Chymotrypsin Hydrolysis of Soybean Protein

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Commercial soybean protein isolates were solubilized by a combination of heat and blending and then hydrolyzed by α-chymotrypsin. The specificity of this enzyme for aromatic amino acids was enhanced by optimizing digestion conditions. The average molecular weight of the soluble digestion products was ~1500. The carboxy-terminal amino acids of the digestion products, determined by carboxypeptidase A digestion, were phenylalanine, tryosine, tryptophan, and leucine, as detected by ascending paper chromatography. Thus this mixture of commercial proteins at high concentration can be hydrolyzed with a high degree of specificity with cleavage after only aromatic amino acids (with only one exception of the nonaromatic amino acid leucine) by using α-chymotrypsin.

This paper describes the hydrolysis of soybean protein, the most economical and readily available commercial protein, using the proteolytic enzyme α-chymotrypsin in order to prepare specific polypeptides, phenylalanine-free hydrolysates for the dietotherapy of the metabolic disease of phenylketonurea. α-Chymotrypsin is known to preferentially cleave proteins at the carbonyl side of aromatic amino acids, although it will also cleave after a number of other amino acids, including leucine, methionine, glutamine, and asparagine (Canfield and Anfinsen, 1963; Needleman, 1970). However, many of the bonds in this second group are split more slowly and are not major sites of cleavage, at least in pure proteins. Studies determining the specificity of α-chymotrypsin have in general used a single peptide or purified protein as the substrate (Needleman, 1970). Thus the specificity of α-chymotrypsin acting on a complex mixture of food proteins such as soybean protein isolate needs to be examined. To prepare phenylalanine-free polypeptides from soybeam protein, it is necessary to enhance the specificity of α-chrmotrypsin for aromatic amino acids, cleaving at each phenylalanyl residue but as few other sites as possible to preserve the length of the polypeptide chains.

This study was carried out to examine the kinetics of the α-chymotrypsin reaction on soybean protein isolates in order to optimize the size of the peptides produced and to identify the C-terminal amino acids in the chymotrypsin hydrolysates in order to determine the conditions yielding the highest specificity for aromatic amino acids.

EXPERIMENTAL PROCEDURE

Preparation of Soybean Protein. Soybean Protein isolate (Supro 710, Ralston Purina, St. Louis, MO), known to have a minimum protein concentration of 92%, was suspended in 50 mM potassium phosphate buffer, pH 7.8 at 5% (w/v) and the pH adjusted to 8.0. The suspension was blended for 3 min in a Waring blender at top speed, then heated at 80 °C for 20 min with stirring, and then blended again as described.

Chymotrypsin Hydrolysis. The solubilized soybean protein mixture was then treated with α-chymotrypsin (Sigma Chemical Co., St. Louis, MO) at a 1:100 or 2:100 enzyme/substrate ratio. Aliquots of 4 mL were removed from the digest at specified intervals, and the reaction was terminated by heating 60 s in a boiling water bath. The samples were allowed to cool and then were centrifuged at 12000 rpm for 30 min in a Sorvall RC-2B centrifuge using an SS34 rotor.

Protein Determination. Protein concentration was determined by two methods: absorbance at 220 nm in a 1-cm light path with a UV spectrophotometer (Hitachi Perkin-Elmer Model 139) and by the Lowry method (Lowry et al., 1951) using bovine serum albumin (Sigma Chemical Co., St. Louis, MO) as the standard.

Determination of Extent of Hydrolysis. Two methods were used to determine the extent of hydrolysis of the soybean protein by chymotrypsin. In the first method, the Lowry assay is used to determine the protein concentration with and with trichloroacetic acid (Cl_3AcOH) precipitation of the proteins. The expression, [(total protein concentration) − (Cl_3AcOH-precipitable protein concentration)]/total protein concentration, is equivalent to the ratio of the peptides soluble in 10% Cl_3AcOH to the total protein (Hsieh et al., 1979) and represents the fraction of protein hydrolyzed since only intact proteins and large peptides are insoluble in 10% Cl_3AcOH. The second method is gel filtration on a column (2.5 × 46 cm) of Sephadex G-25 eluted the buffer 50 mM potassium phosphate, pH 7.8.

Determination of C-Terminal Amino Acids. The carboxypeptidase A reaction is carried out to determine C-terminal amino acids in the polypeptides produced by chymotrypsin. Carboxypeptidase is stored in toluene; the desired amount of enzyme is removed as a slurry and centrifuged, and the toluene discarded. The pelleted crystals of enzyme are dissolved in 1 M LiCl and 1.0 M Tris-HCl, pH 7.8. The enzyme solution is added to the peptide mixture and incubated for varying amounts of time. The reaction is stopped by placing aliquots of the digest in a boiling water bath for 60 s. The amino acids released are identified by ascending paper chromatography on Whatman No. 1 paper with the solvent system butanol–acetic acid–water (12:3:5). The solvent front is allowed to reach the top of the paper (25 cm), and then the sheet is removed from the chromatography tank and allowed to dry. The amino acids are located by dipping the sheet in ninhydrin [0.2% (w/v) ninhydrin in acetone; 3% (v/v) pyridine], allowing it to dry, and then heating at 100 °C for 10 min. To specifically identify phenylalanine and distinguish it from leucine, we dipped the chromatogram in 2% (w/v) $NaHCO_3$ in water and allowed it to dry. All spots fade but phenylalanine, which turns dark blue (Block et al., 1958).

RESULTS AND DISCUSSION

Solubilization of Soybean Protein. Dispersion and solubilization of soybean protein powder is the first necessary step in the hydrolysis. The initial suspension contains large aggregates, each consisting of thousands of soybean protein molecules. Blending leads to a finely dispersed suspension, breaking some of the aggregates. The combination of blending, pH 8.0 treatment, and heating to 80 °C leads to both solubilization and partial unfolding of the soybean proteins. This solubilization and

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Table I. Hydrolysis of Soybean Protein by Chymotrypsin

hydrolysis time, min	10	20	30	40
average molecular weight	1650	1480	1580	1700

partial unfolding are particularly important for the chymotrypsin hydrolysis, since the aromatic amino acids that are the preferred cleavage sites for chymotrypsin tend to be buried in the hydrophobic interior of the protein molecules, making them less accessible to the enzyme.

Chymotrypsin Hydrolysis. Three reaction variables were studied to determine the optimum conditions for hydrolysis of soybean protein by chymotrypsin: enzyme/substrate ratio, reaction temperature, and time. Figure 1 illustrates the course of the reaction using two different levels of enzyme: 1:100 (w/w) and 2:100 (w/w). The two curves run in parallel throughout the reaction time. Thus, the concentration of enzyme is limiting at the lower enzyme/substrate ratio. Figure 2 shows the effect of temperature, comparing the hydrolysis reaction at 25 and 37 °C with a 2:100 enzyme/substrate ratio. The reaction at 37 °C reaches a plateau at 88% hydrolysis between 20 and 30 min. The reaction at 25 °C has not yet reached a plateau by 40 min and is approaching the degree of hydrolysis equivalent to that reached at 37 °C within 20 min. The initial slope of the reaction at 37 °C is steeper than that of the reaction at 25 °C, reflecting the difference in rate. However, the longer period of hydrolysis, the closer the extent of hydrolysis.

For enhancement of the specificity of chymotrypsin for aromatic amino acids, conditions providing maximum cleavage in the shortest time are preferred (Blackburn, 1970). Thus, hydrolysis conditions of 2/100 (w/w) for the enzyme/substrate ratio at 37 °C and 20–30 min were selected. For further analysis of the products of the reaction under these conditions, Sephadex gel filtration elution profiles were obtained for the reaction times 0, 10, 20, 30, and 40 min and are shown in Figure 3. The most obvious difference in the five profiles is the progressive reduction in height of the peak eluting in the void volume. Since G-25 fractionates peptides in the size range 1000–5000 daltons, the void volume peak contains both undigested material and peptide products larger than 5000 daltons. The average molecular weight of the peptide fragments eluting in the included volume of the column was estimated for each sample by the method of Catsimpoolas (1974) and Hsieh et al. (1979). The values are given in Table I. The column was calibrated with the marker peptides, RNase S peptide, bacitracin, Gramicidin, Oxytocin, and insulin chain A. The average molecular weight of the small peptides (<5000) derived from soybean protein remains constant as the hydrolysis proceeds, while the amount of higher molecular weight material decreases (Hsieh et al., 1979). These data imply a mechanism where there is nearly simultaneous cleavage of all readily accessible sites in large or intermediate-sized peptides. This

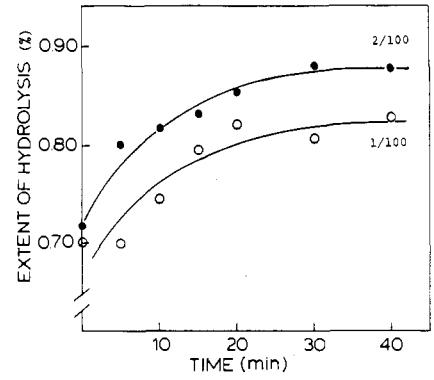

Figure 1. Time course of α-chymotrypsin hydrolysis of soybean protein at 37 °C and two different enzyme concentrations: (○) 1:100 (w/w) enzyme/substrate and (●) 2:100 (w/w) enzyme/substrate.

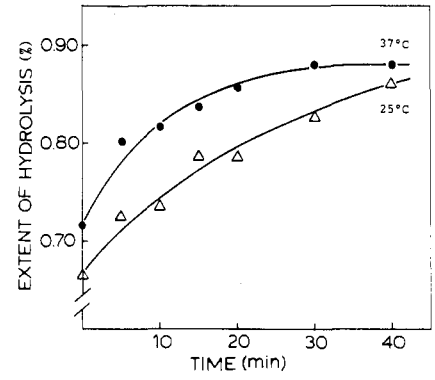

Figure 2. Time course of α-chymotrypsin hydrolysis of soybean protein at 2:100 (w/w) enzyme/substrate and two different temperatures: (△) 25 °C and (●) 37 °C.

conclusion is supported by calculations of the enzyme turnover number (333 bonds cleaved/min) and molar ratio of enzyme/substrate (1 enzyme molecule/3.6 soy protein molecules). There are ~8 specific cleavage sites per soy protein molecule, yielding a ratio of 29 specific cleavage sites for each enzyme molecule. Such a high turnover number and high ratio of enzyme molecules per cleavage site could easily account for simultaneous cleavage of multiple sites in a single soy protein molecule.

Assuming a uniform distribution, one can predict a frequency of one aromatic amino acid for every 14 residues. Thus, the selected conditions of hydrolysis generate peptides of the statistically predicted size with very low concentration of small peptides, thus suggesting that the peptides may terminate with aromatic amino acids.

The above experiments were done on the clear supernatant of the centrifuge hydrolysate. For evaluation of the hydrolysis of the total soybean protein isolate, both pellet and supernatant were examined. By use of the optimum reaction conditions, the amount of protein in each pellet and supernatant was determined for each of a series of

Table II. Soluble and Insoluble Fractions of Hydrolyzed Soybean Protein

	supernatant			pellet			
sample	volume, mL	protein concn, mg/mL	amount of protein, mg	volume, mL	protein concn, mg/mL	amount of protein, mg	sum, mg
control	3.6	26.5	95.4	4.4	10.7	47.1	142.5
0 min	3.5	24.9	87.2	4.5	8.8	39.6	126.8
5 min	3.5	28.2	98.7	4.2	8.3	34.9	133.6
10 min	4.0	28.2	112.8	4.0	8.8	35.2	148.0
15 min	3.7	29.3	108.4	4.4	9.2	40.5	148.9
30 min	3.7	31.8	117.7	4.0	8.1	32.4	150.1
40 min	3.6	30.4	109.4	4.1	9.1	37.3	146.7

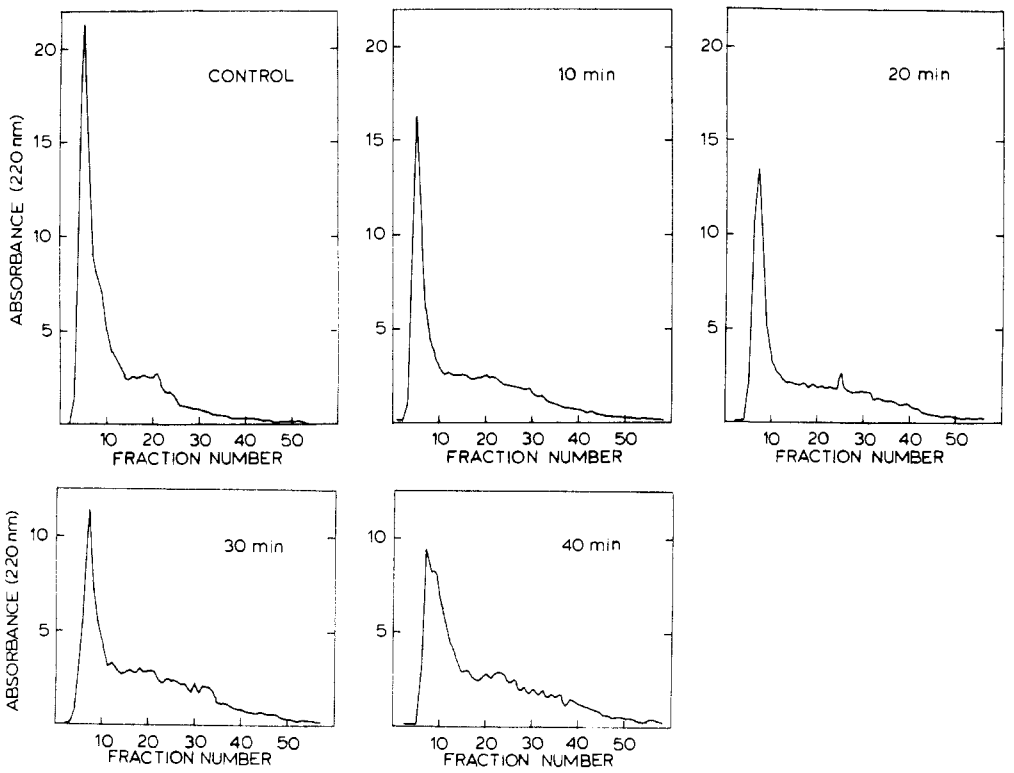

Figure 3. Sephadex G-25 gel filtration of the soluble fraction of α-chymotrypsin digest of soybean protein at different times as indicated. The hydrolysis was carried out at 37 °C and 2:100 (w/w) enzyme/substrate. The control was treated as all other samples except no enzyme was added.

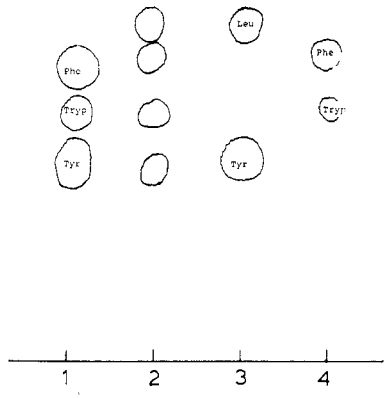

Figure 4. Ascending paper chromatogram fractionating a carboxypeptidase A digest of the soluble portion of an α-chymotrypsin hydrolysis (1000–4000 average molecular weight) and marker amino acids. Track 1: (top to bottom) phenylalanine, tryptophan, and tyrosine. Track 2: carboxypeptidase digest. Track 3: leucine and tyrosine. Track 4: phenylalanine and tryptophan.

aliquots taken at different times (Table II). After 15 min of hydrolysis under this condition, better than 70% of the protein is solubilized and is in the supernatant. Also, there is an increase in the protein content of the supernatant as hydrolysis proceeds, suggesting gradual dispersion of insoluble particles which did not disperse initially. The total protein in each sample is partitioned between pellet and supernatant and is essentially equal to the value for the control. Thus, all the material entering the reaction can be accounted for.

Evaluation of C-Terminal Amino Acids. Carboxypeptidase A is used to cleave the C-terminal amino acids from the peptides prepared by α-chymotrypsin hydrolysis. By use of the pool of polypeptides with a molecular weight of 1000–4000 produced by α-chymotrypsin digestion under optimal conditions, four amino acids were released: tyrosine, tryptophan, phenylalanine, and leucine. The amino acids were identified by comparison with marker amino acids run in adjacent tracks on ascending paper chromatography (Figure 4). Since leucine and phenylalanine are barely resolved in this solvent system, the chromatogram was treated with 2% $NaHCO_3$, which removes the blue color of the ninhydrin from all amino acids but phenylalanine (Block et al., 1958).

The presence of only one nonaromatic amino acid in the pool of amino acids released by carboxypeptides A indicates the high degree of specificity of the α-chymotrypsin reaction. Thus, hydrolysis conditions have been shown to effectively cleave the complex mixture of soybean proteins at the aromatic amino acids and one other amino acid, leucine.

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