Exothermic Transitions of Glycinin Determined by Differential Scanning Calorimetry

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Thermal properties of soy glycinin were investigated in the presence and absence of the reducing agent 2-mercaptoethanol. An endothermic transition was observed without reducing agent, but an exotherm, followed by a relatively small endotherm, was seen in the presence of 0.05 M 2-mercaptoethanol. The presence of the exotherm was dependent on the glycinin concentration and disappeared at 6% protein concentration. The peak maximum temperatures of the endotherms in the presence and absence of 2-mercaptoethanol were similar, indicating the reduction of intermolecular disulfide bonds between acidic and basic polypeptides did not alter the thermal stability of the protein. The reason for the occurrence of the exotherm was investigated and was found to be due to the aggregation of the basic polypeptides. Aggregation, and hence the presence of the exotherm, could be prevented by the addition of soy β-conglycinin. With β-conglycinin present, glycinin exhibited only an endotherm. The exotherm described in this study was considered to be thermally distinct from the exotherm normally attributed to aggregation following denaturation of a protein.

Very little information is available on the thermal properties of glycinin at protein concentrations where gelation does not occur. This would include concentrations below 2.5%, a value Nakamura et al. (1986) found to be the lowest concentration of glycinin needed to form self-supporting gels upon heating. Protein levels below 2.5% have practical value for food applications such as coffee whiteners, infant formulas, and protein-fortified beverages where gelation during thermal processing is undesirable. Thermal properties of proteins are normally investigated by differential scanning calorimetry (DSC), and most of the calorimetric studies of glycinin have been at high (8–10%) protein concentrations. At these values, an endothermic transition is observed where the transition temperature is pH and ionic strength dependent (Hermansson, 1978). Several studies (Hermansson, 1978; Bikbov et al., 1983; Nakamura et al., 1985) have found glycinin to be a thermally stable protein with a denaturation temperature between 84 and 108 °C depending on pH and ionic strength. This heat stability has been interpreted as being due to extensive disulfide bond formation in the molecule and may be one of the factors limiting its use as a functional protein in foods (Kinsella, 1981). Reducing agents, such as 2-mercaptoethanol (2-ME), can readily reduce the intermolecular disulfide bonds between glycinin molecules (Briggs and Wolf, 1957) and potentially lower the denaturation temperature. However, Harwalkar and Ma (1987) have determined the effect of dithiothreitol on the thermal properties of oat globulin and observed little change in the transition temperature. They concluded that hydrophobic interactions, rather than disulfide bonds, contributed to the observed high heat stability of oat globulin when investigated by DSC.

In a preliminary investigation using a glycinin concentration (1.0%) below that required for gel formation, we determined the thermal properties of glycinin in the presence and absence of 2-ME (Zarins and Marshall, 1987). We observed an endotherm in the absence but an exotherm in the presence of 2-ME. Our objectives in the present study were (1) to extend our previous observations to further characterize the exotherm and determine the cause of its appearance and (2) to evaluate the influence of 2-ME on the thermal stability of soy glycinin.

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MATERIALS AND METHODS

Materials. Glycinin was prepared from an aqueous extract of Nutrisoy 7B flakes (Archer Daniels Midland Co., Decatur, IL) by cryoprecipitation (Wolf and Sly, 1967). The crude preparation was further purified by Concanavalin A–Sepharose to remove glycoproteins and Sepharose 6B to remove 2S globulins. The elution buffer for both columns was 0.035 M phosphate, 0.40 M NaCl, pH 7.0. A final purification step involved the use of DEAE–Sepharose where the glycinin was eluted with a NaCl gradient, at an ionic strength of 0.3. A purity of 88% glycinin was determined by calculating the area under the densitometer curves for the acidic and basic polypeptides from a sodium dodecyl sulfate–polyacrylamide gel. A sample consisting of predominantly glycinin basic polypeptides and a sample of β-conglycinin were supplied by W. J. Wolf (Northern Regional Research Center, Peoria, IL).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Soy Globulins. Polypeptide profiles of the soy globulins were obtained by SDS-PAGE, using a 15% gel. SDS-PAGE was carried out with a Tris–glycine buffer system containing 1.0% SDS as described by Fling and Gregerson (1986). The sample buffer contained 4.3% (0.55 M) 2-ME, and 100 μg of protein was added to each sample well on the gel.

Differential Scanning Calorimetry. Protein samples were dissolved in 0.035 M phosphate, 0.40 M NaCl buffer, pH 7.0, in the presence or absence of 0.05 M 2-ME, to the appropriate protein concentration. Differential scanning calorimetry was performed with a Hart Scientific Model 707 calorimeter (Provo, UT). The instrument is designed to acquire thermal data simultaneously on one reference and three sample cells, each having a 1.0-mL capacity. Thermal data are acquired and analyzed by an IBM PC-XT computer using a Xenix multitasking operating system. This operating system permits data collection and data analysis programs to be run simultaneously rather than in sequence. The instrument is calibrated periodically by means of an internal heater while operating isothermally. Calibration constants are obtained for each cell over the temperature range of the instrument. The constants are then stored so that all calorimeter runs are automatically corrected with the calibration constants. Instrument sensitivity is 5 μcal/°C (20.9 J/°C) and refers to the minimum detectable change in heat capacity. The sample cells contained 8.0–30 mg of protein in 0.4–0.8 mL of buffer and were run against an equal weight of buffer in the reference cell. The weight of material in all four cells was within 0.01 g. All samples were prepared at room temperature and the cells equilibrated at 25 °C. The heating rate was 1.0 °C/min over the range of 25–108 °C. Thermal curves were generated by heating the sample to 108 °C, holding for 8 min, cooling the sample from 108 to 25 °C at 1.0 °C/min, and then reheating to 108 °C at the same scan rate. The second heating established a base line for each run, since in

Table I. Transition Temperatures[a] and Calorimetric Enthalpies[a] of Soy Glycinin in the Presence of 0.05 M 2-ME

protein concn, %	transition temp, °C				ΔH, J/g
	T_o	T_m(endo)	T_m(exo)	T_c	
1.0[b]	92.2 ± 0.2	97.8 ± 0.3	NO[d]	103.2 ± 0.4	7.4 ± 0.4
1.0	90.8 ± 0.3	98.2 ± 0.3	91.5 ± 0.2	102.5 ± 0.3	24.0 ± 0.4[c]
2.0	92.0 ± 0.2	98.5 ± 0.2	93.0 ± 0.2	102.5 ± 0.3	24.2 ± 0.3[c]
4.0	89.6 ± 0.2	96.0 ± 0.2	90.4 ± 0.2	101.6 ± 0.2	7.2 ± 0.3[c]
6.0	90.6 ± 0.1	96.9 ± 0.1	NO	102.3 ± 0.2	8.1 ± 0.4
8.0	90.6 ± 0.2	97.5 ± 0.2	NO	102.7 ± 0.3	9.3 ± 0.4

[a] Values given are means ± SEM of duplicate determinations. [b] No 2-ME was added to this sample. [c] Enthalpies measured for the entire thermal process, including both exotherm and endotherm. [d] NO = not observed.

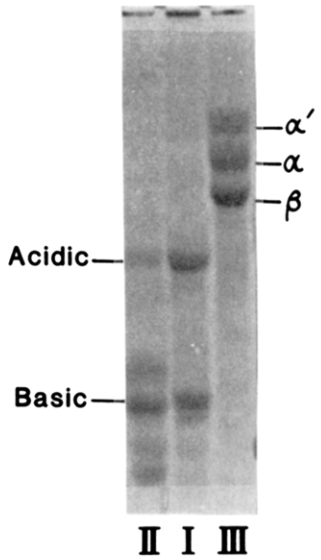

Figure 1. SDS-PAGE gel of soy proteins used in this study: I, glycinin prepared by the authors; II, a protein preparation consisting of predominantly basic polypeptides of glycinin obtained from W. J. Wolf (basic polypeptides); III, β-conglycinin obtained from W. J. Wolf.

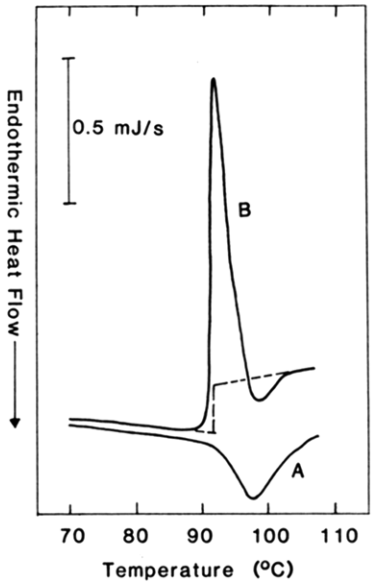

Figure 2. DSC thermal curves of glycinin at 1.0% protein concentration in sample buffer: A, no additions; B, 2-ME added to 0.05 M. The dashed line under B represents the base line used to determine the calorimetric enthalpy for the entire thermal process.

all cases no thermal transitions appeared during the second heating. Base-line substractions were made on all thermal curves, and only corrected thermal curves are shown.

Determination of Transition Temperatures and Calorimetric Enthalpies. Base lines were obtained by extrapolating data on both sides of the thermal transition to the temperature of half-completion of the process. With the base line as reference, the extrapolated onset temperature (T_o), the peak maximum temperature (T_m), and the extrapolated conclusion temperature (T_c) were determined by the procedure described by Lund (1983). Calorimetric enthalpies (ΔH) were determined by planimetry as the area under the thermal transition curves with appropriate base lines.

RESULTS AND DISCUSSION

Polypeptide Profiles of Soy Globulins. Polypeptide profiles of soy globulins are depicted in Figure 1. Glycinin is shown in lane I, with its characteristic two major bands corresponding to the acidic and basic polypeptides. A minor band below the basic polypeptides was not identified. In contrast, the sample known to contain predominately basic polypeptides (referred to in this study as "basic polypeptides") (lane II) shows a major band corresponding to the basic polypeptides and four bands of lesser intensity. One of these minor bands is probably acidic polypeptides as it corresponds to the acidic polypeptide band for glycinin (lane I), although this band corresponds to only 25% of the protein contained in the comparable band in lane I. The other bands may represent degradation products of the acidic polypeptides. Endogenous endopeptidases have been reported in soybeans that preferentially hydrolyze the acidic polypeptides while leaving the basic polypeptides virtually untouched (Bond and Bowles, 1983). These authors found that certain metal ions, such as Ca^{2+} and Mn^{2+}, stimulated enzyme activity. Since the protein elution buffer during Concanavalin A–Sepharose 4B chromatography originally contained Ca^{2+} and Mn^{2+}, these ions may have stimulated endopeptidase activity in this sample (W. J. Wolf, personal communication). Lane III (Figure 1) is a typical profile for soy β-conglycinin, showing the characteristic α, α′, and β subunits.

Thermal Properties of Glycinin in the Presence and Absence of 2-ME. Figure 2 shows the thermal curves obtained for 1.0% glycinin in the presence and absence of 0.05 M 2-ME. In the absence of 2-ME an endotherm was observed. However, when 2-ME was present, an exotherm was seen. The exotherm appeared to be immediately followed by a small endotherm that was partially obliterated by the relatively large size of the exotherm. These results correspond to our previously reported observations (Zarins and Marshall, 1987). The two curves have similar T_o values (Table I), although the exotherm begins at a slightly lower T_o. The similarity between the T_o values for glycinin in the presence and absence of reducing agent (Table I) indicates 2-ME exerts little destabilizing influence on the protein. Therefore, the interchain disulfide bonds appear to play only a minor role in the thermal stability of glycinin. T_m values were considerably different, as the T_m for the exotherm was lower than the T_m for the endotherm seen in the absence or presence of 2-ME (Table I). The ΔH for glycinin in the presence of

Table II. Transition Temperatures[a] of Soy Proteins at 1.0% Concentration in Buffer and in the Presence of 0.05 M 2-ME

sample	transition temp, °C			
	T_o	T_m(endo)	T_m(exo)	T_c
glycinin	90.8 ± 0.3	98.2 ± 0.3	91.5 ± 0.2	102.5 ± 0.3
glycinin + β-conglycinin	76.4 ± 0.3[b]	84.0 ± 0.2[b]	NO[d]	101.7 ± 0.4[c]
		97.3 ± 0.3[c]		
β-conglycinin	76.8 ± 0.2	84.1 ± 0.2	NO	93.2 ± 0.3

[a] Values given are means ± SEM of duplicate determinations. [b] Attributed to β-conglycinin. [c] Attributed to glycinin. [d] NO = not observed.

reducing agent (Table I) included the enthalpies for both the exo- and endothermic processes and was over 3 times greater than the enthalpy for the endotherm observed in the absence of 2-ME.

The presence of an exotherm associated with soy protein has also been observed by Sheard et al. (1987). They found an exothermic transition in soy isolate that occurred at a T_m of 107 °C, or directly after the endothermic transition for 11S globulin. This compares to our T_m of about 92 °C, where our observed exotherm occurred directly before the 11S endotherm. Sheard et al. did not specifically attribute the exotherm to either the 11S or 7S globulin but noted that it did occur at moisture contents above 70%. A direct comparison between their results and ours is difficult because the samples differed in protein composition and concentration, pH, and ionic characteristics of the solvent.

The appearance of an exotherm for soy glycinin in the presence of reducing agent was puzzling because German et al. (1982) had observed only an endothermic transition for 8% protein in the presence of 0.01 M 2-ME. This observation led us to believe the exotherm might be dependent upon the glycinin concentration. A protein concentration study revealed that the presence of the exotherm was indeed concentration dependent as shown in Figure 3. There was a large decrease in the size of the exotherm between 2.0 and 4.0% protein concentration (Figure 3B,C). The decrease can best be assessed quantitatively by the 70% reduction in ΔH for the entire thermal process (Table I). The exotherm completely disappeared at 6.0% and was also not seen at 8.0% concentration (Figure 3D,E), supporting the observation of German et al. (1982) noted above. There also was little change in the transition temperatures over the protein concentration range of 1.0–8.0% (Table I); however, ΔH showed a distinct increase as the glycinin concentration went from 4.0 to 8.0%. Enthalpies of samples with and without reducing agent were compared at 6.0 and 8.0% protein concentration. These values were selected because a comparison at lower protein concentrations would involve an enthalpy contribution from the observed exotherm in the samples with 2-ME. The enthalpies were 56 and 62% greater in the absence of 2-ME at 6 and 8% protein, respectively. Larger enthalpy values were also seen for oat globulin in the absence of 2-ME compared to protein exposed to reducing agent (Harwalkar and Ma, 1987).

We also noted that precipitates formed in the heated samples at 1 and 2% concentration but soft gels were observed in the samples with 4% and higher protein levels.

Molecular Interactions Associated with the Exothermic Transition. In the presence of reducing agent and at neutral pH, soy glycinin is thermally dissociated into acidic and basic polypeptides with the subsequent aggregation of the basic polypeptides (German et al., 1982). Further studies by Damodaran and Kinsella (1982) showed that the thermal aggregation of basic polypeptides required the presence of a reducing agent such as 2-ME. Aggregation, however, could be prevented by the presence of β-conglycinin, as this protein formed a soluble complex

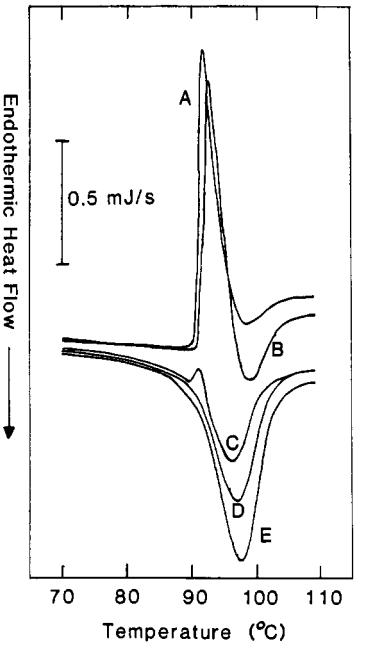

Figure 3. DSC thermal curves of glycinin at different protein concentrations in sample buffer and 0.05 M 2-ME: A, 1.0%; B, 2.0%; C, 4.0%; D, 6.0%; E, 8.0% protein concentration.

with the basic polypeptides through electrostatic interactions. On the basis of the above information, we felt the exotherm seen in Figure 2 might be due to aggregation of the basic polypeptides and sought to test this hypothesis by running the thermal curves of glycinin in the presence of β-conglycinin. The results are shown in Figure 4. Figure 4A represents glycinin only. However, in the presence of β-conglycinin, the exotherm due to glycinin completely disappeared (Figure 4B). Instead, two endotherms were observed. The lower temperature endotherm can be attributed to β-conglycinin, since a comparison of β-conglycinin in Figure 4B to the thermal curve of β-conglycinin only (Figure 4C) showed nearly identical T_o and T_m values (Table II). Apparently, the presence of glycinin had no effect on the thermal stability of β-conglycinin. We believe the higher temperature endotherm is attributable to glycinin because the T_c values for the glycinin and glycinin plus β-conglycinin curves are similar (Table II). In addition, a T_m of 97.3 °C for glycinin in the protein mixture (Table II) is similar to a T_m of 97.8 °C for glycinin in the absence of 2-ME (Table I).

Further evidence that the basic polypeptides of glycinin are contributing to the exothermal process was gathered from the thermal behavior of the basic polypeptides in the absence of 2-ME as shown in Figure 5. Figure 5A showed that this sample exhibited an exotherm similar to that of glycinin heated in the presence of reducing agent (Figure 2B). We recognize that our basic polypeptide sample is not a pure basic polypeptide fraction. However, there appears to be insufficient intact acidic polypeptides attached to the basic chains in this sample to prevent thermal

Table III. Transition Temperatures[a] of Basic Polypeptides and β-Conglycinin at 1.0% Concentration in Buffer

sample	transition temp, °C			
	T_o	T_m(endo)	T_m(exo)	T_c
basic polypeptides	86.4 ± 0.3	94.7 ± 0.2	87.9 ± 0.3	102.1 ± 0.3
basic polypeptides + β-conglycinin	76.4 ± 0.2[b]	84.4 ± 0.3[b] 93.8 ± 0.2[c]	NO[d]	100.6 ± 0.4[c]
β-conglycinin	76.9 ± 0.2	84.0 ± 0.2	NO	92.7 ± 0.3

[a] Values given are means ± SEM of duplicate determinations. [b] Attributed to β-conglycinin. [c] Attributed to basic polypeptides. [d] NO = not observed.

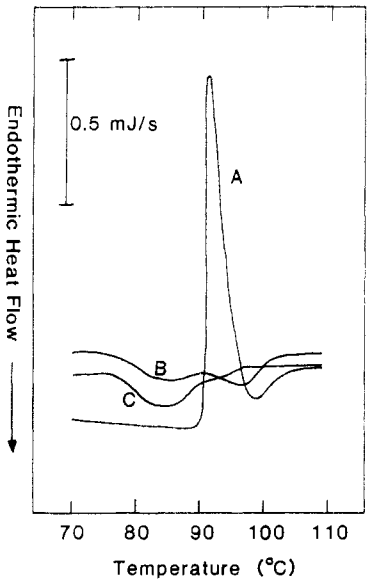

Figure 4. DSC thermal curves of purified soy storage proteins at 1.0% concentration in sample buffer and 0.05 M 2-ME: A, glycinin only; B, glycinin and β-conglycinin; C, β-conglycinin only.

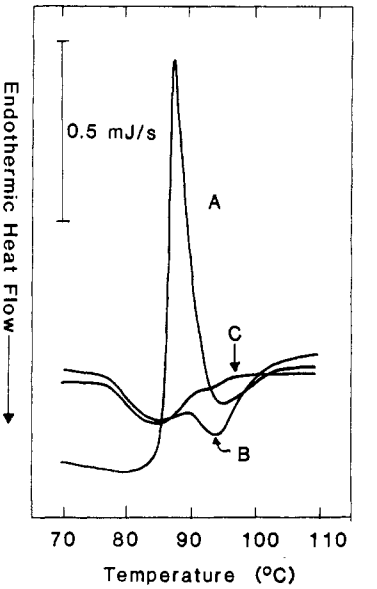

Figure 5. DSC thermal curves of purified soy storage proteins at 1.0% concentration in sample buffer only: A, basic polypeptides only; B, basic polypeptides and β-conglycinin; C, β-conglycinin only.

aggregation. Therefore, reducing agent is not required to chemically separate the acidic and basic chains. Table III gives the transition temperatures for this sample. Compared to the exotherm for glycinin (Tables I and II), the basic polypeptides had a lower T_o and T_m, but both thermal processes had a similar T_c. The ΔH for the basic polypeptides (26.8 J/g) was slightly larger than for glycinin (24.0 J/g).

In the presence of β-conglycinin (Figure 5B), the basic polypeptides behaved in a manner identical to that of glycinin, in that no exotherm was observed. Comparing parts B and C of Figure 5 and comparing the transition temperatures in Table III, the presence of basic polypeptides had no effect on the thermal stability of β-conglycinin.

The data presented in this study indicate that the aggregation of the basic polypeptides of soy glycinin produces an exothermic transition. A recent example of protein aggregation resulting in an exothermic transition was described by Berkowitz et al. (1980) for the polymerization of calf brain tubulin from monomeric subunits. In the present case, the aggregation process appears to be immediately followed by an endotherm that is partially occluded by the larger exotherm. This endotherm had T_m and T_c values similar to T_m and T_c values for the denaturation endotherm of glycinin in the absence of 2-ME (Table I). Therefore, this endotherm could represent the thermal denaturation of glycinin, and at 1.0% protein concentration, this denaturation was found to be irreversible. The effect of protein concentration on the size of the exotherm may be explained as a purely spatial effect. As protein concentration is increased, the glycinin polypeptides come in closer contact with each other. Even though 2-ME has dissociated the acidic from the basic polypeptides, the basic polypeptides become more and more constrained and are hindered from thermally aggregating with each other. Hence, the exotherm is diminished in size. This spatial effect is favored over the notion that the endotherm disappears due to an insufficient amount of 2-ME to reduce all the interchain disulfide bonds at the higher (4.0% and up) protein concentrations. In terms of molar equivalents, 0.05 M 2-ME provides more than sufficient reducing equivalents to reduce all of the interchain disulfide bonds, even at 8.0% concentration.

This study may be the first to attribute an exotherm to a specific soy protein (glycinin) and to a specific molecular event (aggregation) associated with the protein. Under the conditions employed in our experiments, two distinct thermal events (aggregation and denaturation) can be partially separated from each other.

The aggregation of the basic polypeptides described here perhaps represents a special case where the following sequence might occur: native glycinin + 2-ME → acidic + basic polypeptides $\xrightarrow{\Delta}$ basic polypeptide aggregation (exothermic) $\xrightarrow{\Delta}$ protein denaturation (endothermic) $\xrightarrow{\Delta}$ aggregation of the remaining polypeptides (?) (exothermic) → precipitate.

This sequence is different from "normal" protein denaturation and aggregation which could occur for a globular protein like glycinin in the absence of 2-ME: native glycinin $\xrightarrow{\Delta}$ protein denaturation (endothermic) $\xrightarrow{\Delta}$ protein aggregation (exothermic) → precipitate.

Calorimetrically, basic polypeptide aggregation is apparently different from the protein aggregation noted in the second sequence, perhaps in both time and magnitude of occurrence. Both sequences lead to protein precipitation and an increase in turbidity of the protein solutions, which

we observed at the conclusion of the calorimeter runs. However, protein aggregation following denaturation is normally of such small magnitude in relation to the endotherm produced by denaturation that it is generally ignored (Donovan and Ross, 1973).

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Poly-L-lysine and Multioligo(L-methionyl)poly-L-lysine as Nutritional Sources of Essential Amino Acids

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Poly-L-lysine and the corresponding branched-chain polypeptide multioligo(L-methionyl)poly-L-lysine were synthesized by the *N*-carboxy anhydride method, and their nutritional properties were investigated by in vitro and in vivo methods. The extent of modification of poly-L-lysine, consistent with good availability of both amino acids, could be estimated by an in vitro enzyme digestion procedure. Lysine and methionine were found to be rapidly released from the polymer provided that their molar ratio was not beyond 2. Their bioavailability was further confirmed by rat feeding experiments using a 10% basal protein diet supplemented with either free methionine and free lysine or multioligo(L-methionyl)-poly-L-lysine. In both cases, the methionine to lysine molar ratios were 0.85 and 1.30, respectively. The synthetic branched-chain polymer was found to be as effective as the corresponding free amino acids in meeting the rat requirements for growth.

The nutritional value of lower quality food proteins may be improved through fortification with essential amino acids supplied either as the free form of as covalently attached to proteins. As a matter of fact, chemical and enzymatic methods have been successfully applied to food proteins to enhance their content in one or more limiting amino acids over the past few years (Puigserver et al., 1982; Yamashita et al., 1979). More recently, polymerization of L-methionine onto the ϵ-amino groups of casein and β-lactoglobulin by the *N*-carboxy anhydride method resulted in the preparation of modified proteins in which as much methionine as 30% of protein weight was covalently attached in the form of polymethionine of an average chain length of eight residues (Gaertner and Puigserver, 1984a). The bioavailability of covalently attached methionine could be derived from in vitro enzyme digestion experiments in which the successive hydrolysis of methionine polymers by gastric, pancreatic, and intestinal enzymes was quantitatively estimated (Gaertner and Puigserver, 1986). An alternative means to prevent the possible deteriorative reactions occurring in food proteins when they are sup-

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