Molecular-Weight Distribution of Soybean Globulin Peptides Produced by Peptic Hydrolysis

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ABSTRACT

Soybean globulin dispersions (5% w/v) were hydrolyzed with pepsin (enzyme/substrate = 1/100, 2/100) at pH 1.6 at 25°C for various time intervals. Molecular-weight distribution analysis of the buffer-soluble peptides was performed by gel filtration chromatography using a standardized Sephadex G-50 column. There was a rapid decrease in weight-average molecular weight within the first few minutes of hydrolysis. The logarithm of the weight-average molecular weight of the peptides and the hydrolysis time (10 to 70 min) had an approximately linear relationship. This empirical observation can be used to predict the peptic hydrolysis time needed to obtain soluble soybean protein peptides of a desired molecular size.

Hydrolysis of soybean globulins by proteolytic enzymes offers a commercially feasible approach to preparing modified protein products with characteristics desriable for use in human food. In fact, such approaches have been used commercially for processing soybean protein since the early 1950s (Markley 1951). The desirable characteristics in hydrolysates may include increased solubility at a certain pH, improved emulsifying capacity and stability, enhanced flavor, and altered rheological properties of the original material.

It is therefore imperative that soybean protein hydrolysates be characterized physicochemically, so that they can be compared on an objectively defined basis. Molelcular size can be considered one of the important parameters to be determined. Catsimpoolas (1974) developed a method for determining the molecular-weight distribution of buffer-soluble peptides produced by proteolysis and applied it to selected tryptic and peptic hydrolysates of glycinin, the major soybean globulin (Lynch et al 1977a,b). Since enzymatic modification of total soybean globulins precipitable at pH 4.5 is more relevant and practical in terms of food processing, it was considered desirable to extend the applicability of the method to such material. This paper describes the relation between the molecular size of soluble soybean protein peptides, produced at pH 1.6 by pepsin, and hydrolysis time. Such information may have predictive value for obtaining peptides of a desired weight-average molecular weight.

EXPERIMENTAL

Preparation of Soy Globulin Isolate

Defatted soybean meal was prepared from mixed varieties of soybeans by cracking, dehulling, pulverizing, and refluxing with hexane, followed by air-drying. The defatted meal was extracted with water (1:10 w/v ratio of meal-water) by stirring at a low speed (to prevent foaming) for 1 hr at 25°C. The extract was clarified by passing it through several layers of cheesecloth, then centrifuging at 2,000 × *g* for 15 min. The soy globulins were precipitated isoelectrically by acidifying the extract to pH 4.5 with 1*N* HCl. The protein suspension was allowed to stand for 1 hr to complete precipitation and was subsequently centrifuged at 2,000 × *g* for 30 min. The precipitate was washed with water and resuspended in the same solvent. The suspension was adjusted to pH 7.0 with 0.1*N* NaOH and freeze-dried.

Enzymatic Hydrolysis

Soy globulin dispersions (5% w/v) were prepared in pH 1.6 KCl (0.2*M*)-HCl (0.2*M*) buffer. Pepsin from hog stomach mucosa (Sigma Chemical Co., St. Louis, MO, Lot 75C-8220) was used for hydrolysis. The ratio of enzyme to substrate was 1:100 or 2:100. Hydrolysis was done at 25°C for various time intervals. Control samples were prepared and treated in the same manner, but without pepsin. Two-milliliter aliquots were removed from the incubation mixture during hydrolysis. The pH was adjusted to 7.0 using 2*N* NaOH, after which the aliquots were frozen and later freeze-dried.

Molecular-Weight Distribution Analysis

A Sephadex G-50 (Pharmacia, Uppsala, Sweden) column (2.5 × 40 cm), equilibrated with pH 7.6 phosphate buffer (0.1*M*) made 0.05*M* with respect to NaCl, was used. The flow rate was 3.5 ml/min, and 2.7-ml fractions were collected for ultraviolet absorbance measurements at 220 nm (Catsimpoolas and Kenney 1972) with a Beckman Model 25 recording spectrophotometer (Beckman Instruments, Irvine, CA).

Five marker proteins and peptides—pepsin (mol wt 35,000), cytochrome C (mol wt 12,400), insulin (mol wt 5,730), ribonuclease-S-peptide (mol wt 2,200), and bacitracin (mol wt 1,411) (Sigma)—were used to standardize the column. Blue dextran (Pharmacia) and K_2CrO_4 were used as void volume and total volume markers, respectively. The partition coefficient (K_{av}) of each marker was estimated from the equation

$$K_{av} = (V_e - V_o)/(V_n - V_o) \quad (1)$$

where V_o is the void volume, V_n the total volume, and V_e the elution volume of each marker. The K_{av} value for the marker proteins were: 0 for pepsin, 0.30 for cytochrome C, 0.36 for insulin, 0.48 for ribonuclease-S-peptide, and 0.79 for bacitracin. The marker proteins and peptides used in this study obeyed the empirical relationship (Hjerten 1970)

$$-\log K_{av} = R\ M^{2/3} + S \quad (2)$$

where M is the molecular weight of the marker, and R and S are constants obtained by linear regression analysis. In this work, $R = 0.9195 \times 10^{-3}$ and $S = 0.4233 \times 10^{-1}$.

Equations 1 and 2 are combined to obtain the molecular weight, M, of the protein

$$M = \left[\frac{[-\log(\frac{V_e - V_o}{V_n - V_o})] - S}{R}\right]^{3/2} \quad (3)$$

If V_i is the volume of the fraction collected during the i-th time interval, then the molecular weight of the protein in that fraction is M_i, where

$$M_i = \left[\frac{[-\log(\frac{V_i - V_o}{V_n - V_o})] - S}{R}\right]^{3/2} \quad (4)$$

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The protein concentration in any i-th fraction is proportional to its absorbance, A_i, at 220 nm. Therefore, the proportion of the total weight present in the i-th fraction is

$$n_i = \frac{A_i(\Delta V)}{\Sigma A_i(\Delta V)} \quad (5)$$

and

$$\overline{M_w} = \frac{\Sigma A_i M_i}{\Sigma A_i} \quad (6)$$

Combining equations 4, 5, and 6 allows calculation of the weight-average molecular weight (M_w) of an unknown peptide (Markley 1951).

$$\overline{M_w} = \frac{\sum A_i \left[\frac{[-\log(\frac{V_i - V_o}{V_n - V_o})] - S}{R}\right]^{3/2}}{\Sigma A_i} \quad (7)$$

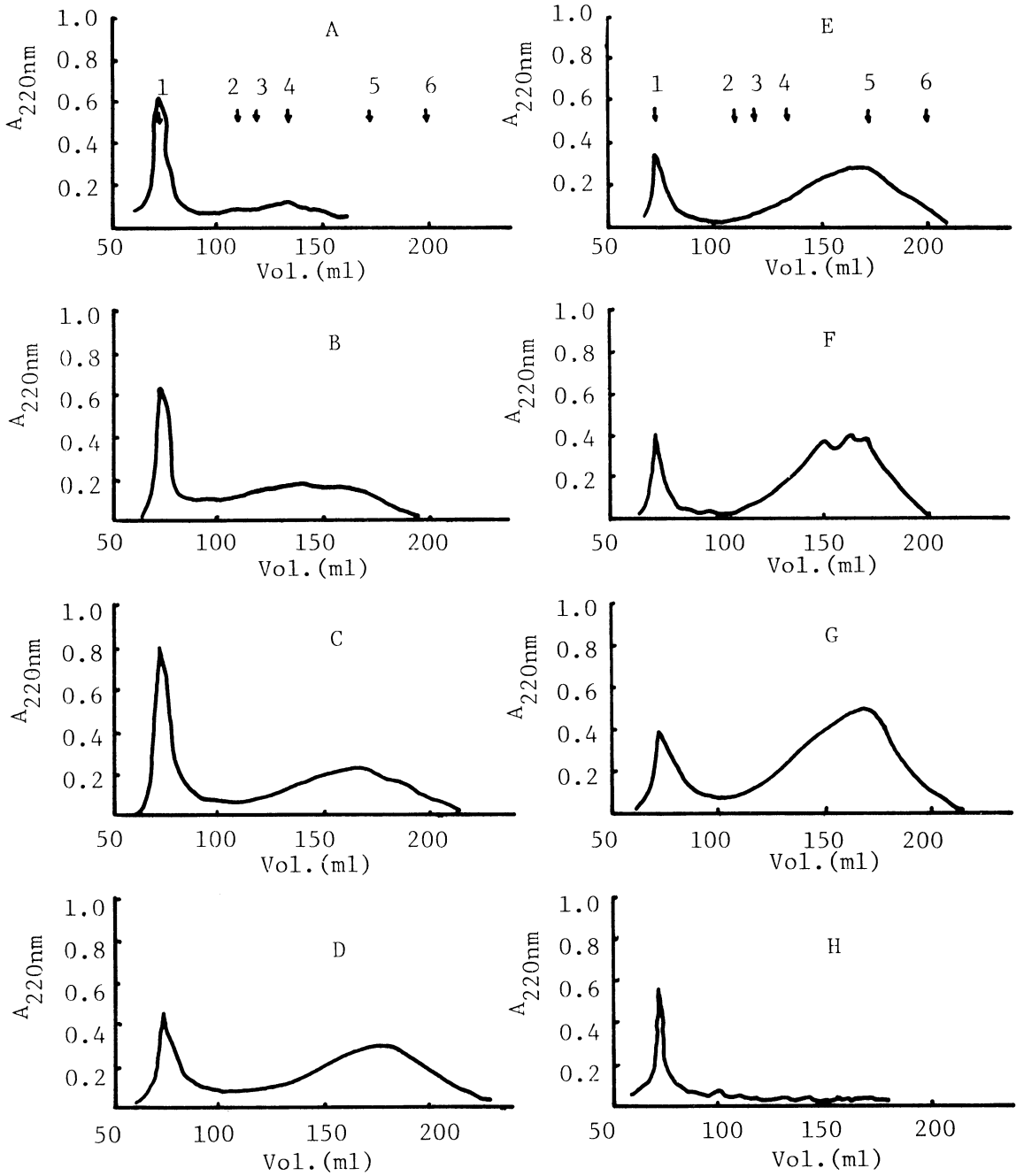

Fig. 1. Elution profiles of soy globulin (5% w/v) dispersion hydrolyzed with pepsin at pH 1.6, 25°C, and at 1:100 enzyme-to-substrate ratio (w/w). The column used is Sephadex G-50 (2.5 × 40 cm) eluted with 0.1 *M* phosphate buffer (pH 7.6) made in 0.05 *M* NaCl. The numbers denote the elution position of the following proteins or peptides: 1, pepsin (mol wt 35,000); 2, cytochrome C (mol wt 12,400); 3, insulin (mol wt 5,700); 4, ribonuclease-S-peptide (mol wt 2,200); 5, bacitracin (mol wt 1,411); and 6, oxytocin (mol wt 1,010). A = 0 min, B = 2 min, C = 20 min, D = 70 min, E = 100 min, F = 120 min, and G = 150 min of hydrolysis time. H = control group at 150 min.

For these experiments, 5 mg of the freeze-dried hydrolysate was dissolved in 2 ml of pH 7.6 phosphate buffer (0.1*M*), made 0.05*M* with respect to NaCl, and stirred at 5°C for 10 min. The soluble material was placed in the Sephadex column for analysis.

Determination of Degree of Hydrolysis

Fifty milligrams of the hydrolysate was dispersed in 5 ml of 5% trichloroacetic acid (TCA) for 20–30 min. The dispersions were separated into supernatant and precipitate with a suction filter. Supernatant (or precipitate) was then transferred to a micro-Kjeldahl digestion flask for nitrogen analysis (Munro and Fleck 1969).

The degree of hydrolysis was defined as the fraction of material soluble in 5% TCA over the soluble material plus the precipitate:

$$DH = \frac{(H)}{(H) + (P)} \tag{8}$$

where DH represents the degree of hydrolysis, (H) is the nitrogen content in the supernatant of the 5% TCA solution, and (P) is the nitrogen content in the precipitate of the 5% TCA solution.

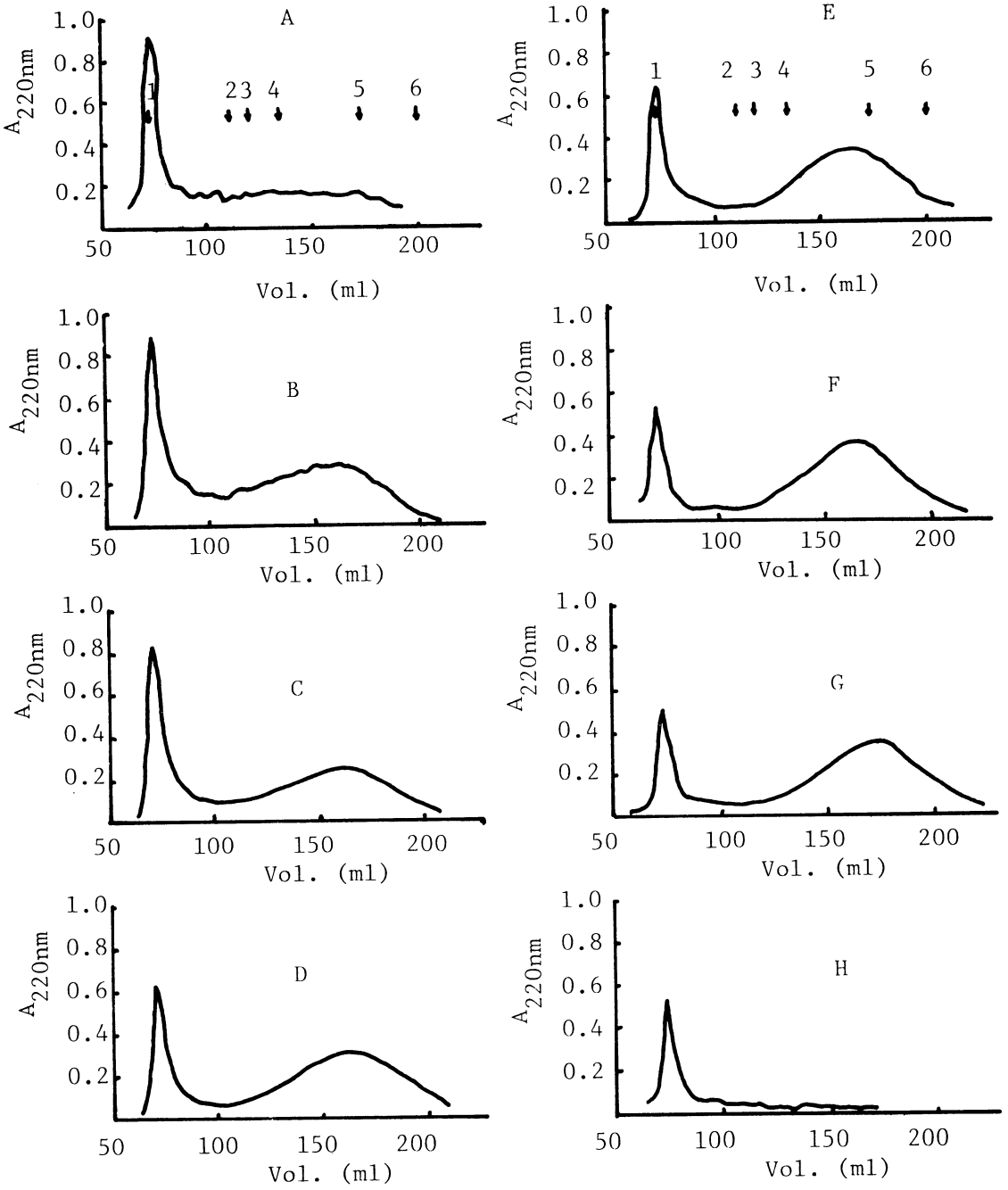

Fig. 2. Elution profiles of soy globulin (5% w/v) dispersion hydrolyzed with pepsin at pH 1.6, 25°C, and at 2:100 enzyme-to-substrate ratio (w/w). The column conditions are the same as in Fig. 1, as are the numbers for the elution position of the compounds. A = 0 min, B = 2 min, C = 10 min, D = 40 min, E = 70 min, F = 120 min, and G = 180 min of hydrolysis time. H = control group at 180 min.

RESULTS

Figures 1 and 2 show typical Sephadex elution profiles of soy globulin peptic hydrolysates. With increasing time of hydrolysis, a shift occurred in the distribution toward the lower molecular-weight peptides in the chromatogram. This distribution was continuous, as would be expected from a mixture of random-size peptides. The $\overline{M_w}$ of each distribution was computed as described above. The material in the void volume was not included in the calculation, as it represented the incompletely hydrolyzed protein core.

Table I shows the change in $\overline{M_w}$ as a function of hydrolysis time. The first few minutes of hydrolysis decreased the $\overline{M_w}$ rapidly. After that, early in the incubation period (ie, 2 to 10 min), the degradation of peptides was still fast. During prolonged hydrolysis (70 to 180 min), the degradation was slow. A graph of log $\overline{M_w}$ vs. the time of initial hydrolysis (20 to 70 min in 1% E/S ratio and 10 to 70 min in 2% E/S ratio) showed a linear relationship (Fig. 3) with correlation coefficients of 0.96 and 0.98, respectively, as estimated by linear regression analysis. The initial and prolonged hydrolyses followed different kinetics.

Calculation of the molecular weight of polypeptides was based on the second peak; the peak at the void volume was excluded

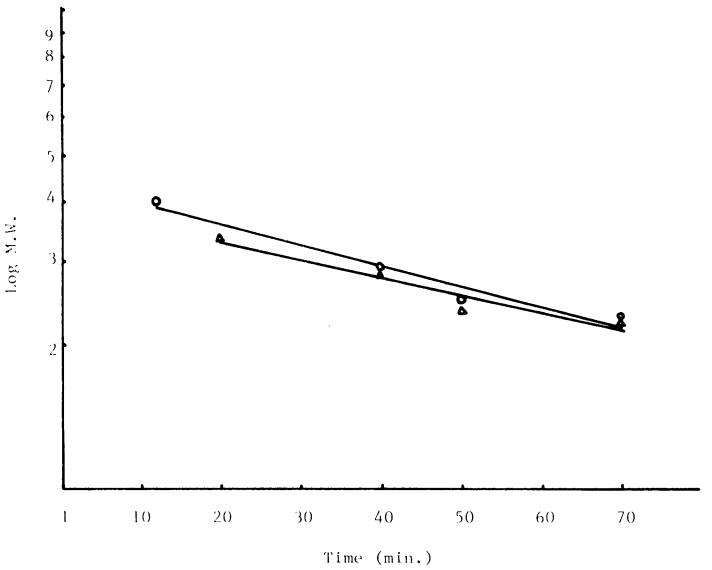

Fig. 3. Plot of log molecular weight vs. hydrolysis time. O , E/S = 2:100; △, E/S = 1:100.

TABLE I
Apparent Weight-Average Molecular Weight ($\overline{M_w}$) Values of Soy Globulin Peptides Produced by Pepsin as a Function of Time

E/S = 1:100		E/S = 2:100	
Time	$\overline{M_w}$	Time	$\overline{M_w}$
2	6,738	2	5,741
5	4,927	5	5,194
10	4,574	10	4,000
20	3,328	40	2,902
40	2,781	50	2,472
50	2,338	70	2,280
70	2,217	90	2,640
90	2,021	100	2,438
		120	2,104
		150	1,928
		180	1,603

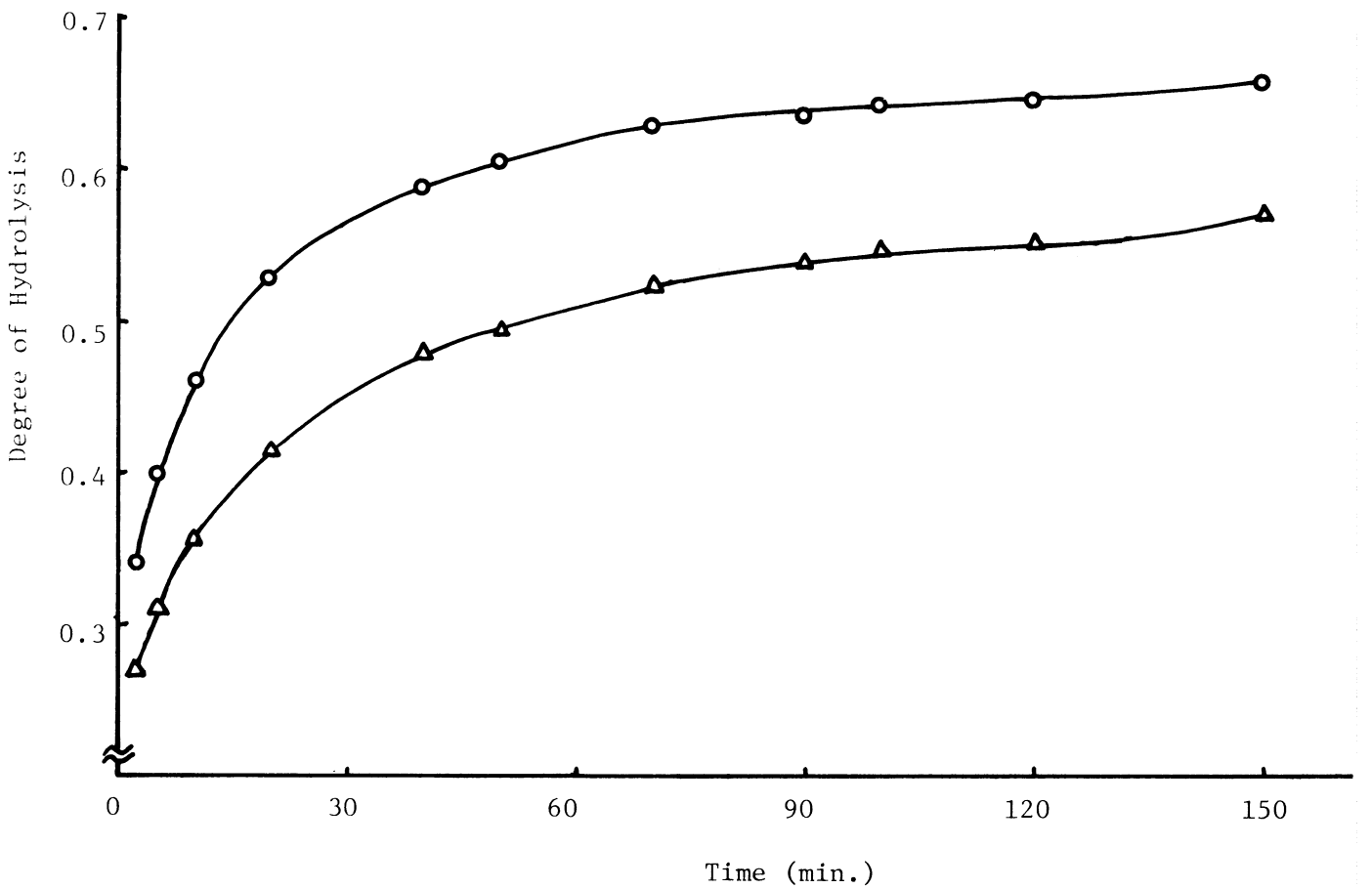

Fig. 4. The extent of peptic hydrolysis of soy globulin determined by total nitrogen. O , E/S = 2:100; △, E/S = 1:100.

because it probably represented material precipitable by 5% TCA. Thus, the second peak should be equivalent to only the fraction soluble in 5% TCA. Figure 4 shows the degree of hydrolysis of the 5% soybean protein dispersion hydrolyzed by pepsin at pH 1.6. After 70 min of hydrolysis, the degree of hydrolysis reached approximately 0.60 and 0.50 for enzyme-to-substrate ratios of 2 and 1%, respectively. The degree of hydrolysis initially increased rapidly, but the rate later decelerated, reflecting the slower rate of change in the $\overline{M}_w$.

DISCUSSION

Previous studies on glycinin (Lynch et al 1977a,b) have shown that, immediately after initiation of tryptic hydrolysis, low-molecular-weight peptide fragments are formed, and ultraviolet absorbance increases at 291–292 nm because of exposure to tryptophan groups. The native, acid-denatured glycinin and its basic subunits are hydrolyzed slowly over a period of several hours, whereas the disulfide bond-reduced acidic subunits, glycinin, and alkali-denatured glycinin are rapidly attacked by trypsin (Lynch et al 1977b). The acidic subunits of glycinin are digested with pepsin at a faster rate than are the basic subunits. The rapid hydrolysis of glycinin by pepsin at pH 2.0 is probably due to the acidic dissociation of the subunits with concurrent hydrolysis of the acidic over the basic units by both pepsin and trypsin, which is probably due to the greater hydrophobic character and thus compactness of the basic subunits (Lynch et al 1977b). In addition, kinetic analysis indicated that enzymatic hydrolysis of glycinin was biphasic, being composed of both a fast and a slow reaction. The fast reaction was completed within 20 min; the slow reaction lasted for 13 to 20 hr. Results also indicated that glycinin contains an inner core that partly retains its structural integrity even after lengthy hydrolysis (Lynch et al 1977a).

This study demonstrates that soy globulin hydrolysates can be characterized with respect to their weight-average molecular weight by Sephadex G-50 column chromatography. For pepsin hydrolysis at pH 1.6, the linear relationship shown in Fig. 3 can be used to obtain peptides of predictable average molecular size. The concentration of enzyme did not change the profiles of the $\overline{M}_w$ of the hydrolysates. The action of other enzymes may also exhibit a mathematically defined relation between $\overline{M}_w$ and hydrolysis time. Other variables—such as temperature, pH, and ionic strength—can be studied and analyzed on a similar basis. In addition, estimation of $\overline{M}_w$ values of peptides produced by proteolysis offers a means of comparing other physical properties (Pour-El 1976, Puski 1975) of interest to food scientists and food processing engineers.

To gain a more comprehensive understanding of the hydrolysis of soybean protein, it is necessary to develop a solvent system that can solubilize all the material without changing the biochemical structure and, at the same time, be compatible with gel filtration materials and detection methods. At present, however, no such solvent system exists for gel chromatography.

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