Factors Affecting Protein Digestibility in Soybean Foods

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Cereal Chem. 72(4):401-405

ABSTRACT

Factors affecting protein digestibility in soybean foods were studied. There was no significant correlation between protein digestibility of the soybean foods examined and their levels of inhibitory activity against trypsin and α-chymotrypsin. On the other hand, a variation in protein digestibility among soybean foods examined was noted: the protein of *yuba* (soy protein-lipid film) was more digestible by protease, but the protein of *kinako* (roasted soy meal) was less digestible. Kinetic analysis revealed a difference in susceptibility of proteins to proteolytic action between *yuba* and *kinako*. The present study concludes that the chemical form of proteins of soybean foods may be an important factor in their protein digestibility.

Soybean (*Glycine max* (L.) Merrill) is an important source of dietary protein around the world, especially in the Far Eastern countries. In recent years, Oriental soybean foods have attracted a lot of attention in the Western countries (Wang 1984, Snyder and Kwon 1987). Soybean is barely accepted as a fresh vegetable, so it is usually subjected to some form of processing before consumption. There are a variety of soybean foods prepared by various traditional processing methods around the world.

The Standard Tables of Food Composition in Japan (RCSTAJ 1982, JSNFS 1984) show a remarkable variation in protein digestibility in humans for various traditional soybean foods: 92% for *ni-mame* (boiled whole soybean); 100% for *yuba* (protein-lipid film); 78% for *kinako* (roasted soybean meal); 93% for *kori-tofu* (freeze-dried soy curd); 91% for *abura-age* (deep-fried soy curd); and 90% for *natto* (fermented whole soybean). Note that the protein digestibility of *yuba* is high, whereas that of *kinako* is low, although the reason for the variation in protein digestibility in various soybean foods is largely obscure.

Soybean contains a number of constituents that adversely affect the utilization of the protein (Rackis 1981, Liener 1994). Among the many biologically active factors in soybean, protease inhibitors have been most extensively investigated. However, it remains uncertain to what extent the protease inhibitors might be responsible for the variation observed in protein digestibility of soybean foods in human subjects (RCSTAJ 1982, JSNFS 1984). Furthermore, it is largely unclear whether some traditional processing methods for soybean products such as *kinako* may lead to reduced susceptibility of the soybean protein to proteolytic action. Now, because a growing interest in soybean and its beneficial effect on human health has become globally evident (Erdman and Fordyce 1989), analysis of factors affecting the protein digestibility of soybean foods is the subject of intense investigation. The present study was undertaken to reveal factors affecting protein digestibility in soybean foods.

MATERIALS AND METHODS

Materials

Soybean (*Glycine max* (L.) Merrill var. Tsurunoko) seed was obtained from Takii Co. (Kyoto, Japan) and used for analysis. Boiled whole soybean, *ni-mame* in Japanese, was prepared under essentially the same conditions as described by RCSTAJ (1982): an aliquot of the soybean seed was immersed overnight at room temperature and then cooked in boiling water for about 1 hr, then lyophilized. Six different kinds of fresh soybean foods, commercial products in Japan, were selected for this investigation: *yuba,* the dried soy protein-lipid film that rises to the surface on heating of soy milk; *kinako,* roasted soybean meal; *kori-tofu,* freeze-dried soy curd; *abura-age,* deep-fried soy curd; *natto,* whole soybean fermented by *Bacillus natto;* and *okara,* the edible residue left after squeezing soy milk during preparation of soybean curd. Food samples were lyophilized, ground with an electric mill, and then stored at −35°C before analysis. The protein content (% dwb, N × 5.71) (FAO 1947) of soybean samples examined was determined by the micro-Kjeldahl method (AOAC 1984): 35.4% soybean seed; 58.7% *yuba*; 34.3% *kinako*; 44.9% *kori-tofu*; 32.0% *abura-age*; 42.0% *natto*; and 26.1% *okara*.

Inhibitory Activity Against Trypsin and α-Chymotrypsin

The activity of trypsin (EC 3.4.21.4, Type I from bovine pancreas, 10,900 units/mg of solid, Sigma Chemical Co., St. Louis, MO) with benzoyl-D,L-arginine *p*-nitroanilide (BApNA) (Nacalai Tesque, Inc., Kyoto, Japan) as the substrate was assayed by the method of Erlanger et al (1961). Trypsin inhibitory activity was determined in quadruplicate according to the method of Kakade et al (1974) with slight modification. Ground soybean samples were extracted with eightfold (v/w) volumes of 0.2*M* NaCl at room temperature for 2 hr by rotary shaking, followed by centrifugation at 15,000 × *g* for 15 min.

An aliquot of each supernatant obtained from soybean samples was assayed for trypsin inhibitory activity. An appropriate volume of the extracts from soy samples (up to 0.5 ml), which caused 40–60% enzyme inhibition, was pipetted into test tubes and adjusted to 0.5 ml with 0.2*M* NaCl. The inhibitor solution to be examined was preincubated with 0.5 ml of trypsin solution (25 μg/0.5 ml) for 10 min at 37°C; its remaining enzyme activity with 4.0 μmol of BApNA was then assayed at 37°C for 15 min in 0.2*M* Tris-HCl buffer (pH 8.2) in a total volume of 4.0 ml. The activity of α-chymotrypsin (EC 3.4.21.1, Type II from bovine pancreas, 52 units/mg of solid, Sigma) was assayed according to Bundy (1962) with benzoyl-L-tyrosine *p*-nitroanilide (BTpNA) (Nacalai Tesque) as the substrate.

α-Chymotrypsin inhibitory activity was determined in quadruplicate by essentially the same procedure as described for trypsin inhibition. The inhibitor solution to be examined was preincubated with 0.5 ml of α-chymotrypsin solution (50 μg/0.5 ml) for 10 min at 37°C; its remaining enzyme activity with 4.0 μmol of BTpNA was then assayed at 37°C, for 15 min in 0.2*M* Tris-HCl buffer (pH 7.6) in a total volume of 4.0 ml. One unit of enzyme activity was defined as the amount of enzyme that released *p*-nitroaniline equivalent to an absorbance value of 1.0, according to the definition reported by Prabhu et al (1984). One unit of inhibitory activity is the amount of inhibitor that suppressed one unit of enzyme activity (Prabhu et al 1984).

Two different types of trypsin inhibitor preparations, i.e., Kunitz (from soybean, Sigma) and crude Bowman-Birk, were subjected to pepsin digestion with an enzyme-to-protein ratio of 1:10 in 0.1*M* HCl-KCl buffer solution (pH 2.0) for 3 hr at 37°C. Acetone-insoluble trypsin inhibitor fraction was isolated from

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soybean seed according to the procedure of Birk (1961) and was used as crude Bowman-Birk inhibitor preparation. After incubation with pepsin, remaining trypsin inhibitory activity in the preparations was assayed.

In Vitro Proteolytic Digestion of Food Samples

In vitro proteolytic digestion of food samples was performed with pepsin alone or with pepsin plus pancreatin. Pepsin (EC 3.4.23.1., powder purified by crystallization from porcine stomach mucosa, 3,000 units/mg of solid, Sigma) digestion was performed in triplicate by incubating 0.5 g each of lyophilized and ground samples with pepsin in 0.05*M* HCl-KCl buffer (pH 1.5) at 37°C with an enzyme-to-protein ratio of 1:5 for appropriate intervals. After digestion, trichloacetic acid (TCA) was added to each reaction mixture at a final concentration of 3% and the mixture was allowed to stand for 30 min. After the suspensions were centrifuged at 3,000 × *g* at 10 min, the supernatants were assayed for released peptide using both the colorimetric procedure with phenol reagent (Lowry et al 1951) and the colorimetric procedure with 2,4,6,-trinitrobenzenesulfonate (Habeeb 1966).

Pepsin plus pancreatin digestion was performed in six replicate determinations by the procedure described previously (Ikeda et al 1986, 1991). One gram each of the ground samples was first incubated with α-amylase (Type XI-A, Sigma) at pH 6.8 at 37°C for 30 min. After incubation, the digestion mixtures were adjusted to pH 1.0 with 2*M* HCl and then incubated with pepsin for 3 hr at 37°C. After incubation, the digestion mixtures were adjusted to pH 8.0 with 2*M* Tris and incubated for an additional 20 hr with pancreatin (from porcine pancreas, Sigma). The enzyme-to-protein ratio, except for 500 IU of α-amylase per gram of food, was 1:10. Sodium azide was added to the pancreatin digestion mixture to a final concentration of 0.025% to prevent growth of microorganisms. After digestion, a 4-ml aliquot of the soluble digesta was added to a test tube containing 1 ml each of 10% sodium tungstate and 0.67*N* sulfuric acid (Scheffner 1967, Kam and Shipe 1984). Tubes were allowed to stand for 10 min, then centrifuged at 3,000 × *g* for 15 min. The supernatant obtained was assayed for peptide with 2,4,6,-trinitrobenzenesulfonate (Habeeb 1966). Protein digestibility (%) by pepsin plus pancreatin was calculated as: % Protein digestibility/pepsin plus pancreatin = content of free peptide released upon the digestion of 1 g of food/the content of the total protein of 1 g of food before digestion × 100% (Ikeda et al 1986).

Electrophoresis

Proteins of soybean samples were extracted with 0.1*M* Tris-HCl buffer (pH 8.0) for 1 hr at 4°C with stirring, followed by centrifugation at 15,000 × *g* for 30 min. The result of extraction with the same flour-to-buffer ratio for all the samples was that the color of the protein bands from some samples, such as *kinako* and *kori-tofu,* became very pale when compared with samples such as raw soybean meal; therefore, they could not be compared with each other. In addition, the maximum volume of sample solution that could be applied to electrophoresis was limited. Therefore, a flour-to-buffer ratio of extraction was selected based on each concentration of soluble protein of soybean samples: 1:50 for raw soybean meal; 1:20 for *ni-mame*; 1:12.5 for *yuba*; 1:7 for *abura-age*; 1:6.25 for *kori-tofu*; and 1:5 for *kinako*. After centrifugation, a 5-μl aliquot of each supernatant was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5% acrylamide according to the procedure of Laemmli (1970). Protein was stained with Coomassie Brilliant Blue R-250 and then destained with 7% acetic acid solution. Bovine serum albumin, trypsinogen, β-lactoglobulin, and lysozyme were used as molecular weight marker proteins.

Other Analyses

Protein concentration was assayed by the method of Lowry et al (1951). Free sulfhydryl group was determined by the procedure of Ellman (1959). Data were subjected to statistical analysis of variance, and the significance of difference was determined by the *t*-test.

RESULTS AND DISCUSSION

Inhibitory Activities Against Trysin and α-Chymotrypsin of Soybean Foods

Table I shows inhibitory activities against trypsin and α-chymotrypsin of various soybean foods. There was a variation in trypsin inhibitory activity among soy foods examined. There was also a variation in α-chymotrypsin inhibitory activity among soy foods examined. A high correlation between the trypsin inhibitory activity and α-chymotrypsin inhibitory activity in the soy foods examined was observed (correlation coefficient = 0.998). Raw soybean meal exhibited the highest inhibitory activity against the two enzymes. *Yuba* and *kori-tofu* had a relatively higher level of inhibitory activity against the two enzymes when compared with other soybean foods, whereas *kinako* had less inhibitory activity. The Standard Tables of Food Composition in Japan (RCSTAJ 1982, JSNFS 1984) show a remarkable variation in protein digestibility in humans for various soybean foods. The protein digestibility of *ni-mame* is 92%; *yuba* is 100%; *kinako* is 78%; *kori-tofu* is 93%; *abura-age* is 91%; and *natto* is 90%. There was no significant ($P > 0.05$) correlation of protease inhibitory activities of various soybean foods (Table I) to in vivo protein digestibility in humans (RCSTAJ 1982, JSNFS 1984). In fact, the nutritional effects of protease inhibitors in humans is the subject of much controversy (Liener 1986, 1994). It remains particularly uncertain whether or not soybean protease inhibitors could disturb normal human pancreatic function, i.e, cholecystokinin release (Calam et al 1987, Holm et al 1988). In addition, it is still unclear whether or not there is a clear relationship between protein quality of plant foods and their protease inhibitor level (Liener 1976). Kakade et al (1973) reported that soybean extract, from which the trypsin inhibitor had been removed by affinity chromatography, was still resistant to proteolytic attack. Thus, it is noteworthy that there was substantially no relationship between protease inhibitor levels of various soybean foods (Table I) and in vivo protein digestibility in humans (RCSTAJ 1982, JSNFS 1984). On the other hand, protease inhibitory activity of soybean was found to be, at least in part, inactivated through pepsin digestion: ~80% of the original trypsin inhibitory activity of the Kunitz inhibitor preparation disappeared during pepsin digestion, whereas ~77% of the original trypsin inhibitory activity of isolated Bowman-Birk inhibitor preparation persisted even after incubation with pepsin at pH 2. Obara and Watanabe (1971) have reported similar partial inactivation of soybean trypsin inhibitors under acidic conditions at 37°C. Krogdahl and Holm (1981) have reported that Kunitz protease inhibitor purified from soybean is rapidly inactivated by incubation with human gastric juices, whereas purified lima bean inhibitor is resistant to peptic digestion.

That the protease inhibitor may have little effect on the protein digestibility of soybean foods is suggested by three points: 1) a very low level of protease inhibitory activity (Table I), 2) no relationship to in vivo protein digestibility in humans, and 3) possible partial inactivation in stomach.

TABLE I
Inhibitory Activities Against Trypsin and α-Chymotrypsin of Various Soybean Foods[a]

Foods Examined	Inhibitory Activity (IU/10 g of food)	
	Trypsin	α-Chymotrypsin
Raw soybean meal	13,462 ± 646	1,596 ± 181
Ni-Mame	169 ± 9.4	2.3 ± 2.4
Yuba	611 ± 27	143 ± 1.0
Kinako	22 ± 4.4	4.2 ± 0.9
Kori-tofu	629 ± 41	122 ± 1.9
Abura-age	127 ± 5.6	12 ± 1.3
Natto	46 ± 6.0	2.7 ± 1.6
Okara	590 ± 24	125 ± 1.3

[a]Values are means ± standard deviation ($n = 4$).

Susceptibility to Proteolytic Action of Proteins of Soybean Foods

In addition to protease inhibitor, there has been concern regarding susceptibility to proteolytic action of proteins per se in various soybean foods. Table II shows protein digestibility by pepsin plus pancreatin of various soybean foods. *Yuba* and *ni-mame* exhibited a high protein digestibility by pepsin plus pancreatin. On the other hand, *kinako* exhibited the lowest protein digestibility among all the soybean foods examined, except for raw soybean meal. There was a high relationship of the observed protein digestibility by pepsin plus pancreatin of soybean foods (Table II) to the reported in vivo protein digestibility in humans of soybean foods (RCSTAJ 1982, JSNFS 1984) (correlation coefficient = 0.920). This finding indicated that in vitro studies on protein digestibility of soybean foods (Table I) may agree with the in vivo studies in humans (RCSTAJ 1982, JSNFS 1984). In addition, there was no significant ($P > 0.05$) relationship between protease inhibitor levels of soybean foods (Table I) and their observed digestibility by pepsin plus pancreatin (Table II).

Figure 1 shows the effect of time on pepsin digestion of *yuba* and *kinako*. Analyses with 2,4,6-trinitrobenzenesulfonate (Fig. 1A) and with phenol reagent (Fig. 1B) both showed a higher amount of peptide released from *yuba* than that from *kinako,* indicating that the protein of *yuba* was more easily digested by pepsin action than protein of *kinako*. Also, some in vitro procedures (both single and multiple enzyme systems) used to estimate protein digestibility in foods have been developed and discussed (Satterlee et al 1981). Each of these procedures may have inherent characteristics on estimation. Analysis of protein digestibility in soybean foods with pepsin (Fig. 2), unlike that with trypsin or α-chymotrypsin, may give reasonably adequate results without interference from the protease inhibitors in the soybean foods (Table I). Digestion of food proteins by pepsin with a broad specificity (Fruton 1976) affords some information regarding susceptibility to proteolytic action of the proteins (Scheffner et al 1956, Oka et al 1965, Bietz et al 1970, Hansen and Johnston 1976, Masson et al 1986). On the other hand, digestion by pepsin plus pancreatin may be an accurate approximation for protein digestibility in foods (Akeson and Stahmann 1964, Saunders et al 1973, Gauthier et al 1982). Interestingly, analyses by both procedures (pepsin alone [Fig. 2] and pepsin plus pancreatin [Table I]) equally indicated a marked difference in the digestibility of protein for *yuba* and *kinako,*.

Figure 2 is a reciprocal plot of pepsin digestion of *yuba* and *kinako*. Apparent K_m and V_{max} of *kinako* were estimated to be 8.0×10^{-2} g of protein/ml and 2.5×10^{-2} g of peptide/ml/min, respectively. Apparent K_m and V_{max} of *yuba* were estimated to be 3.0×10^{-2} g of protein/ml and 2.5×10^{-2} g of peptide/ml/min, respectively. The kinetic analysis indicates that *yuba* has a smaller K_m value than does *kinako* with a similar V_{max} value, suggesting that there may be a qualitative, but not quantitative, difference in a chemical form of protein for *yuba* and *kinako*. The present findings (Table II, Figs. 1 and 2) suggest that the conformation of the *yuba* protein may make it more susceptible to proteolytic action. Thus, based on scanning electron microscope studies, Okamoto (1976) proposed a model of *yuba* protein that has an unfolding, stretched structure by hydrophobic binding with lipid. Protein unfolding increases susceptibility of proteins to protease action (Privalov 1979). In addition, Fukushima (1968) reported that most soybean proteins are globular molecules that are resistant to proteolytic attack unless their internal structure is disrupted. These previous studies strongly support the present finding on the high protein digestibility of *yuba* (Table II, Figs. 1 and 2). On the other hand, it appears that the chemical form of the *kinako* protein may be less susceptible to proteolytic action (Table II, Figs. 1 and 2).

Table III shows velocity of pepsin digestion of various soybean foods. There was a significant ($P < 0.05$) difference in velocity of pepsin digestion among soybean foods examined. The protein of *yuba,* especially, exhibited a significantly high velocity in pepsin digestion. The proteins of *kinako,* raw soybean meal, and *natto* exhibited a low velocity. Except for raw soybean meal and *natto,* there was a high correlation of the observed velocity of pepsin digestion of soybean foods to that reported in vivo digestibility in humans of soybean foods (RCSTAJ 1982, JSNFS 1984) (correlation coefficient = 0.913). However, *natto,* which is a fermented food, initially contained a remarkably high level of TCA-soluble peptide before pepsin digestion as compared with the nonfermented other foods examined (data not shown). Therefore, *natto,* exhibited a high pepsin plus pancreatin digestibility, expressed as the ratio of the content of free peptide to the original protein content of the foods (Table II). In contrast to Table II, *natto,*

TABLE II
Protein Digestibility by Pepsin Plus Pancreatin of Various Soybean Foods[a]

Foods Examined	Protein Digestibility by Pepsin Plus Pancreatin (%)
Raw soybean meal	39.7 ± 3.6 c
Ni-Mame	86.0 ± 0.8 a
Yuba	86.5 ± 3.0 a
Kinako	62.8 ± 3.3 b
Natto	83.1 ± 5.2 a

[a] Values are means ± standard deviation ($n = 6$). Means within the same column that are not followed by the same letter are significantly different at $P < 0.05$.

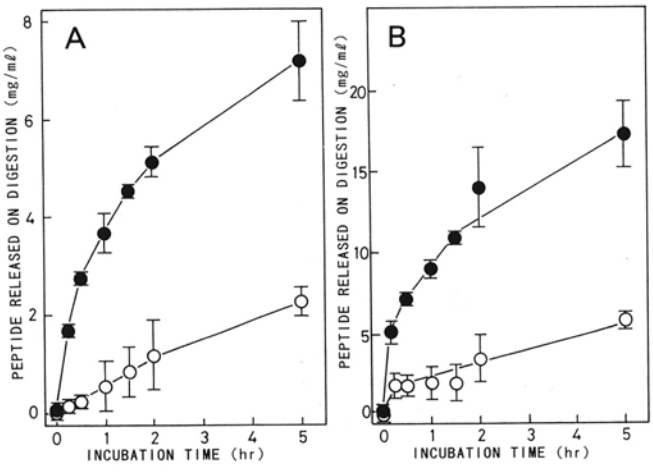

Fig. 1. Pepsin digestion of *yuba* (●) and *kinako* (○). Peptide released on digestion assayed with 2,4,6-trinitrobenzenesulfonate (**A**) and with phenol reagent (**B**). Vertical lines are standard deviation.

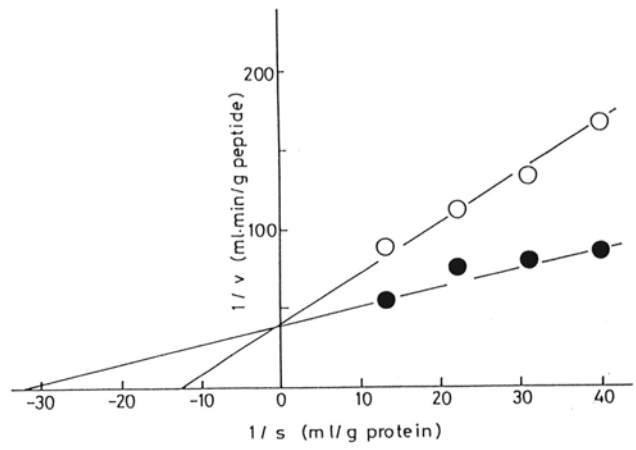

Fig. 2. Reciprocal plot of pepsin digestion of *yuba* (●) and *kinako* (○). s = Total protein concentration (mg/ml of reaction suspension). v = Velocity of pepsin digestion as expressed by milligrams of peptide released per minute during digestion. Peptide released on digestion was assayed with phenol reagent.

TABLE III
Velocity of Pepsin Digestion of Various Soybean Foods[a]

Foods Examined	Velocity of Pepsin Digestion (μg peptide released/ml/min)
Raw soybean meal	114 ± 11 d
Yuba	445 ± 67 a
Kinako	125 ± 54 c
Kori-Tofu	262 ± 2 b
Abura-Age	198 ± 32 c
Natto	26 ± 27 e

[a]Peptide released on digestion was determined by the procedure with phenol reagent. Values are means ± standard deviation ($n = 3$). Means within the same column that are not followed by the same letter are significantly different at $P < 0.05$.

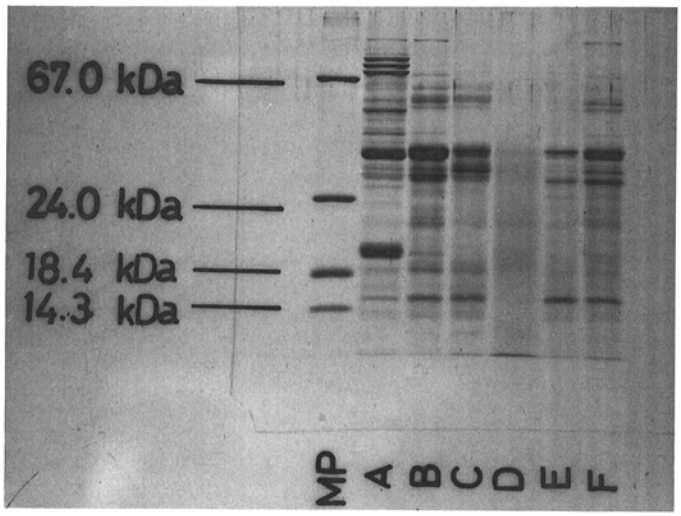

Fig. 3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of various soybean foods. Lane MP, molecular weight marker proteins; lanes A to F (respectively): raw soybean meal; *ni-mame*; *yuba*; *kinako*; *kori-tofu*; and *abura-age*.

exhibited a low velocity of pepsin digestion (Table III), expressed as the amount of peptide released per minute during digestion. This difference in the protein digestibility of *natto* may be due to a large amount of soluble peptide initially present in *natto*. A similar phenomenon was observed with soy sprout (data not shown). In addition to *yuba* and *kinako* (Figs. 1 and 2), these findings for both *natto* and soy sprout also suggest that a chemical form of the protein in soybean foods may be an important factor affecting protein digestibility. Furthermore, there was no significant ($P > 0.05$) correlation between protease inhibitor levels of soybean foods (Table I) and the observed velocity of pepsin digestion (Table III).

Electrophoretic Analysis

Figure 3 shows SDS-PAGE patterns of various soybean foods. There was a variation in protein components among soybean foods examined. About 20 distinguishable protein components in raw soybean meal are indicated with some minor components on SDS-PAGE (lane A in Fig. 3). *Ni-mame* (lane B in Fig. 3) and *yuba* (lane C in Fig. 3) exhibited relatively similar SDS-PAGE patterns. Interestingly, *abura-age* (lane F in Fig. 3), even though processed at high temperature, also exhibited a pattern relatively similar to that of *ni-mame* and *yuba*. On the other hand, SDS-PAGE showed that the majority of *kinako* protein was insoluble (lane C in Fig. 3). *Kinako* extract had lower levels of soluble protein and free sulfhydryl group (data not shown). The insolubilization of protein in *kinako* (Fig. 3) may be closely associated with low digestibility of the protein (Figs. 1 and 2) (RCSTAJ 1982, JSNFS 1984). In this connection, heating-induced behavior of soy protein has been extensively studied (Circle et al 1964, Catsimpoolas and Meyer 1970, Kinsella 1979, Mori et al 1986). However, the behavior of *kinako* soy protein upon heating at high temperature under dry conditions is not well understood.

In conclusion, this study suggests that the chemical form of the proteins of soybean foods may be an important factor responsible for protein digestibility.

ACKNOWLEDGMENTS

We wish to express our sincere gratitude to Kyoden Yasumoto, professor at the Research Institute for Food Science, Kyoto University, for many valuable suggestions during the course of this investigation.

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[Received October 19, 1994. Accepted April 27, 1995]