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Fractionation and characterization of buckwheat proteins

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Summary

Buckwheat proteins have been fractionated into the albumin, globulin, prolamin and glutelin fractions according to the method of Javornik *et al.* and their properties were compared with those of the fractions of wheat proteins. The percentages of the protein contents of each fraction to the total protein content of buckwheat were 19.3% for the albumin, 49.1% for the globulin, 3.3% for the prolamin and 14.2% for the glutelin, although about 70% of the wheat proteins is fractionated into the prolamin and glutelin fractions. The buckwheat albumin and globulin fractions were rich in lysine. The fractions are responsible for the high level of lysine in the whole grain. The prolamin proteins contained the high levels of glutamic acid and proline and were poor in lysine. By sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the main components in the fractions of the buckwheat proteins were shown to be some proteins with low molecular masses for the albumins, six proteins with molecular masses ranging from 60 to 25 kDa for the globulins, several proteins with molecular masses around 38 kDa for the prolamins and some proteins with molecular masses around 45 kDa for the glutelins. Among the buckwheat proteins, the 25-kDa protein in the globulin fraction was observed to be the major protein. The amino acid composition and SDS-PAGE of the buckwheat prolamins were shown to be very similar to those of the wheat prolamins.

Key words : buckwheat, protein, albumin, globulin, prolamin, glutelin.

Introduction

Buckwheat (*Fagopyrum esculentum*) is rich in lysine and is one of the well-known

sources of proteins with high biological values[1-3]. In general, buckwheat is milled and utilized for the preparation of noodle, pancake or porridge. Recently, it has been renewed as one of the popular vegetarian diets[4]. Buckwheat is not a true cereal, but since it is similar to cereal with regard to starchy endosperm and oily embro[5], it has been classified with the cereal[3].

The proteins in wheat, which is representative of the cereals, have been extensively studied and understood well. Some investigations[6-10] on the fractionation, amino acid composition and electrophoresis analyses of buckwheat proteins on polyacrylamide gels have been reported, but any systematically detailed studies on buckwheat proteins have not been reported. It is very important for further utilization of the buckwheat proteins that they are fully understood.

In the present study, we have investigated systematically chemical and physiochemical properties of the buckwheat proteins, which have been fractionated into albumins, globulins, prolamins and glutelins. The immunological analyses of the proteins in each fraction of buckwheat were also performed with rabbit antisera against the globulin proteins. Furthermore, the comparison of the properties of the fractionated buckwheat proteins with those of the fractionated proteins of wheat is described.

MATERIALS AND METHODS

Materials

Buckwheat (Shinano No. 1) and wheat (Norin No. 27) flours were obtained from Asahi Seifun (Sakurai, Nara, Japan), peroxidase-conjugated anti-rabbit IgG was purchased from Organo Teknika (West Chest, PA, U.S.A.) and nitrocellulose membrane from Bio-Rad (CA, U.S.A.). All the other materials used were of analytical grade.

Fractionation of proteins in buckwheat and wheat flours

The fractionation of proteins in buckwheat and wheat flours was done according to the method of Javornik *et al.*[11]. First of all, each flour (100 g) was defatted with *n*-butanol-petroleum ether by the method of Shewry *et al.*[12] and the proteins in the defatted flours were suspended in 10% NaCl for 1 h at room temperature. The NaCl-soluble proteins were obtained by a 30-min centrifugation at 20,000×*g* and filtration with a Millipore filter (1.2 μm) (Millipore, MA, U.S.A.), after which the proteins were dialyzed against water overnight. The precipitate formed was collected by centrifugation at 20,000×*g* for 30 min and used as the globulin fraction in this work. The supernatant obtained by the centrifugation was designated the albumin fraction. Both of the two fractions were flitered with a Millipore filter and lyophilized.

The NaCl-insoluble proteins of buckwheat were incubated at room temperature for 1 h with 800 ml of 70% ethanol and the extract was obtained by centrifugation. After filtration through a Millipore filter, the filtrate was dialyzed against water. The precipitate formed

was collected by centrifugation, dried and used as the prolamin fraction in this paper.

The residue obtained from 70% ethanol suspension by centrifugation was further extracted with 0.2% NaOH. After filtration of the extract, the filtrate was adjusted to pH 4.2 with 2 M HCl and allowed to stand at 4℃ for 1 h. The precipitate formed was collected by centrifugation, washed twice with water and lyophilyzed. The proteins obtained were used as the glutelin proteins.

The NaOH-insoluble materials separated from the NaOH-soluble proteins by centrifugation were designated the residue fraction in this paper.

In order to investigate the effect of NaCl concentration on the extraction of the albumin and globulin proteins in buckwheat and wheat flours, the proteins in the flours were incubated for 1 h at room temperature with 0, 3, 5 and 10% NaCl. The extracts were prepared by a 30-min centrifugation at 20,000×g and filtered with a 1.2μm Millipore filter. After dialysis of the filtrates against water overnight, the supernatants, the albumin proteins, and the precipitates, the globulin proteins, were prepared by centrifugation and freeze-dried. The albumins and globulins obtained with 0, 3, 5 and 10% NaCl are designated 0s, 3s, 5s and 10s albumins and globulins, respectively, in this paper.

Preparation of antisera against the buckwheat globulin proteins

The globulins obtained with 10% NaCl as described above were used as the immunogen. Rabbits were subcutaneously injected with a mixture of a complete Freund's adjuvant and the globulin proteins (2 mg/rabbit) in phosphate-buffered saline (PBS) containing 8M urea. At the intervals of two weeks, the animals were subcutaneously immunized two times with a mixture of an incomplete Freund's adjuvant and the antigen (5 mg/rabbit). One week after the third injection, the rabbits were killed, the blood was taken out and the sera were prepared. The sera obtained were used as the antisera against the buckwheat globulin proteins in the present study.

Immunodiffusion

The immunodiffusion of the albumins and globulins was done according to the method of Takeoka *et al.*[13].

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

Proteins were mixed at a concentration of 10mg/ml with the sample buffer[14] and heated in a boiling water bath for 5 min. The samples were electrophoresed on 15% polyacrylamide gels by the method of Laemmli[14]. The proteins separated on the gels were stained with 0.1% Coomassie Brilliant Blue R250 in methanol / acetic acid / water (5:1:4, by vol.) and destained with methanol / acetic acid / water (3:1:6, by vol.).

The immunoblotting was performed essentially by the method of Towbin *et al.*[15]. Proteins were separated on a 15% polyacrylamide gel as described above. The proteins on the

gel were electrophoretically transferred onto a nitrocellulose membrane. After blocking the membrane with 1% BSA in 20 mM Tris-HCl buffer (pH 7.4) containing 0.15 M NaCl and 0.05% Tween 20 (buffer A), the membrane was incubated at 37℃ for 1 h with the antisera against the globulin proteins. The bound antibody was reacted with peroxidase-conjugated anti-rabbit IgG, diluted 1:2000 with buffer A containing 1% bovine serum albumin (BSA). The immunocomplexes on the membrane were detected by a 20-min incubation at room temperature with 4-chloro-1-naphthol (0.3 mg/ml) and 0.03% H_2O_2 as the substrates in 50 mM Tris-HCl buffer (pH 7.3).

Kjeldahl and amino acid analysis of the proteins in each fraction

In order to determine the amounts of the proteins in each fraction, the freeze-dried preparations of each fraction were subjected to the micro Kjeldahl method[16], and the nitrogen-protein conversion factors used were 6.31 for buckwheat and 5.71 for wheat[17].

For amino acid analysis of the proteins in each fraction, the proteins were hydrolyzed for 20 h at 110℃ in 6M HCl in sealed tubes under vaccum. The amino acid analyses were performed using a IRICA A-8700 amino acid analyzer.

RESULTS

Fractionation of buckwheat proteins

Proteins in defatted buckwheat flour were succesively fractionated with 10% NaCl, 70% ethanol and 0.2% NaOH according to the method of Javornik *et al.*[11]. In order to compare the buckwheat proteins with wheat proteins, the wheat proteins were fractionated in the same manner as described for the fractionation of buckwheat proteins. The results were summarized in Table 1. About a half of the total buckwheat proteins was fractionated into

Table 1. Yields and protein contents of the fractions obtained from buckwheat and wheat flours

Fraction	Buckwheat		Wheat	
	Yield (%)[a]	Protein content (%)[b]	Yield (%)	Protein content (%)
Albumin	4.2[c]	19.3	1.2	3.4
Globulin	3.6	49.1	1.3	8.6
Prolamin	1.9	3.3	4.1	58.9
Glutelin	4.4	14.2	4.8	13.5
Residue	n.d.[d]	11.6	n.d.	10.3
Total protein		97.5		94.7

a Yields are expressed as the percentages of the lyophilized material obtained after dialysis against water to the weight of whole buckwheat or wheat.

b Protein contents are expressed as the percentages of the protein content of each fraction to the total proteins in buckwheat or wheat. The protein contents of buckwheat and wheat were 11.9% and 11.4%, respectively.

c Values are expressed as the means of triplicate determinations.

d not determined.

the globulin fraction, followed by the albumin and glutelin fractions. The amount of the prolamin proteins was shown to be very small in the buckwheat proteins. The profile of the fractionation of the buckwheat proteins was quite different from that of the wheat proteins. In wheat, the prolamin fraction contained about 60% of the total wheat proteins and only small amounts of the wheat proteins were fractionated into the albumin and globulin fractions.

Amino acid composition of the proteins in each fraction

The amino acid composition of the proteins in each fraction obtained from buckwheat flour is shown in Table 2. The amino acid composition of the albumin proteins in buckwheat was very similar to that of the globulin proteins, except that the glutamic acid content of the latter proteins was two fold more than that of the former. In the prolamin proteins, glutamic acid was shown to occur predominatly, followed by proline. When the prolamin fraction was prepared with 50% *n*-propanol instead of 70% ethanol, the *n*-propanol-soluble proteins gave the amino acid composition similar to that of the prolamin proteins obtained with 70% ethanol as shown in Table 2. Furthermore, the acid hydrolysate of the prolamin fraction contained high level of NH_3 (24.5%), which would be originated from the amide-NH_2 of glutamine and asparagine, indicating that about 70% of glutamic acid determined may be glutamine because the aspartic acid content of the fraction was very small. In the glutelin fraction of buckwheat, glutamic acid were also contained predominantly, followed by proline, leucine and alanine in the decreasing order. However, the prolamin

Table 2. Amino acid composition of the proteins in each fraction obtained from defatted buckwheat flour

Amino acid	Albumin	Globulin	Prolamin A[b]	Prolamin B[c]	Glutelin
			mol%[a]		
Asp[d]	9.1	9.9	3.5	2.4	6.5
Thr	3.8	4.6	3.3	2.7	4.7
Ser	5.4	6.7	5.5	5.2	6.8
Glu[d]	7.9	16.7	33.4	34.6	21.7
Pro	3.6	4.1	12.9	15.1	7.8
Gly	10.6	9.5	6.7	4.8	9.6
Ala	6.4	7.4	4.7	3.9	7.1
Cys	1.3	0.4	1.2	1.0	0.7
Val	5.7	5.6	4.4	4.9	5.4
Met	1.8	0.9	0.3	0.5	1.0
Ile	4.8	4.4	5.0	4.7	4.5
Leu	7.0	7.6	6.9	7.2	7.9
Tyr	1.9	2.0	2.1	2.0	2.4
Phe	3.3	5.0	6.0	4.5	4.2
Lys	6.7	4.5	1.6	0.9	3.6
His	2.5	1.8	1.9	1.6	1.7
Arg	8.1	8.7	2.6	2.1	4.7

a Data are expressed as the means of duplicate determinations.
b Prolamin A was prepared with 70% ethanol.
c Prolamin B was prepared with 50% *n*-propanol.
d Aspartic acid and glutamic acid values include asparagine and glutamine, respectively.

Table 3. Amino acid composition of the proteins in each fraction obtained from defatted wheat flour

Amino acid	Albumin	Globulin	Prolamin	Glutelin
		mol%[a]		
Asp[b]	8.9	8.0	2.6	5.1
Thr	4.3	4.5	2.3	3.8
Ser	5.8	6.2	5.5	6.1
Glu[b]	13.7	15.7	36.6	25.9
Pro	7.5	5.5	14.1	10.3
Gly	9.7	10.3	3.8	8.3
Ala	8.8	8.9	3.6	7.3
Cys	1.5	0.6	0.5	0.1
Val	7.7	8.0	5.4	6.2
Met	1.7	1.3	1.3	0.2
Ile	4.0	5.0	5.1	4.9
Leu	7.9	8.5	7.9	8.7
Tyr	2.7	2.3	2.1	2.1
Phe	3.7	4.2	4.3	4.0
Lys	4.3	4.6	1.0	2.9
His	2.5	2.6	1.9	2.0
Arg	5.4	8.0	2.3	2.3

a Data are expressed as the means of duplicate determinations.
b Aspartic acid and glutamic acid values include asparagine and glutamine, respectively.

and glutelin proteins were observed to contain only small amounts of lysine and arginine. The amino acid compositions of the fractions obtained from wheat were also shown in Table 3. Generally, the amino acid composition of each fraction from wheat was very similar to that from buckwheat.

Effect of NaCl concentration on the extraction of the albumin and globulin proteins from buckwheat flour

The effect of salt concentration on the extraction of the albumin and globulin proteins

Table 4. Yields and protein contents of the albumins and globulins obtained from defatted buckwheat flour with various concentrations of NaCl.

Fraction[a]		NaCl concentration (%)			
		0	3	5	10
Albumin					
	Yield	3.7[b]	4.6	5.4	6.7
	Protein content (%)	15.8	18.3	22.1	20.2
Globulin					
	Yield	2.1	3.2	3.8	3.6
	Protein content (%)	39.1	42.3	50.5	49.2

a The buckwheat flour (1g) was suspended in 10 ml of 0, 3, 5 and 10% NaCl and stirred for 1 hr at room temperature. After a 30-min centrifugation at 20,000×*g*, the supernatants obtained were filtered with a Millipore filter before dialysis against water as described in Materials and Methods.
b Values are expressed as the means of triplicate determinations.

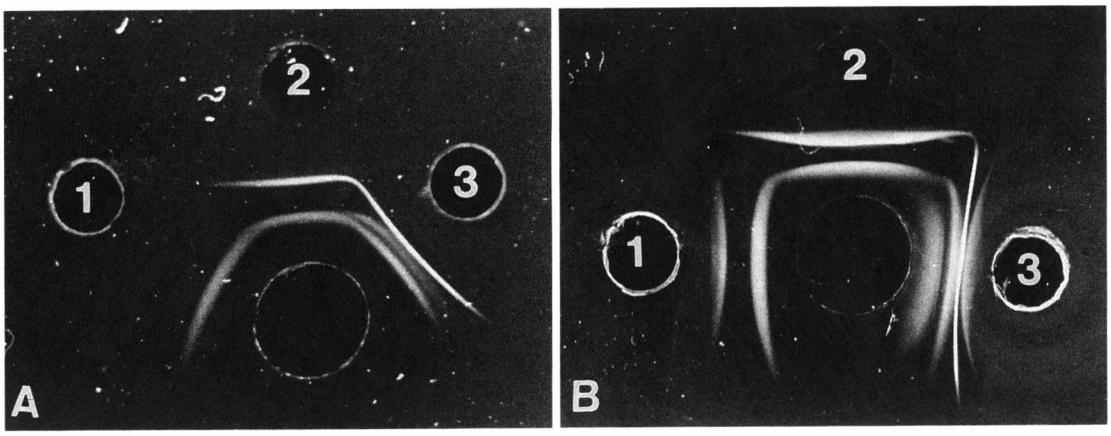

Fig. 1. Immunodiffusion of the albumins and globulins prepared from buckwheat flour with various concentrations of NaCl. (A) Well 1, 0s-albumins; 2, 5s-albumins; 3, 0s-globulins. (B) Well 1, 3s-albumins; 2, 5s-albumins; 3, 5s-globulins. The center wells contained the antisera raised against the buckwheat globulins.

from buckwheat flour was investigated by the use of the Kjeldahl method and immunological techniques. The protein contents of the albumin and globulin fractions obtained with 0, 3, 5 and 10% NaCl were summarized in Table 4. In parallel with the concentration of NaCl, both of the fractions increased in the protein content of each fraction lyophilized. At a NaCl concentration of 5%, the amounts of the albumin and globulin proteins reached to a maximum, indicating that the NaCl concentration of 10% was sufficient to obtain the maximal amounts of both the proteins. The proteins prepared with 0, 3 and 5% NaCl were subjected to immunodiffusion (Fig. 1A). Interestingly, the albumin obtained with water (0% NaCl) showed one precipitate line in the agarose gel, while the 3% NaCl-soluble albumin proteins exhibited another precipitate line on the antigen side. On the other hand, the globulin proteins obtained with water gave two precipitate lines and those obtained with 5% NaCl showed four lines. The two precipitate lines of the four lines were fused with the two precipitate lines formed with 5s-albumins (Fig. 1B). These results indicate that both of the albumin and globulin proteins obtained with 10% NaCl share common proteins. This possibility was further examined by immunoblotting analyses as described below.

SDS-PAGE of buckwheat proteins

The buckwheat proteins were examined by SDS-PAGE (Fig. 2). The albumin fraction obtained with 10% NaCl gave the five main bands of the proteins with molecular masses of 25, 16, 15, 13, and 11 kDa (Fig. 1A, lane 3). However, when the albumin proteins were prepared with water instead of 10% NaCl, the 25 kDa protein was not observed in the SDS-PAGE of the albumin proteins. The globulin fraction gave the bands of proteins with molecular masses of 62, 50, 43, 41, 35, and 25 kDa on its SDS-PAGE (Fig. 2A, lane 4). Among them, the 25-kDa protein was shown to be the major component in the globulin fraction. The globulin fraction contained small amounts of the proteins with molecular masses ranging from 16 to 11 kDa, although the albumin fraction was also observed to con-

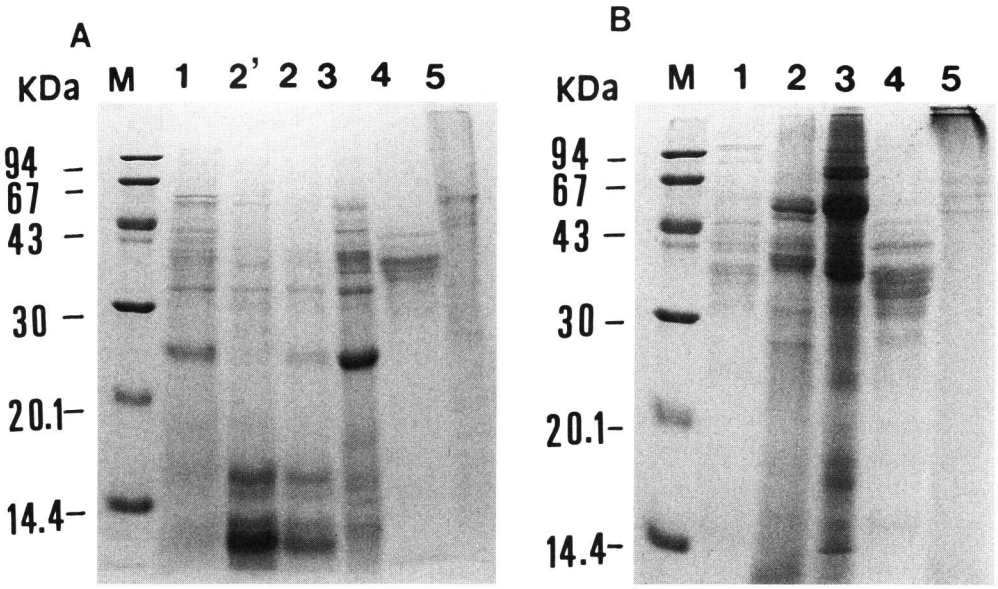

Fig. 2. SDS-PAGE of the proteins in each fraction obtained from buckwheat (A) and wheat (B) flours. Lane M, the standard marker proteins; 1, total flour; 2', albumins obtained with water; 2, albumins obtained with 10% NaCl; 3, globulins; 4, prolamins; 5, glutelins. The standard marker proteins used are as follows: phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and α-lactoalbumin (14,400).

tain similar proteins as described above. The prolamin fraction was observed to consist mainly of several polypeptides with molecular masses ranging from 45 to 36 kDa. In the glutelin proteins, the 55-, 45-, 43- and 28-kDa proteins were shown to be the main components.

Immunoblotting

The above-mentioned immunodiffusion of the albumins and globulins indicates that both the proteins were crosscontaminated. Therefore, we investigated the crossreactivity of the proteins in each fraction of buckwheat with the antisera against the buckwheat globulin proteins, using an immunoblotting technique. As shown in Fig. 3, many proteins in the globulin fraction were immunostained with the antisera. Of the albumin proteins, some proteins, especially the 16-kDa protein, were strongly immunoblotted by the antisera. However, no protein in the prolamin and glutelin fractions was observed to be im-

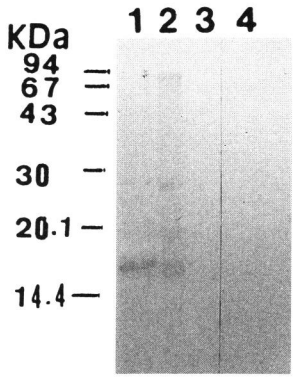

Fig. 3. Immunoblot of the proteins in each fraction of buckwheat. The proteins in each fraction were electrophoresed on a 15% polyacrylamide gel, and the proteins on the gel were electroblotted on a nitrocellulose membrane. The membrane was immunostained with the rabbit antisera against the buckwheat globulins. Lane 1, albumin proteins obtained with 10% NaCl; 2, globulins; 3, prolamins; 4, glutelins.

munostained by the antisera.

DISCUSSION

In order to characterize proteins in foods, the proteins have been fractionated into the albumin, globulin, prolamin and glutelin fractions according to the solvent method, which was originally devised by Osborne[18]. For example, with regard to wheat proteins, 5, 10, 69, and 16% of the wheat proteins were shown to be separated into the albumin, globulin, prolamin and glutelin fractions, respectively[5]. The outstanding characteristic of the wheat proteins is that the major part of the proteins is fractionated into the prolamin fraction. Several investigators carried out the fractionation of buckwheat proteins and reported that the globulins are the major proteins in the buckwheat proteins[11, 19, 20]. Our results are compatible with those reported by the investigators.

The amino acid composition of the buckwheat albumin fraction was very similar to that of the globulin fraction, except that the level of glutamic acid in the albumin fraction was lower than that in the globulin proteins. The lysine contents of both the fractions were high. These results show that the high lysine content of the whole grain of buckwheat originates from the proteins in the albumin and globulin fractions. Interestingly, the buckwheat prolamin fraction showed that the molar percentages of glutamic acid and proline were 33.4% and 12.9%, respectively (Table 1). The glutamic acid and proline contents of the 50%–*n*–propanol–soluble fraction were shown to be the same as those of the prolamins obtained with 70% ethanol. Also, several investigations on the amino acid composition of buckwheat proteins have been reported, but all the results reported are the data obtained with the whole buckwheat proteins, except for the data reported by Skerritt[19]. In contrast to our data, Skerritt[19] reported the molar percentage of glutamic acid in 70% ethanol–soluble proteins is 14.8% and that of proline is 9.6%. We repeated three times to prepare the prolamin proteins from buckwheat and to analyze the amino acid composition of the proteins, but the same results were obtained. Since the prolamin fraction is a minor component of buckwheat proteins, the contamination of a small amount of the globulin proteins, the major fraction of buckwheat proteins, would cause a marked decrease in the glutamic acid and proline contents of the prolamin fraction. In the preparation of the fractions, we filtered the supernatants obtained by centrifugation with a Millipore filter in order to remove fine particles in the supernatants, although Skerritt did not clarify the supernatants prepared with any filter. The difference between our data and Skerritt's data concerning the glutamic acid and proline contents of the buckwheat prolamin proteins may arise from those in cultivars and extraction procedures such as extraction time and solvent. All of the known prolamins such as gliadin in wheat[19], hordein in barley[21] and zein in corn[22] contain high levels of glutamic acid (mainly as glutamine) and proline. The present finding that glutamic acid and proline occur at high concentrations in the buckwheat prolamin fraction supports the hypothesis that the prolamin proteins in cereals contain high

levels of glutamine and proline[23].

The proteins in each fraction were examined by SDS-PAGE (Fig. 2). The albumin fraction was shown to consist mainly of the polypeptides with molecular masses ranging from 16 to 11 kDa. The globulin fraction comprised of the six main proteins. Of the main proteins, the 25-kDa protein was observed to be the most predominant component in the globulin fraction, namely the 25-kDa protein is the major protein in the buckwheat proteins. The immunodiffusion analyses showed that the albumin fraction shared some proteins in the globulin fraction (Fig. 1). Furthermore, the immunoblotting analyses revealed that some proteins in the albumins, including the 25- and 16-kDa protein, shared those in the globulin fraction. These observations demonstrate that some proteins in the albumin and globulin fractions of buckwheat preprared by the present study were crosscontaminated. The prolamin fraction was shown to mainly consist of the proteins with molecular masses around 38 kDa and to contain no component contaminated from the globulin fraction (Figs. 2 and 3). The protein profile on the SDS-PAGE of the buckwheat prolamin fraction is very similar to that observed with the wheat prolamin, gliadin[19]. However, our SDS-PAGE pattern was quite different from that obtained by Skerritt[19]. This may reflect the above-mentioned difference between our data and Skerritt's data concerning the glutamic acid and proline contents of the buckwheat prolamins.

In summary, we fractionated the buckwheat proteins into the albumin, globulin, prolamin and glutelin fractions. The proteins in each fraction were examined in detail by amino acid analysis, SDS-PAGE and immunological techniques. The major part of the buckwheat proteins was recovered in the globulin fraction. The globulin fraction was shown to contain the 25-kDa protein as the outstanding component in the total buckwheat proteins. Furthermore, the buckwheat prolamin proteins were rich in glutamic acid and proline and poor in lysine. Together with these results, SDS-PAGE of the prolamin proteins shows that both of the buckwheat and wheat prolamins are very similar with regard to the amino acid composition and the protein components of the prolamin fraction. This paper is the first report showing that the buckwheat proteins have been systematically characterized. At present, further investigations on the purification and characterization of the 25-kDa protein, the major proteins in the globulin fraction, are actively in progress.

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