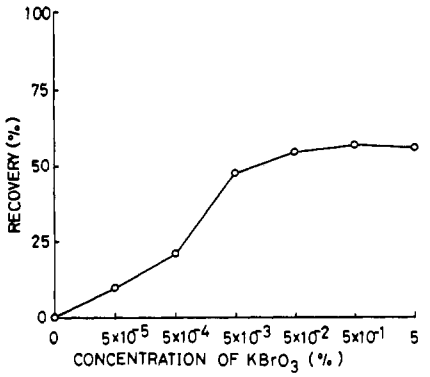

Figure 5. Effect of $KBrO_3$ concentration on the recovery of added hydrogen peroxide from larval fish. Hydrogen peroxide was added to boiled and half-dried larval fish at 1 ppm and was extracted with various concentrations of $KBrO_3$ solution.

Table III gives comparative data of hydrogen peroxide residues in foods and drinks determined by this method and the improved 4-AA method. In most samples except milk and milk products, the residue levels determined by both methods were in good agreement, but in milk and milk products the values of the improved 4-AA method were higher than those obtained by this method. We are now conducting experiments to determine the causes of their discrepancy.

This method requires only 20 min for one analysis of solid food and about 10 min for liquid food. The minimum detectable level is 0.1 ppm for the former and 0.01 ppm for the latter. Accordingly, as a routine analysis this is a time-saving method and sufficiently sensitive.

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Heterogeneity and Properties of Heat-Stable Ovalbumin from Stored Egg

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The changes in the properties of ovalbumin during storage were examined, especially with regard to the heat stability. Two fractions of heat-stable ovalbumin were separated from the stored egg. One was a minor new protein which was slightly acidic compared with the fresh ovalbumin. Another major ovalbumin could not be distinguished in properties, except for the stability to heat denaturation, from native ovalbumin, since the major ovalbumin from the stored egg showed the same behaviors in electrophoresis and chromatography as did the fresh ovalbumin. These results show that the transformation of ovalbumin to the heat-stable form cannot be explained by the change in the net surface charge.

The occurrence of the more stable form of ovalbumin (S-ovalbumin), first reported by Smith (1964), is one of the most interesting changes in the white, as well as the thinning, during the storage of eggs. No distinct difference in physical and chemical properties, however, was found by Smith and Back (1965, 1968b) between native ovalbumin and S-ovalbumin, except for the difference in the stability to heat or denaturing agents (Smith and Back, 1968a).

Recently, during the transformation of ovalbumin to *S*-ovalbumin, slight conformational changes were detected by a Raman difference spectroscopy (Kint and Tomimatsu, 1979) and changes in the surface charge were reported (Nakamura et al., 1980). However, the mechanism of the conversion is still a matter of speculation.

In the present study, we examined the correlation between the heat stability and other chemical or physical properties of ovalbumin from the fresh or stored eggs and found that two types of heat-stable ovalbumin, more acidic ovalbumin and ovalbumin with the same surface charge as the fresh ovalbumin, were formed during storage.

MATERIALS AND METHODS

Source and Storage of Eggs. Eggs from White Leghorn (Babcock) were supplied from the National Institute of Animal Industry (Chiba, Japan). Only the eggs with type AA ovalbumin of genetic variants (Lush, 1964) were used to avoid confusion in the interpretation of the results. Shell eggs were stored on a plastic tray at 30 ± 2 °C for 1, 2, 3, and 4 weeks. This temperature was chosen to accelerate the formation of heat-stable ovalbumin. The pHs of the white from fresh eggs and stored eggs were 8.52–8.80 and 9.35–9.62, respectively.

Fractionation of Ovalbumin. Eggs were broken carefully and egg white was separated. Egg white was homogenized with a Polytron (Kinematica, Switzerland) at scale 5 for 15 s at 5 °C, dialyzed against deionized water, and lyophilized. The dried sample (1500 mg) was dissolved in 0.05 M imidazole hydrochloride buffer (pH 6.20) containing sodium azide (200 mg/L) and centrifuged at 15000 rpm for 60 min at 15 °C. The supernatant was equilibrated with the same buffer for at least 24 h and fractionated by ion-exchange chromatography on DEAE-Sephacel (Pharmacia Fine Chemicals). The column was 21

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× 420 mm. Elution was performed initially with 100 mL of the equilibration buffer and subsequently by a linear gradient with 1000 mL of the buffer, increasing NaCl concentration from zero to 0.15 M at a flow rate of 30 mL/h. The fraction size was 10 mL/tube. The eluted fractions were dialyzed against deionized water and lyophilized. The ovalbumin fractions were rechromatographed for the purification by the same way as above except that the column size and the amount of elution buffer were half. The pooled fractions were exhaustively dialyzed against deionized water and lyophilized. Proteins were stored at −18 °C until use.

Heat Stability of Whole Egg White and Ovalbumin. The heat stability was represented as the solubility of protein at pH 4.7 after the heat treatment essentially according to Gibbs (1954). Protein was dissolved in distilled water, and the pH was adjusted to 7.00–7.10 with 0.1 N NaOH or HCl. One milliliter of the solution (1%), previously centrifuged to remove insoluble materials, was poured into a glass test tube (15 × 105 mm), covered with parafilm, heated in a water bath controlled previously at 73.5, 85.0, or 95.0 ± 0.1 °C, and kept for the desired time. Then, 10 mL of cold "stopping solution" (0.5 M NaCl in 0.1 M acetate buffer, pH 4.70) was added to the protein solution. After the solution was mixed and allowed to stand for 20 min in a ice water bath, the insoluble protein was centrifuged off. The concentration of the soluble protein was determined by the ultraviolet absorption at 280 nm in a 10-mm cell, correcting for scattering by subtracting the absorption at 320 nm. The corrected absorption was expressed as a percentage of absorption of the unheated protein solution treated with the same way.

Electrophoresis and Isoelectric Focusing. Polyacrylamide gel electrophoresis was performed essentially based on the method of Davis (1964) in a slab gel (135 × 145 mm, 2 mm thick) with a constant current of 20 mA/plate. After electrophoresis, protein was stained with Coomassie brilliant blue R 250 (CB). Isoelectric focusing was carried out on a Pharmacia Model FBH-3000 flat-bed electrophoretic apparatus by using polyacrylamide gel (115 × 230 mm, 1 mm thick) containing 2% Pharmalyte (pH range 4–6.5; Pharmacia Fine Chemicals) at a constant power of 10 W for 3 h. Electrode solutions were 0.04 M DL-glutamic acid (anode solution) and 0.2 M L-histidine (cathode solution). After focusing, the pH of the gel surface was determined directly at 5-mm intervals with a microelectrode (SE 1600GC, Fuji Kagakukeisoku). The gel was fixed in 10% trichloroeacetic acid solution containing 5% sulfosalicylic acid for 30 min, then washed with the mixed solution of methanol, acetic acid, and water (3:1:6) for 30 min, stained with CB for 3 h, and destained.

Other Analyses. Amino acids were analyzed after hydrolysis of proteins with 6 N HCl for 20 and 72 h at 110 °C in sealed evacuated tubes with a Hitachi 835 automatic amino acid analyzer. The values for threonine and serine obtained at the two times of hydrolysis were extrapolated to zero time. The phosphorus content was determined by the method of Rouser et al. (1966). Double immunodiffusion was carried out according to Ouchterlony and Nilsson (1978). Rabbit antiserum was raised against whole egg white protein. Sedimentation analyses were performed at 25 °C with a Hitachi Model UCA-1A at 60 000 rpm. Protein samples were dissolved in 0.05 M imidazole hydrochloride buffer (pH 6.20) containing 0.1 M NaCl at a concentration of 0.6%.

RESULTS

Electrophoretic Profiles and Heat Stability of White from Stored Eggs. The changes in electrophoretic profiles of the white during storage are shown in Figure 1. In the ovalbumin fraction, the fast migrating band (d in Figure 1) preceding ovalbumin A_1 (c in Figure 1) appeared during storage of 2 weeks, and the intensity of the band increased with the increase of the storage time. Also, the band of conalbumin (a in Figure 1) and ovoglobulin fraction (b in Figure 1) became blurred after storage.

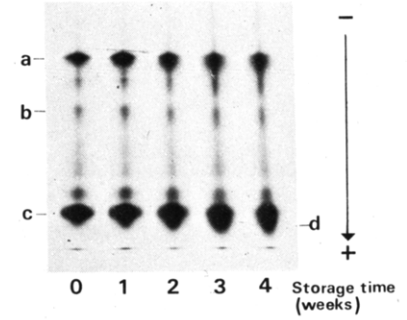

Figure 1. Electrophoretic patterns of white from the fresh and the stored (30 °C) eggs. (a) Conalbumin; (b) ovoglobulin fraction; (c) ovalbumin fraction; (d) newly appeared protein during storage.

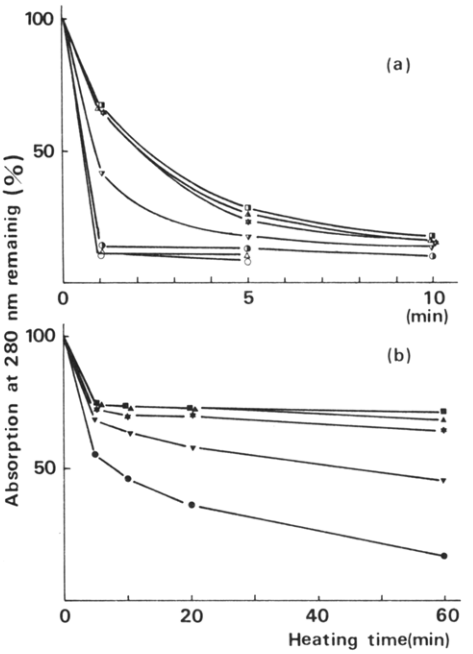

Figure 2. Solubility changes of the fresh and the stored egg white with time of heat treatment. (a) Heated at 95 °C (open symbols) or 85.0 °C (half-closed symbols); (b) heated at 73.5 °C (closed symbols). (○, ◑, and ●) Fresh egg white; (▽ and ▼) white from eggs stored for a week; (★ and ☆) white from eggs stored for 2 weeks; (△, ▲, and ▲) white from eggs stored for 3 weeks; (□ and ■) white from eggs stored for 4 weeks.

Practically no difference in heat stability was found between the fresh white and the white from the stored eggs on heating at 95 or 85 °C for 10 min (Figure 2a), but when the whites were heated at 85 °C for 1 min or 73.5 °C for 20 min and 60 min (Figure 2b), marked differences were detected between the fresh white and the white stored for 2 weeks or more. The white stored for 1 week showed intermediate stability. A 25–30% loss of ultraviolet absorption was shown on heating even for 5 min at 73.5 °C (Figure 2b). It was probably due to the heat denaturation of conalbumin and the other proteins, whose heat stability was reduced by the interaction with conalbumin (Johnson and Zabik, 1981).

Isolation of Ovalbumin from Stored Eggs. The representative elution patterns of the fresh and 3-week-

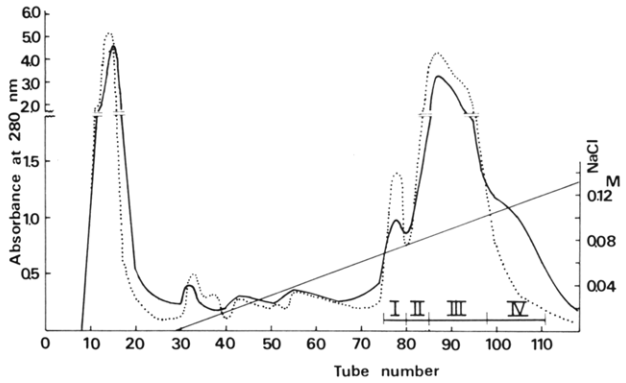

Figure 3. Fractionation of egg white proteins on DEAE-Sephacel. (···) Fresh egg white; (—) egg white from eggs stored for 3 weeks.

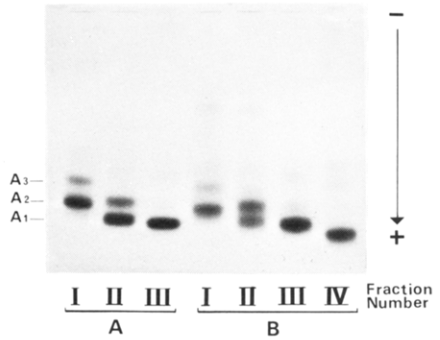

Figure 4. Electrophoretic profiles of the fractions separeated on DEAE-Sephacel. (A) Fresh eggs; (B) eggs stored for 3 weeks.

stored egg white on DEAE-Sephacel are shown in Figure 3. The shoulder (fraction IV), eluted at a concentration of 0.1 M NaCl, was noted in the stored white, though other minor differences were found between the fresh white and the stored white.

The electrophoretic profiles of the fractions underlined in Figure 3 (I, II, III, and IV) are shown in Figure 4. Fractions I, II, and III from the fresh contained ovalbumin A_3 plus A_2, ovalbumin A_2 plus A_1, and ovalbumin A_1, respectively. No difference was found in the mobilities of the corresponding fractions (I, II, and III) between the fresh white and the stored white. Fraction IV, characteristic of the stored white, was consistent in mobility with the fast migrating component which appeared in the stored white (Figure 1). Ovalbumin A_1 from the fresh white (0W-OA_1) and the fractions III and IV from the stored white were purified by rechromatography on DEAE-Sephacel and used in the subsequent experiments.

Chemical and Physical Properties of Ovalbumins from Stored Eggs. 0W-OA_1 and the fractions III and IV from the white stored for 3 weeks had similar amino acid composition and phosphorus content as shown in Table I. Moreover, the antiserum raised against whole egg white protein formed a single precipitine line, as shown in Figure 5, with 0W-OA_1 and fractions III and IV in double immunodiffusion. Thus, fractions III and IV were similar ovalbumins and therefore designated 3-weeks-stored ovalbumin A_1 (3W-OA_1) and 3-weeks-stored α-ovalbumin A_1 (3W-α-OA_1), respectively.

The sedimentation coefficients (s_{obsd}) of 0W-OA_1, 3W-OA_1, and 3W-α-OA_1 were 3.55, 3.53, and 3.60 S, respectively. The patterns of the polyacrylamide gel isoelectric focusing of 0W-OA_1, 3W-OA_1, and 3W-α-OA_1 are shown in Figure 6. The isoelectric point of 0W-OA_1 and 3W-OA_1 were 4.62 and that of 3W-α-OA_1 ranged from 4.52 to 4.60. The minor component (pI = 4.70) found in 0W-OA_1 and

Table I. Amino Acid Composition and Phosphorus Content of the Fractions Separated on DEAE-Sephacel

	fraction III from fresh eggs[a] (0W-OA_1)	fraction III from eggs stored for 3 weeks[a] (3W-OA_1)	fraction IV from eggs stored for 3 weeks[a] (3W-α-OA_1)
		mol %	
Asp	8.81	8.49	8.01
Thr	3.60	4.00	4.06
Ser	8.81	8.92	8.77
Glu	13.32	14.03	13.56
Pro	3.37	3.46	3.30
Gly	5.21	5.04	4.95
Ala	9.28	9.11	9.27
$^1/_2$-Cys	1.41	1.54	1.76
Val	7.25	7.46	7.85
Met	4.62	4.31	4.41
Ile	6.03	6.27	6.32
Leu	8.62	8.54	8.62
Tyr	2.59	2.65	2.61
Phe	5.60	5.31	5.36
Lys	5.48	5.34	5.44
His	1.92	1.77	1.84
Arg	4.07	4.04	3.87
Trp	ND[b]	ND[b]	ND[b]
		residues/mol	
phosphorus	1.98	1.89	1.87

[a] These protein samples were obtained by rechromatography of the respective fractions (Figure 3) on DEAE-Sephacel. [b] Not determined.

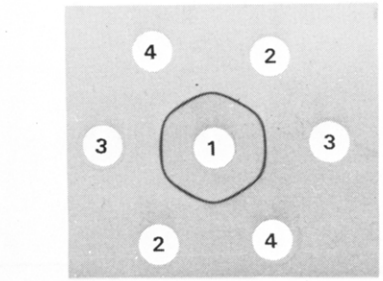

Figure 5. Double immunodiffusion analyses of the fractions separated on DEAE-Sephacel. (1) Anti whole egg white protein antiserum; (2) fraction III from fresh egg (0W-OA_1); (3) fraction III from eggs stored for 3 weeks (3W-OA_1); (4) fraction IV from eggs stored for 3 weeks (3W-α-OA_1).

in 3W-OA_1 was ovalbumin A_2.

Heat Stability of Ovalbumins. The heat stability of the three ovalbumins (0W-OA_1, 3W-OA_1, and 3W-α-OA_1) is compared in Figure 7a,b. When heated at 95 °C, 0W-OA_1 and 3W-OA_1 were rapidly denatured, but at 85 °C, the stored ovalbumins were only partly denatured (Figure 7a). When the ovalbumins were heated at lower temperature (73.5 °C), the marked differences in heat stability were shown between the fresh and the stored ovalbumins (Figure 7b). Almost all of 3W-OA_1 and 3W-α-OA_1 remained soluble in the "stopping solution" even after 1 h of heat treatment, while the solubility of 0W-OA_1 decreased proportionally to the heating time.

DISCUSSION

Notable changes were observed in the electrophoretic profiles of the ovalbumin fractions in the egg white stored for 2 weeks or more (Figure 1). Fast migrating components (Figure 1d) preceding ovalbumin A_1 (Figure 1c) appeared during storage, though some changes were also observed in conalbumin and ovoglobulin fractions (Figure 1a,b). The intensity of the new bands increased with increase of the storage time. The egg white proteins stored for 2 weeks

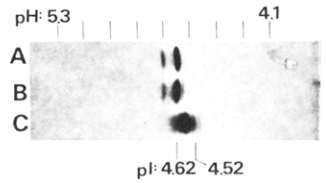

Figure 6. Isoelectric focusing of ovalbumins. (A) 0W-OA_1; (B) 3W-OA_1; (C) 3W-α-OA_1.

or more showed notable heat stability (Figure 2b). These results were suggestive of the correlation between the increase in heat stability and the occurrence of the new protein bands which appeared in the electrophoretic patterns. Then, we tried to isolate ovalbumin A_1 from the fresh eggs (0W-OA_1) for the control and two proteins from the 3-weeks-stored eggs, a ovalbumin fraction with the same electrophoretic mobility as 0W-OA_1 and a new protein migrating faster than 0W-OA_1.

For the fractionation of ovalbumins with varied degree of phospohorylation from whole egg white, one-step procedures, without prior salting out with sodium sulfate (Kekwick and Cannan, 1936), were reported (Rhodes et al., 1958; Mandeles, 1960; Herbert, 1967), but they are rather complexed and incomplete for the purification. So, we devised the ion-exchange chromatography with a linear NaCl gradient elution system (Figure 3) and were able to obtain the three proteins described above (fraction III from the fresh and the stored white and fraction IV from the stored white). As the two proteins isolated from the stored white were consistent chemically and immunologically with 0W-OA_1 (Table I and Figure 5), they were designated 3W-OA_1 for the major ovalbumin with identical chromatographic behavior and electrophoretic mobility with 0W-OA_1 and 3W-α-OA_1 for the minor new protein with the fast mobility in electrophoresis. Almost all of ovalbumin A_1 in the stored egg was recovered in these two proteins, 3W-OA_1 and 3W-α-OA_1. No significant difference was found in sedimentation coefficient of the three kinds of ovalbumins. 3W-OA_1 had an identical isoelectric point (pI = 4.62) with 0W-OA_1. 3W-α-OA_1 was more acidic and heterogeneous (pI = 4.52–4.60) (Figure 6) as assumed from the chromatographic and electrophoretic behavior (Figures 3 and 4).

3W-OA_1 and 3W-α-OA_1 were unambiguously stable to heat compared with 0W-OA_1 (Figure 7), though these stored proteins might be contaminated with a small amount of ovalbumin susceptible to heat denaturation or the intermediates to S-ovalbumin (Donovan and Mapes, 1976).

Evans et al. (1958) and Koehler (1974) reported that the electrophoretic mobilities of egg white proteins increased during storage, but they did not refer to heat stability. Meehan et al. (1962) related the heat stability of stored white to the broadening of the peak in the ion-exchange chromatographic profile of ovalbumin, but no further investigation was made.

During storage of egg for 3 weeks, ovalbumin A_1 was, thus, transformed into at least two kinds of S-ovalbumin, 3W-OA_1 and 3W-α-OA_1. 3W-OA_1 was the major and had an identical isoelectric point with that of 0W-OA_1, while 3W-α-OA_1 was more acidic and heterogeneous. The minor ovalbumin fractions, ovalbumins A_2 and A_3, were not investigated but they might change similarly during storage. The mechanism of the formation of the acidic ovalbumin, 3W-α-OA_1, during storage was not examined in the present work, though the deamidation, the masking of amino groups, or the exposure of carboxyl groups might be related.

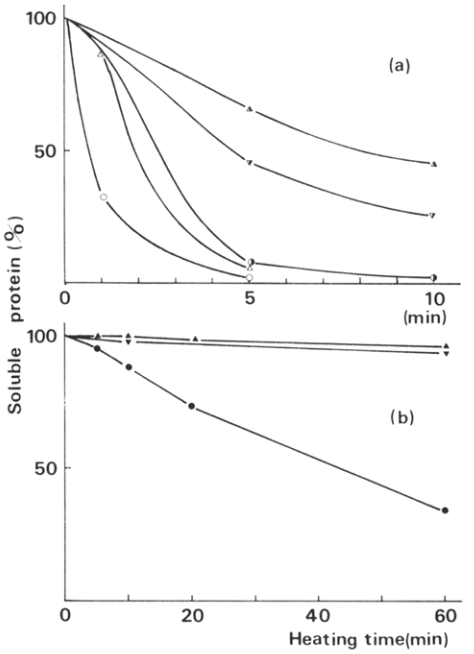

Figure 7. Solubility changes of ovalbumin with time of heat treatment. (a) Ovalbumins heated at 95 °C (open symbols) or 85.0 °C (half-closed symbols); (b) ovalbumins heated at 73.5 °C (closed symbols). (○, ◑, and ●) 0W-OA_1; (△, ▲, and ▲) 3W-OA_1; (▽ and ▼) 3W-α-OA_1.

Smith and Back (1965, 1968a,b) failed to show any difference in the chemical and physicochemical properties between ovalbumin and heat-stable ovalbumin (S-ovalbumin) except for the differences in the stability to heat or denaturing agents. Kint and Tomimatsu (1979) reported that slight (3–4%) conformational changes, α helix to antiparallel β sheet, were involved in the conversion of ovalbumin to S-ovalbumin. Nakamura et al. (1980) observed the broadening of the peak or band in ion-exchange chromatography or isoelectric focusing of S-ovalbumin prepared from ovalbumin by heating at 55 °C for 16 h at pH 9.9 and the minor differences in the titration curves between S-ovalbumin and ovalbumin. They concluded that the changes in the surface charge occurred during the transformation of ovalbumin to S-ovalbumin. In our present study, however, it is shown that the conversion of ovalbumin to the heat-stable form cannot be explained by the changes in the net surface charge since 0W-OA_1 and 3W-OA_1 showed the same behaviors in electrophoresis and chromatography though they were entirely different in stability to heat.

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Determination of Isoflavones in Soybean Flours, Protein Concentrates, and Isolates

Arthur C. Eldridge

The individual and total isoflavone content in commercial soybean protein products has been determined by high-performance liquid chromatography. Dehulled, defatted soybean flours contain the following mean isoflavone content (mg/100 g): daidzin, 61.7; glycitein 7-β-glucoside, 12.9; genistin, 119.8; daidzein, 32.8; genistein, 26.7. The same isoflavones were found in soybean protein concentrates and soybean protein isolates but in decreased amounts.

Soybeans contain isoflavones (Naim et al., 1974) that have several known activities, including estrogenic (Drane et al., 1980; Kitts et al., 1980), fungitoxic (Wyman and VanEtten, 1978), and antioxidant (Pratt and Birac, 1979) properties. Because of the ever increasing use of soybean protein products in foods and feeds, it is necessary to know the concentration of these biologically active compounds in various commercial products. Only one report in the literature (Naim et al., 1974) gives any quantitative data on the concentration of isoflavones in soybeans. Therefore, this study has been conducted to determine the amount of these compounds in soybean flours, protein concentrates, and isolates.

MATERIALS AND METHODS

Samples. A dehulled, defatted soybean flour was prepared in the laboratory (Eldridge et al., 1971) from Amsoy soybeans that were grown in 1978. In addition, one sample of commercial soybean meal and eight texturized soybean flours were obtained from various manufacturers. Five commercial samples of soybean protein concentrates (products containing a minimum of 70% protein) were obtained from four manufacturers. Three processors each use a different procedure for the preparation of their concentrates (Circle and Smith, 1972). Five soybean protein isolates (products containing a minimum of 90% protein) were procured from one manufacturer.

Trade names and sources of samples are given in Table I. All samples were ground to pass a 60–80-mesh screen.

Preparation of Extracts. Ground defatted soybean flour was extracted with several solvents to determine the most suitable solvent for dissolving the soybean isoflavones. Solvents investigated were 50%, 80%, and absolute ethanol, 50%, 80%, and absolute methanol, ethyl acetate, and acetonitrile. Refluxing with 80% methanol gave the most reproducible results and maximum extraction.

Table I. Identity of Samples Used in the Study

samples	trade name or description	source[a]
flours		
A	hexane, defatted Amsoy variety, 1978 crop	1
B	Nutrisoy 7B	2
C	unflavored TVP	2
D	Textratein	3
E	Centex 300	4
F	Centex 300 SL	4
G	Centex 400	4
H	Centex 400 SL	4
I	Mira Tex	5
J	Promote III, SL	6
concentrates		
K	Response	4
L	Food protein concentrate	7
M	Pro Con 2000	5
N	Promosoy 100	4
O	GL-301	6
isolates		
P	Edi Pro N	8
Q	Edi Pro A	8
R	Supro 610	8
S	Supro 620	8
T	Supro 710	8

[a] 1, Northern Regional Research Center; 2, Archer-Daniels-Midland Co.; 3, Cargill, Inc.; 4, Central Soya Co.; 5, A. E. Staley Manufacturing Co.; 6, Griffith Laboratories, Inc.; 7, Swift and Co.; 8, Ralston Purina Co.

In the analysis of soybean products, *n*-butyrophenone, which served as an internal standard, was dissolved in 80% aqueous methanol, and an accurate volume was added to the sample. A 1-g sample with 25 mL of 80% aqueous methanol containing the internal standard was heated (boiling) on a steam bath for 4 h, cooled, and filtered through a Type AP prefilter followed by a Type HA, 0.45-μM filter (Millipore Corp., Bedford, MA).

Chromatography. The previously published chromatographic procedure (Eldridge, 1982) was followed, using a linear methanol gradient from 25 to 50% in 20 min followed by an isocratic hold period of at least 30 min. Response factors for the individual isolated glucosides and

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