterations of amide I, II and III bands (Fig. 3, spectra A and B). As is seen from Fig. 3, the intensities of the amide I and II bands decreased in the denatured samples in comparison with those of the

TABLE 2
Amide I, II and III band positions (in cm^{-1}) and relative intensity ratios to 1450 cm^{-1} band

Sample	Amide I		Amide II		Amide III	
	Band position	*R*	Band position	*R*	Band position	*R*
Native ovalbumin	1651	10.5	1532	4	1311	1
					1239	5
Denatured ovalbumin	1656	7	1532	3	1315	1
					1240	4
Native bovine albumin	1656	10.5	1542	2.8	1240	2
Denatured bovine albumin	1660	9	1531	3.3	1233	1.5
Native γ-globulin	1646	10	1534	4.5	1235	3
Denatured γ-globulin	1655	5	1535	4	1235	2.5

TABLE 3

X-Ray diffraction spacings in native and denatured OVA

NN	Samples	Interplanar spacings, d (Å); very strong (vs), strong (s); medium (m) weak (w) and broad (br) intensities
A	Native OVA (powder)	9.39 (m), 4.38 (vs)
B	Native granulated OVA	9.81 (m); 4.55 (vs); 4.15 (w)
C	OVA (powder) denatured overnight at 100°C	9.60 (m), 4.67 (vs); 3.83 (w)
D	Granulated OVA denatured overnight at 100°C	9.20 (m), 4.67 (vs); 3.77 (w)
E	Granulated OVA heated at 100° 1 h	9.85 (m); 3.94 (vs)
F	Granulated OVA heated at 100° 5 days	9.85 (m); 4.11 (vs)
G	OVA denatured with urea (1:1)	10.04 (m)

native OVA. Also the intensity ratio of the amide II band to the amide I band increased in both denatured samples relative to the native one. In the denatured protein amide I and II bands are almost of the same intensity (Fig. 3, spectra B and C). Such spectral behavior is purportedly associated with the disruption of hydrogen bonds through heat denaturation and reflects a decrease in α-helix content of the denatured protein, according to the findings of Kato *et al.* (17). They showed the dependence of the intensity ratio of the amide II band to the amide I band on the α-helix content. The influence of disruption of hydrogen bonds on enthalpy changes in DSC was also reported by some authors (18). As can be seen from curve C in Fig. 3, GdnHCl also induced alterations in protein conformation. These samples show that the intensity of the amide II band is the same as that of the amide I band, indicating that the α-helix content of the treated ovalbumin decreases in comparison with the native one.

Table 2 indicates differences in the peak position and peak intensity ratios (R) of native and denatured samples. R-Values were obtained as the ratio of the band's intensity to the CH_2 deformation vibration (1450 cm^{-1}) by the baseline method (Fig. 3, dotted line). As may be seen, the frequency of the amide I band (curve B) shifted 5 cm^{-1} more on the high-frequency side than curve A. Ovalbumin spectra showed alterations in the 1350–1200 cm^{-1} region (i.e. the amide III band): namely, the difference in intensity ratio between the 1359 and 1450 cm^{-1} bands decreased, and the distinction between the 1311 and 1239 cm^{-1} peaks increased in the B spectrum. The peak at 1240 cm^{-1} was converted from a sharp intensive band to a broad one and the intensity of 1311 cm^{-1} band decreased, suggesting that the α-helix and β-structure were disordered upon heat treatment. These results correspond to those of Kaiden *et al.* (16), who assigned the broad band in the 1300–1250 cm^{-1} region to an α-helix; the relatively sharp band in the 1240–1230 cm^{-1} region to a β-sheet and a broad, medium intensity band in the 1270–1240 cm^{-1} region to a disordered structure.

The spectral changes in bovine albumin were similar to the alterations in ovalbumin spectra (Table 2). The spectra of γ-globulin differed from ovalbumin spectra (Fig. 4). A clear sharp band at 1235 cm^{-1}, corresponding to a β-sheet, became broader and smaller after denaturation compared to native γ-globulin (Fig. 4). It supposes a decrease in the β-conformation of γ-globulin. Also, the intensity ratio of the peak at 1450 cm^{-1} to that at 1400 cm^{-1} increased after denaturation. However, the intensity ratio of the amide II to amide I bands did not change drastically. Such a difference can be

TABLE 4

Thermodynamic data of native and dry-heated ovalbumin[a]

Samples	T (°C)	ΔH (J/g)	ΔH (kcal/mol)	ΔS (kcal/mol K)	%D
Native granulated OVA (dry)	68.5	116.1	1248.7	3.70	
Granulated OVA heated at 80°C 1 h	68.0	95.6	1028.2	3.01	18
Granulated OVA heated at 80°C overnight	67.5	92.5	994.9	2.92	20
Granulated OVA heated at 100°C 1 h	67.5	90.4	972.2	2.85	22
Granulated OVA heated at 100°C overnight	66.2	66.0	709.8	2.12	43
Granulated OVA heated at 100°C 5 days	63.2	60.1	646.2	1.91	48
Native granulated OVA with glycerol	72.0	108.1	1162.6	3.43	
Granulated OVA with glycerol heated at 100°C 1 h	64.3	40.3	433.4	1.31	63

[a] T_d, temperature of the denaturation; %D, percent of denaturation calculated from DSC experiment.

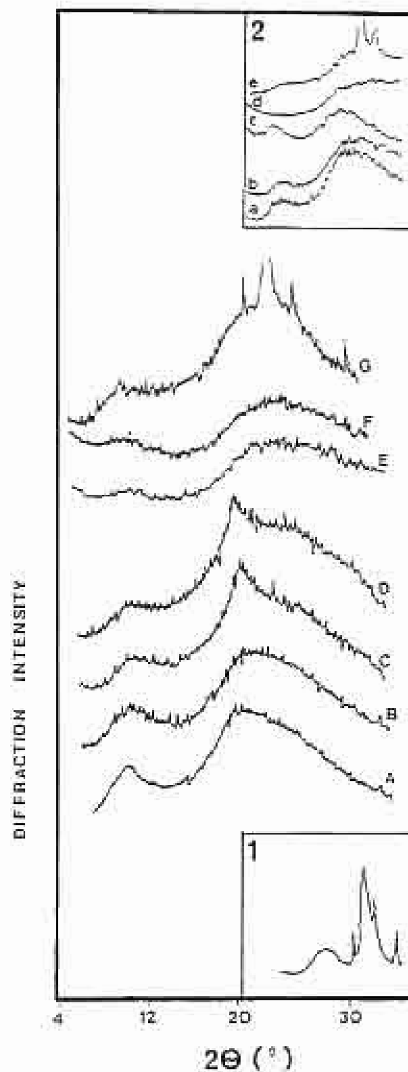

FIGURE 5

X-Ray diffraction patterns of native and denatured ovalbumin: A, B, C, D, E, F and G respectively (A) native powder, (B) native in granules, (C) powder after denaturation at 100 °C overnight in closed conditions, (D) granulated protein after denaturation at 100 °C overnight in closed conditions, (E) granulated protein after denaturation at 100 °C for 1 h in opened conditions, (F) granulated protein after denaturation at 100 °C during 5 days in opened conditions, (G) powder mixed with urea (1:1). Insert 1: X-ray diffractogram of urea. Insert 2: X-ray diffraction patterns of native and denatured BA: (a) native in granules, (b) granulated BA heated at 100 °C 1 h, (c) granulated BA heated at 100 °C overnight, (d) granulated BA heated at 100 °C 5 days, (e) powdered mixed with urea (1:1).

attributed to the fact that the β-sheet is the main ordered structure of γ-globulin, compared to ovalbumin. All results on peak position are included in Table 2.

X-Ray diffractometry

The treatment of OVA at 100 °C slightly changed the crystal patterns (Fig. 4, A and C). Native powder and

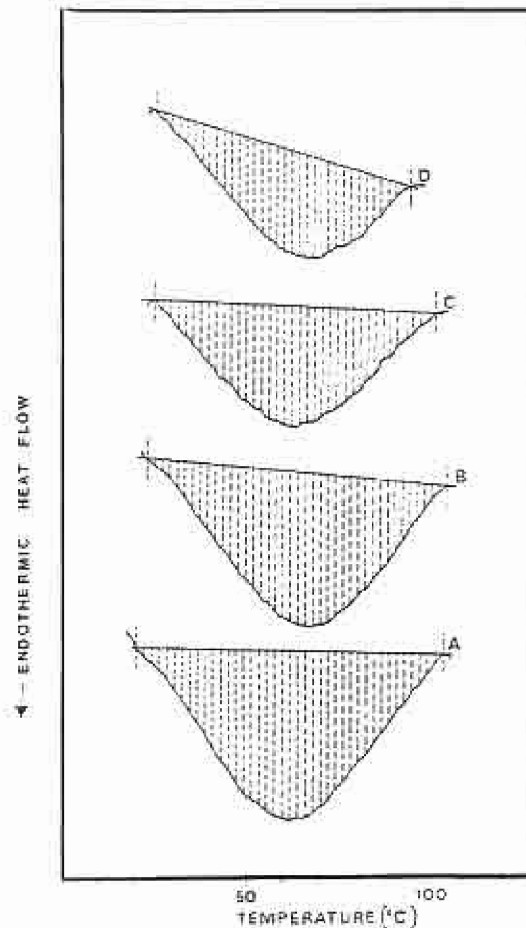

FIGURE 6

DSC thermograms of granulated OVA treated in non-closed conditions: (A) native OVA, (B) OVA heated at 80 °C overnight, (C) OVA heated at 100 °C overnight, (D) OVA heated at 100 °C 5 days.

native granulated OVA showed very strong peaks at 4.38 and 4.55 Å and medium peaks at 9.39 and 9.81 Å.

In granulated OVA a weak peak also appears at 4.15 Å. The number of spacings changed very slightly in heat-denatured samples. Upon denaturation with urea, the peaks were present around 4.67 Å, showing a drastic change from the native samples.

The relative crystallinity was measured taking into consideration two prominent peaks around 4.50 and 9.50 Å. The crystallinities of samples A–G (see details in Table 3) were, respectively, 100, 100, 82, 88, 86, 78 and 72% (Table 3). Slightly different results of relative crystallinity were received for bovine albumin. The crystallinities samples of a–e (Fig. 5, insert 2) were, respectively, 100, 73, 66, 34 and 28%. The spacing values of these samples were similar to OVA and were shown to be in the range 9.60–9.85 and 3.80–4.67 Å. From the results which are shown it may be concluded that the crystallinity is connected with the degree of

TABLE 5

Thermodynamic data of native and dry-heated albumin[a]

Samples	T (°C)	ΔH		ΔS (kcal/mol K)	%D
		(J/g)	(kcal/mol)		
Native granulated BA (dry)	66.2	106.3	1676.0	4.9	
Granulated BA heated at 80 °C 1 h	55.[illegible]	105.1	1673.9	4.9	
Granulated BA heated at 80 °C overnight	65.3	105.9	1670.5	4.9	
Granulated BA heated at 100 °C 1 h	63.4	94.5	1490.2	4.4	11
Granulated BA heated at 100 °C overnight	62.1	79.8	1258.6	3.8	25
Granulated BA heted at 100 °C 5 days	61.8	56.9	897.6	2.7	46
Native granulated BA with glycerol	65.1	113.9	1796.7	5.3	
Granulated BA with glycerol heated at 100 °C 1 h	63.2	42.2	665.7	1.98	63

[a] T_d. Temperature of the denaturation: %D, percent of denaturation calculated from DSC experiment.

denaturation, as well as with the molecular weight of these proteins. The larger percent denaturation corresponds to the smaller crystallinity.

Dry-heated OVA

Dry ovalbumin was heated for different periods of time in non-closed conditions and compared with the data obtained after denaturation of OVA in ampules. Figure 6 and Table 4 demonstrate DSC thermograms and thermodynamic data. These results indicate a significant increase of percent denaturation of ovalbumin with increasing heating time. A decrease in ΔH and T_d and broadening of the peak were noted upon OVA heating. A peak broadening, as well as a decrease in ΔH, indicates denaturation (19). As is seen from Table 4, an increase in the temperature and time of heat treatment caused a decrease in enthalpy and a slight shift in T_d to the lower value. After incubation during 5 days at 100 °C denaturation reached 48%. However, in the presence of 11% of water (Table 1) denaturation reached 57% after 12 h incubation at 100 °C. Addition of glycerol (7.5%) reduced the enthalpy by 63% after 1 h incubation at 100 °C. These results are in agreement with the conclusions of Nose *et al.* (4), which revealed that thermal denaturation of solid ovalbumin was sensitive to the water and glycerol content. However, Nose *et al.* (4) did not observe thermal denaturation in dry solid ovalbumin even after 3 h heating at 100 °C. Figures 6 and 7 represent thermograms of different proteins before and after denaturation at various conditions. All sample proteins show a gradual decrease in the extent of denaturation with increase in temperature (Tables 4–6). However, the percentage of denaturation was maximal in the case of γ-globulin. Denaturation in bovine albumin was not observed upon heating at 80 °C overnight. This result may be attributed to the different denaturation temperatures of the tested proteins. However, the extent of denaturation in the presence of water or glycerol was quite similar in all cases. Probably different molecular weights of proteins did not influence the degree of thermal denaturation in the presence of additives, such as water and glycerol, which increase the extent of thermal denaturation.

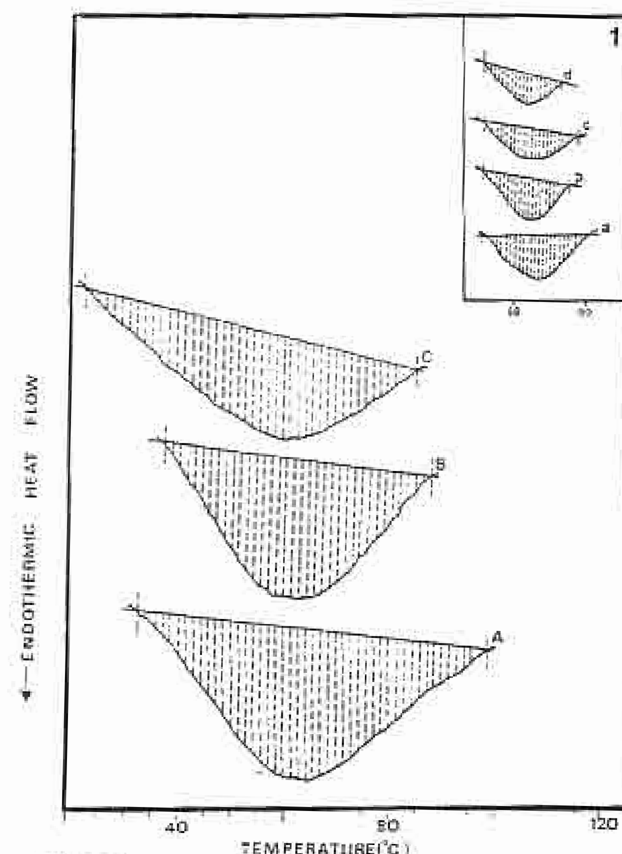

FIGURE 7

DSC thermograms of granulated BA treated in non-closed conditions: (A) native BA, (B) BA heated at 100 °C overnight, (C) OVA heated at 100 °C 5 days. Insert 1: DSC thermograms of granulated γ-G treated in non-closed conditions: (a) native γ-G, (b) γ-G heated at 80 °C overnight, (c) γ-G heated at 100 °C overnight, (d) γ-G heated at 100 °C 5 days.

According to Wagner and Anon (18), thermal protein denaturation involves the rupture of one disulfide

TABLE 6

Thermodynamic data of native and dry-heated γ-globulin[a]

Samples	T (°C)	ΔH		ΔS (kcal/mol K)	% D
		(J/g)	(kcal/mol)		
Native granulated γ-G (dry)	73.7	130.6	4682.1	13.5	
Granulated γ-G heated at 80 °C 1 h	67.7	87.4	3131.6	9.2	33
Granulated γ-G heated at 80 °C overnight	62.3	84.1	3015.1	8.99	36
Granulated γ-G heated at 100 °C 1 h	61.9	71.2	2552.6	7.6	46
Granulated γ-G heated at 100 °C overnight	60.6	63.3	2269.4	6.8	52
Granulated γ-G heated at 100 °C 5 days	59.5	51.2	1835.6	5.5	61
Native granulated γ-G with glycerol	64.5	173.4	6216.5	18.4	
Granulated γ-G with glycerol heated at 100 °C 1 h	60.3	69.5	2491.6	7.5	60

[a] T_d, Temperature of the denaturation; % D, percent of denaturation calculated from DSC experiment.

bond (which contributes a ΔH of 25 kcal/mol and a negligible ΔS), and the rupture of n hydrogen bonds ($\Delta H = 4$ kcal/mol and $\Delta S = 0.012$ kcal/mol/per protein molecule). So, the number of broken hydrogen bonds can be calculated as:

$$n = \Delta S/0.012 \text{ and } n = (H - 25)/4$$

where n is the number of broken hydrogen bonds and ΔS is the entropy and ΔH the enthalpy of denaturation.

With ΔH of native ovalbumin and of the heat-treated one 1248.7 and 646.2 kcal/mol, respectively, we can approximately evaluate that 308 and 158 hydrogen bonds were broken during the conformational transition of native protein and of previously heated ovalbumin, respectively. Consequently, 49% of hydrogen bonds were ruptured during heat pretreatment in non-closed conditions. These results are in harmony with recent work of Murphy and Gill (20), who demonstrated that hydrogen bonding is the main stabilizing force in protein stability. In the case of bovine albumin and γ-globulin 55 and 41% of hydrogen bonds, respectively, were ruptured during heat pretreatment (5 days at 100 °C).

Table 7 represents the susceptibility to α-chymotrypsin of ovalbumin heated in the dry state during various periods of time. Susceptibility to proteolysis gradually increases with time of heat treatment and indicates the changes in protein conformation towards a less stable structure during the heating (Table 7). These results correspond to the conclusions of Imoto *et al.* (21), who suggested that susceptibility to proteases is proportional to the flexibility of protein structure and is very sensitive to changes in protein conformation. For visuality, the rate curves of protein hydrolysis with chymotrypsin are shown in Fig. 8.

TABLE 7

Susceptibility to chymotrypsin treatment of ovalbumin (non-closed conditions)

Samples	Extent of digestion (%)
Native granulated OVA	61
Granulated OVA heated at 80 °C 1 h	62
Granulated OVA heated at 80 °C overnight	66
Granulated O VA heated at 100 °C 1 h	65
Granulated OVA heated at 100 °C overnight	70
Granulated OVA heated at 100 °C 5 days	76

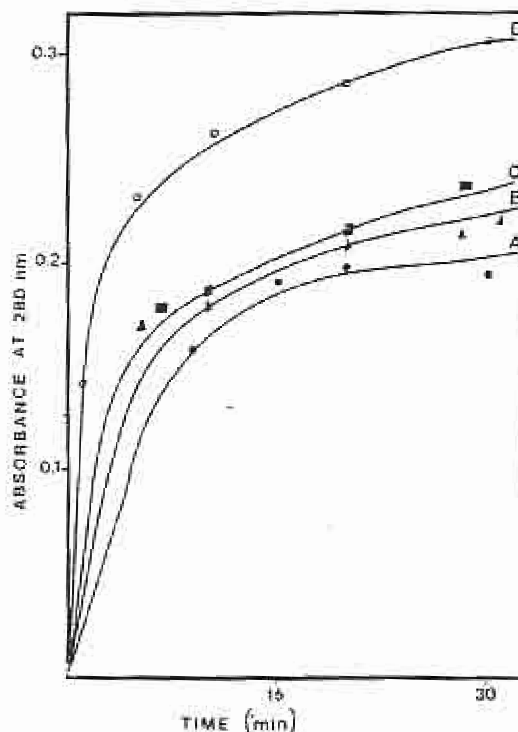

FIGURE 8

Proteolysis of OVA with α-chymotrypsin: native OVA (A), OVA heated at 80 °C 1 h (B), OVA heated at 100 °C overnight (C), OVA heated at 100 °C 5 days.

CONCLUSIONS

We developed a new application previously unknown in the literature, based on DSC and X-ray studies, by analogy with earlier investigations of starches and proteins (22, 23). DSC has proved to be useful for the analysis of solid-state ovalbumin and can give a quantitative estimation of protein denaturation. Comparison of OVA, BA and γ-G demonstrated the possible application of DSC to the analysis of different proteins. OVA and BA showed similar extents of denaturation; however, γ-G, which significantly differed in molecular weight, revealed the maximal conformational changes. In the presence of water or glycerol different molecular masses did not influence the degree of denaturation. A good correlation between DSC, X-ray diffraction, fluorescence, IR spectra and susceptibility to α-chymotrypsin of native and heated granulated proteins was obtained. All abovementioned methods confirmed the existence of alteration in protein structure upon heating.

REFERENCES

1. Stein, P.E., Leslie, A.G.W., Finch, J.T. & Carrell, R.W. (1991) *J. Mol. Biol.* **221**, 941–959
2. Imai, T., Nohdomi, K., Acarturk, F. & Otagiri, M. (1991) *J. Pharm. Sci.* **80**, 484–487
3. Torrado-Duran, J.J., Torrado-Valeiras, J.J. & Cadorniga, R. (1991) *Drug Dev. Ind. Pharm.* **17**, 1305–1323
4. Nose, T., Taguchi, T. & Inoue, E. (1979) *Nippon Kagaku Kaishi* **4**, 455–460
5. Kato, A., Ibrahim, H.R., Watanabe, H., Honma, K. & Kobayashi, K. (1990) *J. Food Sci.* **55**, 1280–1283
6. Kato, A., Ibrahim, H.R., Watanabe, H., Honma, K. & Kobayashi, K. (1990) *J. Agric. Food Chem.* **38**, 32–37
7. Shriver, J.W. (1990) *Arch. Biochem. Biophys.* **283**(2), 472–475
8. Gruen, L.C., Guthrie, R.E. & Blagrove, R.J. (1987) *J. Sci. Food Agric.* **41**, 167–178
9. Miyazawa, K., Ogawa, M. & Mitsui, T. (1984) *Int. J. Cosmetic Sci.* **6**, 33–46
10. Walbridge, D.G., Knutson, J.R. & Brand, L. (1987) *Anal. Biochem.* **161**, 467–478
11. Arntfield, S.D., Ismond, M.A.H. & Murray, E.D. (1987) *Int. J. Peptide Protein Res.* **29**, 9–20
12. Hizukuri, S. (1978) in *Starch Science Handbook* (in Japanese), (Nakamura, M. and Suzuki, S., Eds.), Asakura Shoten, Tokyo, Japan, pp. 209–209
13. Nara, Sh., Mori, A. & Komiya, T. (1978) *Starch/Stärke* **30**, 111–114
14. Xie, D., Bhakuni, D. & Freire, E. (1991) *Biochemistry* **30**, 10673–10678
15. Zemser, M., Friedman, M., Katzhendler, J., Greene, L.L., Minsky, A. & Gorinstein, S. (1994) *J. Protein Chem.* 13 (2), 261–274
16. Kaiden, K., Matsui, T. & Tanaka, S. (1987) *Appl. Spectrosc.* **42** (2), 180–184
17. Kato, K., Matsui, T. & Tanaka, S. (1987) *Appl. Spectrosc.* **41** (5), 861–865
18. Wagner, J.R. & Anon, M.C. (1985) *J. Food Sci.* **50**, 1547–1550
19. Wright, D.J., Leach, I.B. & Wilding, P. (1977) *J. Sci. Food Agric.* **28**, 557–557
20. Murphy, K.P. & Gill, S.J. (1991) *J. Mol. Biol.* **222**, 699–709
21. Imoto, T., Fukuda, K. & Yagishita, K. (1976) *J. Biochem.* **80**, 1313–1318
22. Gorinstein, S. (1975) *J. Agric. Food Chem.* **23**, 45–47
23. Gorinstein, S. & Lii, Ch.-Yi. (1992) *Starch/Stärke* **44**, 461–466

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