Effects of Extrusion upon Soy Concentrate Solubility

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Thermoplastic extrusion is an important process used to impart structural integrity and the first level of meatlike texture to soy meal, flour, or concentrate. Although it has been assumed that the structure of soy extrudate is a result of alterations occurring in the protein fraction, the molecular changes that occur within the extruder are poorly understood. By analogy with spun soy fibers, intermolecular disulfide bonding has been invoked as one plausible mechanism that contributes to structure formation. No direct evidence showing this has been published, and one report that intermolecular peptide bonding is largely responsible for extrudate structure has appeared. This study provides evidence that intermolecular disulfide bonding is an important factor contributing to extrudate structure, at least for low-temperature (~150 °C) extrusion. No evidence of significant intermolecular peptide bond formation was found. A discussion of the relative importance of those forces (covalent and noncovalent) leading to the insolubility of low temperature extruded soy protein is given.

In the manufacture of soy protein meat analogues, most of the protein must be made insoluble and given structural integrity and viscoelastic properties similar to those of meat. Thermoplastic extrusion (Harper and Harmann, 1973) is the process most often chosen to induce such structure and texture in soy meal or concentrate. It has long been assumed that the soy is insolubilized and made part of a macroscopic structure due to molecular changes in the protein fraction (Cumming et al., 1972). These changes are clearly complex, involving alteration of both covalent and noncovalent interactions, and are poorly understood at the molecular level. This paper reports data intended to shed light on the nature of the changes in covalent bonding that occur during extrusion. The data also allow a comparison of the relative importance of covalent and noncovalent interactions to the insolubilization of the protein by the extrusion process.

Intermolecular disulfide bonding of proteins has been used as a likely explanation of the covalently bonded structure induced by thermoplastic extrusion (Jenkins, 1970; Cumming et al., 1973). This explanation would agree with careful studies of the covalent bonding changes that accompany the formation of structure in spun soy fibers. Both Kelley and Pressey (1966) and Chiang and Sternberg (1974) clearly showed that spun soy fibers are held together by intermolecular disulfide bonds. Wolf (1970) has also studied the aggregation of dissolved soy protein molecules by heat-induced scission of intramolecular disulfide bonds followed by formation of intermolecular disulfide linkages. Finally, a patent has been issued for improving extruded soy granule texture by the addition of elemental sulfur or reactive disulfhydryl compounds to the protein (Jenkins, 1970).

No detailed studies (of the type reporting the nature of bonding in spun soy fibers) exist to show the relative importance of noncovalent interactions, intermolecular disulfide bonds, and other covalent bonds in extruded soy protein. But analogy with the evidence quoted above argues that it is plausible that intermolecular disulfide bonding is important in the formation of the structure of soy protein extrudate. Work by Burgess and Stanley (1976), however, disputes this conclusion. Their study argues that intermolecular peptide bonds, formed during extrusion, dominate the structure of soy protein extrudate.

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Because the direct support for the importance of disulfide bonds as the main covalent interaction contributing to extrudate structure is weak and because Burgess and Stanley have proposed a much different mechanism for the formation of structure during extrusion, we undertook a study of the forces responsible for the insolubilization of soy protein upon extrusion.

MATERIALS AND METHODS

Defatted soy meal (54.5% protein on a moisture-free basis) was obtained from Central Soya. The soy meal was extracted 3 times with a 70% ethanol/30% water solution (10 mL/g soy meal) and oven-dried to produce a soy concentrate (70.0% protein on a moisture-free basis). A portion of this material was extruded with a laboratory-scale extruder (Model 2003, C. W. Brabender Co.). Extrusion parameters were: Compression ratio = 4/1; L/D (length/diameter) ratio = 20/1; speed = 70 rpm; product temperature = 135 °C.

Measurements of the solubility of the original soy concentrate and of the extruded soy concentrate were performed in two buffer systems with and without the reagents urea, sodium sulfite, and acrylonitrile (alone and in combination).

In measuring the solubility, 2 g of each protein sample were homogenized in 100 mL of solvent. Homogenization was performed using a Polytron at maximum speed for 5 min. The samples were then centrifuged for 30 min at 10000g. The precipitate was separated from the supernatant and washed twice with water. In cases where the solvent contained urea, the precipitates were dialyzed against distilled water for 1 week at 4 °C. The precipitates were dried, weighed, and analyzed for nitrogen by the Kjeldahl assay. The fraction of nitrogen dissolved was calculated as

$$((N_{init} - N_{pr})/N_{init}) \times 100 \quad (1)$$

where N_{init} is the mass of nitrogen present in the 2 g of sample before the experiment (0.218 g of N for the initial concentrate; 0.220 g for the extruded concentrate) and N_{pr} is the mass of nitrogen remaining in the precipitate.

The fourteen solvents used to extract the samples were as follows: 1, 2.6 mM KH_2PO_4 and 32.5 mM K_2HPO_4, pH 7.6 (buffer I); 2, 0.0182 M $NaHCO_3$ and 0.0318M Na_2CO_3, pH 10.6 (buffer II); 3, buffer I adjusted to be 8 M in urea; 4, buffer II adjusted to be 8 M in urea; 5, solvent 3 adjusted to be 0.1 M in Na_2SO_3; 6, solvent 4 adjusted to be 0.1 M in Na_2SO_3; 7, solvent 5 adjusted to be 0.1 M in acrylonitrile;

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Table I. Percentage of Total Protein Dissolved by Different Solvents[a]

	reagent(s)				% of total protein dissolved							
					native concentrate				extruded concentrate			
					buffer 1		buffer 2		buffer 1		buffer 2	
none	none	urea	Na_2SO_3	acrylonitrile	run 1	run 2	run 1	run 2	run 1	run 2	run 1	run 2
1	X				63	60	67	64	30	26	30	29
2		X			86	78	81	82	53	52	66	70
3			X		67	67	lost	68	33	47	41	37
4				X	61	64	66	63	27	30	28	30
5			X	X	85	84	83	81	79	78	83	83
6		X	X		88	92	89	89	97	97	94	93
7		X	X	X	94	94	94	92	96	97	96	96

[a] Buffer 1 is a pH 7.6, phosphate buffer. Buffer 2 is a pH 10.6, carbonate buffer.

8, solvent 6 adjusted to be 0.1 M in acrylonitrile; 9, buffer I adjusted to be 0.1 M in Na_2SO_3 and 0.1 M in acrylonitrile; 10, buffer II adjusted to be 0.1 M in Na_2SO_3 and 0.1 M in acrylonitrile; 11, buffer I adjusted to be 0.1 M in Na_2SO_3; 12, buffer II adjusted to be 0.1 M in Na_2SO_3; 13, buffer I adjusted to be 0.1 M in acrylonitrile; 14, buffer II adjusted to be 0.1 M in acrylonitrile. For those solvents containing acrylonitrile, the acrylonitrile was added immediately before homogenization so that no sulfonation of the acrylonitrile occurred prior to the dissolution step.

Sulfhydryl and disulfide analyses were performed using the method of Robyt et al. (1971). DTNB [5,5-dithiobis-(2-nitrobenzoic acid)], 2-mercaptoethanol, and dithiothreitol were obtained from Calbiochem. The extinction coefficient at 412 nm for the CNT (3-carboxylato-4-nitrothiophenolate) group assayed in this method was measured to be $(1.2 \pm 0.1) \times 10^4$.

To determine free sulfhydryl groups in the protein, 0.1 mL of buffer (1 M in Tris and 1 M in phosphate, pH 8.1) and 0.5 mL of 2 mM DTNB (buffered with 10 mM phosphate, pH 8.1) were added to 2.0 mL of sample containing $(0.5\text{–}10.0) \times 10^{-5}$ M sulfhydryl; the reaction was allowed to proceed 30 min at room temperature (23 °C). The number of sulfhydryl groups per molecule of protein was computed from the absorbance measurements and the extinction coefficients of protein and CNT at 280 and 412 nm, respectively.

To determine disulfide groups in the protein, the pH of the reaction solution from the determination of free sulfhydryl groups was increased from 8.1 to 10.5 by carefully adding 0.1 N sodium hydroxide and allowing the reaction to proceed for 1–2 min. The pH was readjusted to 8.1 by adding 0.1 N hydrochloric acid, and the increase in absorbance was measured at 412 nm. A DTNB-reagent blank was prepared to correct for the formation of CNT from the alkaline cleavage of DTNB. After correction of the DTNB blank, the resulting absorbance is a measure of the total sulfhydryl content of the protein, i.e., free sulfhydryl plus the sulfhydryl groups derived from the disulfide groups.

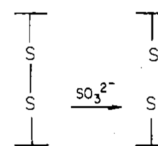
The number of disulfide groups can be computed by the formula

$$\text{no. of disulfide groups} = (\text{no. of CNT after pH 10.5} - 2(\text{no. of CNT before pH 10.5}))/2.0 \quad (2)$$

CHEMISTRY OF SOLUTION PROCESS

The buffers alone should dissolve protein molecules that remain in their native states. The buffered urea samples will, in addition, dissolve denatured (but not highly aggregated) molecules and small aggregates (e.g., protein monomers insolubilized by hydrogen bonding or hydrophobic interactions or small aggregates of protein molecules joined by intermolecular covalent bonds). The sodium sulfite is added to cleave disulfide bridges that make large aggregates insoluble, even in urea. The cleaved protein segments should then be soluble in buffer or in buffer plus area.

Sulfite acts according to (Greenstein and Winitz, 1961)



The free sulfhydryl groups may reoxidize to form new intermolecular disulfide bridges during this process. Burgess and Stanley (1976) (using mercaptoethanol to reduce the disulfide, thus forming two free sulfhydryls per disulfide) failed to guard against reoxidation. Here the effects of reoxidation are minimized by adding excess acrylonitrile, which cyanoethylates the protein sulfhydryl groups by

$$R\text{–}SH + CH_2\text{=}CH\text{–}CN \rightarrow R\text{–}S\text{–}CH_2CH_2CN$$

This reaction is quantitative in less than 2 min and proceeds without detectable side reaction between the thiol and acrylonitrile (American Cyanamid Company 1959). A competing sulfonation reaction between the sulfite and the acrylonitrile is, under these circumstances, about 10 times slower than the reaction with the thiol (Morton and Landfield, 1952).

RESULTS

Earlier work (Cumming et al., 1972, 1973) showed that extrusion produces large aggregates of soy protein molecules that cause the texturized product to be insoluble. If this insoluble protein can be dissolved by reagents whose chemical effects are known, then one can infer the nature of the forces leading to insolubilization. Here, we are using urea to disrupt insolubilization due primarily to noncovalent forces (hydrogen bonding and hydrophobic interactions). Sodium bisulfite in combination with acrylonitrile disrupts insolubilization due to intermolecular disulfide bridges.

Table I gives the percentages of nitrogen extracted from extruded soy concentrate and from the soy concentrate used as the starting material for the extrudate for each of the 14 solvent systems listed earlier. The table lists duplicate determinations (runs 1 and 2) for each experiment. The average deviation of the duplicate determinations from their means is ±2.3% of the mean. The maximum deviation from the mean was ±17.5% of the mean.

Between 60 and 66% of the nitrogen-containing material (assumed to be protein) in the unprocessed concentrate is dissolved by the buffers. A total of 78–80% of the protein is dissolved by the buffers with urea. A total of

Table II. Fraction of Soy Protein Soluble and Insoluble Due to Noncovalent Forces, Formation of Small Aggregates, and Formation of Large Aggregates

	native soy concentrate		extruded soy concentrate	
	buffer 1	buffer 2	buffer 1	buffer 2
% protein soluble in simple buffers	61.5	65	27.5	29
% protein insoluble by noncovalent forces	9.5	10.75	18	13
% protein insoluble due to disulfide covalent forces	12	11.25	44	28.25
% protein insoluble due to a combination of both disulfide covalent and noncovalent interactions	11	5.75	7	25.75
% indeterminate	6	7.25	3.5	4

92–94% of the protein is dissolved by buffer with urea, sodium sulfite, and acrylonitrile and 81–85% is dissolved by buffer with only sodium sulfite and acrylonitrile.

For the extruded concentrate, only 26–30% of the protein can be dissolved by the buffers alone. Addition of urea raises the fraction dissolved to 52–53% with the pH 7.6 buffer and 66–70% with the pH 10.6 buffer. Further, addition of sodium sulfite and acrylonitrile raises the soluble fraction of the protein to 96–97% of the original amount.

It should finally be noted that, in the presence of 8 M urea, reoxidation of the free sulfhydryls is unimportant, in that the addition of acrylonitrile to samples containing Na_2SO_3 does not affect the fraction dissolved. In the absence of urea, addition of acrylonitrile greatly increases the solubility of the protein (from 67 to 81–81% for unprocessed concentrate; from 33–47 to 78–83% for extruded concentrate), implying that reoxidation is important in the absence of urea.

DISCUSSION

The major result of this work is the finding that, for our extrudate, all but 3–4% of the protein can be solubilized by using urea and a disulfide cleaving reagent. This amount of totally insoluble material is the same for extruded soy concentrate as for the unprocessed soy concentrate. Further, extrusion decreases (1) the amount of molecularly soluble (i.e., in buffer alone) protein from 60–66 to 25–30% and (2) the amount of urea-soluble protein (that insolubilized by hydrogen bonding/hydrophobic interactions) from 78–86 to either 52–53 (pH 7.6) or 65–70% (pH 10.6). The amount of protein that can be solubilized by buffer with sodium sulfite and acrylonitrile is only marginally decreased, from 81–85 to 78–83%.

Thus, the major forces responsible for insolubilization and supramolecular structure in these samples would appear to be hydrophobic interactions, hydrogen bonding, and covalent intermolecular disulfide bridges. It is unnecessary to involve other covalent interactions to explain the structue of this soy protein extrudate.

A semiquantitative measure of the relative importance of these forces can be inferred from the solubility data. Rather arbitrarily, one can define four states of the protein: (1) buffer-soluble protein = that protein that is soluble in solvents 1 and 2 alone. (2) protein that is insoluble in simple buffers due primarily to noncovalent interactions = protein that is insoluble in buffers containing disulfide-cleaving reagent without urea (solvents 9 and 10) but that can be made soluble by adding urea (solvents 7 and 8). (3) protein that is insoluble in simple buffers due primarily to covalent (disulfide) interactions = protein soluble in buffers that contain both urea and disulfide-cleaving agents (solvents 9 and 10) minus that protein that is soluble in buffered urea alone (solvents 3 and 4). (4) Protein that is insoluble in simple buffers due to a combination of covalent and noncovalent interactions = protein made soluble by adding urea alone to the buffers (solvents 3 and 4) minus that protein counted in state 2 above = protein soluble in buffer containing disulfide cleaving agents alone (solvents 9 and 10) minus that counted in state 3 above. The fourth state includes buffer-insoluble protein, which can be solubilized by the action of either urea or disulfide-cleaving agents.

The results are calculated from the means of the two runs for each condition as

$$\text{state } 1 = \text{line } 1, \text{ Table I}$$

$$\text{state } 2 = (\text{line } 7, \text{ Table I}) - (\text{line } 5, \text{ Table I}) \quad (3)$$

$$\text{state } 3 = (\text{line } 7, \text{ Table I}) - (\text{line } 2, \text{ Table I}) \quad (4)$$

$$\text{state } 4 = (\text{line } 5, \text{ Table I}) - (\text{line } 1, \text{ Table I}) - \text{state } 2 \quad (5)$$

$$\text{state } 4 = (\text{line } 2, \text{ Table I}) - (\text{line } 1, \text{ Table I}) - \text{state } 3 \quad (6)$$

Table II lists the fraction of protein that is insoluble due to purely noncovalent interactions, purely covalent interactions, and a combination of these two types of forces as derived from the data in Table I. These fractions should be viewed as qualitative indications of the relative importance of these structures, rather than as quantitative estimates, because they are obtained from indirect measurements. Even viewed with such caution, the data support the hypothesis that the insolubilization and formation of structure caused by extrusion are strongly influenced by an increase in covalent bonded molecular aggregation due to intermolecular disulfide bridging.

Finally, these results disagree with those of Burgess and Stanley (1976), who must invoke formation of intermolecular peptide bonds to explain their data. Our first hypothesis to explain the disagreement was that free sulfhydryls formed by the disulfide cleavage procedure (using 2-mercaptoethanol) reoxidized during their experiment because the sulfhydryl groups formed thereby were not blocked by a procedure such as cyanoethylation.

While we find such reoxidation to be important in the absence of urea, our sulfhydryl cleavage results indicate very little reoxidation (i.e., the values are unaffected by cyanoethylation) when urea is present. Burgess and Stanley use disulfide cleaving reagents only in the presence of urea, so that the difference between their results and those reported here is probably not due to experimental artifact.

One must conclude that the differences reflect a difference in cross-linking mechanism between the high-temperature extrusion of Burgess and Stanley (150–190 °C) and our lower temperature extrusion (110–150 °C). Melius (1975) has reported that thermal polymerization by peptide bond formation requires a temperature of at least 180 °C.

Our studies also do not show the pronounced decrease in disulfide level (to form free sulfhydryl) reported by Stanley and Burgess. Disulfide and free sulfhydryl levels were analyzed by the method of Robyt et al. (1971). Table III presents the disulfide and sulfhydryl concentrations in the starting concentrate and the extruded concentrate reported here and by Burgess and Stanley (1976). Low-temperature extrusion does not appear to decrease the

Table III. Disulfide and Sulfhydryl Concentrations in Native and Extruded Soy Concentrate[a]

	native soy concentrate	extruded soy concentrate
this work (140 °C extrusion)		
(1) -S-S- content, mol/mg	22.7×10^{-8}	19.6×10^{-8}
(2) -SH content, mol/mg	0.5×10^{-8}	4.1×10^{-8}
Burgess and Stanley (1976) (178 °C extrusion)		
(1) -S-S- content, mol/mg	4.5×10^{-8}	0.9×10^{-8}
(2) -SH content, mol/mg	3.3×10^{-8}	48.9×10^{-8}

[a] This work was done on a sample extruded at 140 °C. Burgess and Stanley (1976) used the extrudate formed at 178 °C. The expected value is about 20×10^{-8} mol of disulfide/mg of native protein (Wolf and Cowan, 1975).

structural role of disulfide units as was found for high-temperature extrusion.

In summary, low-temperature (≲150 °C) thermoplastic extrusion forms structured protein primarily by intermolecular disulfide bridging accompanied by changes in noncovalent bonding. Higher temperature (≳180 °C) extrusion may produce protein polymerization through formation of intermolecular peptide bonds.

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Investigations of Carotenoid Reactions on Micro-Cel C

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The reactivity of carotenoids with Micro-Cel C, a common chromatographic adsorbent, has been investigated further. α-Carotene was substantially converted to 4-hydroxy-α-carotene. Neither 4,4′-dihydroxy-α-carotene nor 3′-hydroxy-α-carotene was formed, suggesting that the reaction was limited to positions allylic to the conjugated double bond system. β-Apo-8′-carotenal also underwent hydroxylation at the allylic 4-position of the β-ring, while retinal and β-ionone did not react. Lycopene reacted to such an extent that none of it remained unchanged. Other important characteristics of the reaction were determined by using β-carotene as the substrate. The hydroxylation process was stopped when Micro-Cel C was washed with acetone or methanol prior to mixing with β-carotene. Used Micro-Cel C could be recycled after removing the acetone from the first extraction of pigments. Drying the Micro-Cel C at 100 °C for 20 h decreased the amount of isocryptoxanthin produced but increased the level of dehydro-β-carotene 200-fold. The water content of the adsorbent was shown to play a key role in the reaction. The reaction was also shown to be solvent dependent. Petroleum ether was the best solvent while ethyl ether, chloroform, and acetone were inhibitors to different degrees. Methanol and ethanol changed the course of the reactions.

Micro-Cel C has been routinely used as a liquid chromatographic adsorbent in the separation and analysis of carotenoids, particularly the xanthophylls. It was found that β-carotene underwent substantial (65%) hydroxylation at the position 4 carbon upon exposure to this adsorbent when in the presence of a nonpolar solvent such as petroleum ether (Rodriguez et al., 1976). The extent of the conversion was directly dependent on the amount of Micro-Cel C, and the highest rate of accumulation of isocryptoxanthin occurred within the first 15 min. This type of reaction was not observed in other adsorbents such as silica gel, kieselguhr, Celite, alumina, HyfloSupercel, and MgO.

It is of interest, therefore, to determine what other carotenoids react with this adsorbent and the extent to which this reaction takes place.

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EXPERIMENTAL SECTION

Apparatus. A high-performance liquid chromatograph (HPLC; Waters Associates, Milford, MA) equipped with a μ-porasil silica column and varible-wavelength detector

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