Isolation and Enzymatic Digestion of Body Complex of Soybean Seed

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The body complex of the soybean seed (BCSS) was isolated from the single cells (27.2%) by a sequential procedure of autoclaving with water, cellulase digestion for the primary cell wall, pectinase digestion for the secondary cell wall, and defatting with hexane washing. Its characteristics were then investigated. The defatted BCSS (DBCSS) consisted of protein (76.5%) and mannose-rich carbohydrates (3.2%). Screening of the food-processing protease for the digestion of DBCSS was carried out, and a kind of alkaline protease was selected. The inner protein of DBCSS was easily extracted with 0.1 M sodium carbonate buffer, pH 10, and the insoluble shell of the body complex (SDBCSS) was left. SDBCSS consisted of hydrophobic amino acid-rich protein. SDBCSS was easily digested by the selected alkaline protease. SDBCSS was dissolved by boiling with sodium dodecyl sulfate−mercaptoethanol, and it was found to consist of a protein of ∼3 kDa. The high enzymatic digestion including the selected protease for soybean seed and defatted soybean meal was carried out; both were extracted and digested with a yield of >99.5%. The final indigestible residue was found as paired hexagonal and filamentous organs of the soybean cells.

INTRODUCTION

The soybean is an important food seed that contains many good-quality proteins and oils for foods and feeds. Digestion and extraction are generally essential for food processing and the use of agroproducts. Many trials were also made for the defatted soybean (soybean meal), but its complete digestion and extraction were difficult. Fischer et al. examined an extraction using enzymes of the soybean meal in detail (*1*, *2*). Ohida et al. also studied an extraction of the defatted soybean (*3*). The former shows that an advanced extraction is possible by using some proteases and the cellulolytic enzyme with a high-temperature−humidity condition as for the soybean meal (*1*). A perfect enzymatic digestion is very difficult, and some fractions were extracted by an alkaline extraction; the latter reports that a residue would be mainly a cellulose-rich composition (*3*). It is reported that the extraction from soybean is difficult because it is a complex of carbohydrates and proteins for the defatted soybean (*1*, *2*, *4*). This is not limited to the soybean. Proteins and carbohydrates are functionally interacted with each other in plants (*5*). As for the previous studies, the extractions and the enzymatic digestions were done using defatted soybean meal.

Therefore, although the chemical analysis of the residue was carried out, soybean organs were not studied in detail. There is little research on the compositions of the organs of the soybean seed or the soybean cell. It would be useful in the investigation of the compositions of the organs and their high digestions to use the whole soybean. We did the stepwise extraction of the soybean and the single cell formation of the soybean or examined the enzymatic digestion of the soybean milk residue, okara (*6*, *7*). A secondary cell wall of soybean consisted of protein, pectin, and hemicelluloses, and it was shown that a selected pectinase could digest the secondary cell wall and that the digestion of the residual soybean body complex was difficult (*6*, *7*). However, the paper shows that an enzyme extraction can be easily done if an effective enzyme is found. In this study, the indigestible body complex of the cells was isolated, and the digestion using food-processing enzymes was investigated; the characteristics of the body complex were studied. An effective alkaline protease can be selected, and the high digestion of the soybean and the defatted soybean could almost be done as a result (>99%). The high enzymatic digestion for soybean was established by using the series of autoclaving treatments with formation of a single cell and cellulolytic and proteolytic enzymes. This result would be useful knowledge for using soybeans and the meal or to investigate the location of the food function in soybeans.

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Enzymatic Digestion of Body Complex of Soybean Seed

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MATERIALS AND METHODS

Soybean and Deffated Soybean Meal. Soybeans (*Glycine max* L.), cultivated in the United States, and the soybean meal were gifts from Showa Sangyou Co., Ltd., Tokyo, Japan.

Oil Body Complex of Soybean Seed. The soybean seed was dehulled and autoclaved with 5-fold water (121 °C, 10 min). The water extraction was removed, and the single cells of the soybean were prepared by pasting (*6, 7*). Digestion of the primary cell walls with cellulase and digestion of the secondary cell walls with pectinase were carried out using a method described in the previous papers (*6, 7*). The oil drops on the supernatant were removed, and the body complex of the soybean seed (BCSS) was obtained on the filter paper. The obtained BCSS was freeze-dried and then defatted at room temperature using hexane dipping and washing (DBCSS).

Enzymes. Cellulase (for food processing, 5000 units/g) and glucanase were kindly provided by Daiwa-Kasei Co., Ltd. (Osaka, Japan), and pectinase (Pectinex Ultra SP; 26000 units/mL) was obtained from Novozyme Japan. Prolaser FG-F, *Bacillus subtilis*; protease S, *Bacillus stearothermophilus*; bromelain F (bromelain); pancreatin F (pancreatin); papain W-40 (papain); protease P3G, *Aspergillus melleus*; umamizyme (*Aspergillus oryzae* peptidase); and protease A, *Aspergillus oryzae*, were gifts of Amanozyme Co., Ltd. (Nagoya, Japan). Protease N, *Bacillus subtilis*; protin FN, *Aspergillus* sp. metalloproteinase; protin PC10F, *Bacillus* sp. (metalloproteinase); thermoase PC10F, *Bacillus thermoproteolyticus*; protin AC10F, *Bacillus* sp. (serineproteinase); and protin FA (carboxyprotease) were gifts from Daiwa-Kasei Co., Ltd. The reagents of trypsin, pepsin, and pancreatin were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were of reagent grade.

Screening of Protease for Enzymatic Digestion of DBCSS. The buffer systems were 0.1 M phosphate buffer for pH 7−8, 0.1 M acetic acid buffer for pH 5, and 0.1 M sodium carbonate buffer for pH 10 for the enzymatic digestion. A 1% solution of DBCSS aq (100 μL) and 1% of each protease were mixed and reacted at 36 °C overnight. The turbidity and the reacted DBCSS were observed visually and by light microscopy.

Estimation of Sugar and Protein. The amount of uronic acid was measured according to the 3-phenylphenol method (*8*). The total sugar was estimated by using the phenol−sulfuric acid method (*9*). The reducing sugar was estimated according to the Nelson−Somogyi method (*10*). Protein was estimated with the Lowry method (*11*). Each amount was calculated using a colorimetric standard curve with D-galacturonic acid, D-glucose, and serum albumin as the standards.

HPLC Analysis. The digestion or extraction was analyzed by HPLC. Ten microliters of the supernatant or the dissolved freeze-dried samples (0.5%) was applied using an analytical HPLC system (DP8020, Toso, Tokyo, Japan) consisting of a molecular sizing column (TSK-Gel G3000SWXL, 7.8 × 300 mm, Toso), a difference refraction indicator RI8020 (Toso), and an optical photometer UV8020 (Toso) for detection.

Light Microscopic Observation. The microscopic observations and photographs were done using an Olympus model BH-21 (Olympus Optical Co., Ltd., Tokyo, Japan) light microscope and a digital DP-II microscope photography device.

Staining and Observation. Toluidine blue O and Coomassie Brilliant Blue (CBB) were used for the staining reagent of DBCSS. The polysaccharides were stained by PAS dyeing (*12*).

SDS-PAGE Electrophoresis. SDS-PAGE was done according to the Laemmli method (*13*). The electrophoresis equipment used was an AE-9631M/P with an attached concentration slope gel (5−20 and 10−20%) (manufactured by ATTO, Tokyo, Japan). The standard molecular marker was the Kaleidoscope Prestained Standards from Bio-Rad. The buffer system was Tris−glycine or Tris−tricine, pH 7 and 8.

Neutral Sugar Analysis. Analysis of the neutral sugars was done using the alditol−acetate method (*14*). The composition of the neutral sugar was analyzed by a GC system with a capillary column of DB-225 (J&W Scientific Co., 0.25 mm × 30m) connected to a Yanaco G-2800 (Yanaco Co., Ltd., Kyoto, Japan). The standard solution was adjusted with a 1% solution of each of the seven kinds of sugars: L-rhamnose, L-fucose, L-arabinose, D-xylose, D-mannose, D-galactose, and D-glucose.
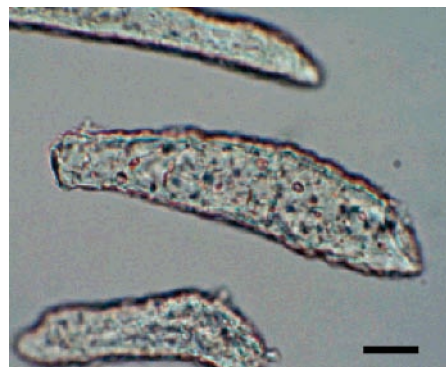

Figure 1. Light microscopy photograph of body complex of soybean seed (BCSS). The BCSS was isolated from the single cells after sequential formation procedures of single cells by autoclaving, cellulase digestion for the primary cell wall, and pectinase digestion for the secondary cell wall. Black bar represents 5 μm.

Alkaline Solubilization of DBCSS and DBCSS. DBCSS was added to 0.1 M sodium carbonate buffer, pH 10, and SDBCSS was added to 1 M NaOH, and then the mixture was boiled for 20 min. Each mixture was neutralized by 1 M HCl, and the supernatant was obtained and dialyzed overnight. The solution was then freeze-dried.

Solubilization of SDBCSS. Alkali solublization of SDBCSS was done using the same procedure for the solubilization of DBCSS as described above. Solubilization of SDBCSS using SDS−mercaptoethanol was as follows: SDBCSS (1%) was dispersed in SDS−mercaptoethanol (1%) and heated in a sealed tube at 100 °C for 24 h. The supernatant was treated with 80% ethanol, and the precipitate was washed with 80% ethanol. The precipitate was resolved in 10 mM phosphate buffer, pH 8.0, and was applied on a column of TOSO-HW50 (2.5 × 42.5 cm) equilibrated with the same buffer. The protein peak was collected and freeze-dried.

Amino Acid Analysis of SDBCSS. The freeze-dried powder of the alkali solublization of SDBCSS (2%) and 6 M HCl were mixed in a hydrolysis tube, and the mixture was hydrolyzed at 110 °C for 22 h in vacuo. The sample and authentic samples were analyzed by an amino acids analyzer (Hitachi model L-8500 Tokyo, Japan).

RESULTS

Preparation of Body Complex of Soybean Seed. The dehulled soybean was autoclaved, and the primary cell wall and the secondary cell wall of the formed single cells were digested by cellulase and pectinase using previously described methods (*6, 7*). The residual body complex (BCSS) was obtained by centrifugation. The partial soybean oil drops were collected and floated, and BCSS was centrifuged to the bottom. The BCSS was free of the cell walls. The BCSS was defatted by hexane at room temperature, and DBCSS was obtained in a yield of 27.2 wt % from the dehulled soybean. The oil content of the BCSS was 62.1 wt %.

Microscopic Observation of BCSS. Figures 1 and **2** are the microscopy photographs of the obtained BCSS and DBCSS, respectively. The obtained BCSS was clearly stained with CBB and toluidine blue O. The inner content that had seeped out from the pressed BCSS was observed. The oil body of the soybean was also well maintained. DBCSS was mechanically pressed (150 kg/cm^2, 5 min) and observed. The DBCSS had a shell with small squamation structures (**Figure 2**).

Composition of DBCSS. The obtained DBCSS was dissolved in 1 M NaOH by boiling for 20 min and neutralized with 1 M HCl, and then the components were analyzed. **Table 1** shows the analytical compositions of DBCSS from the dehulled soybean seed and soybean meal. Protein was the main component. The amounts of total sugar and uronic acid of DBCSS
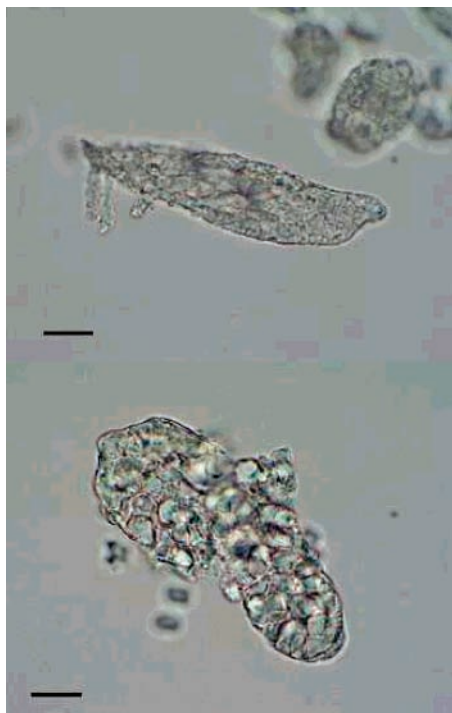
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Figure 2. Light microscopy photographs of mechanically pressed DBCSS. BCSS was deffated by hexane, and DBCSS was prepared. DBCSS was pressed at 150 kg/cm^2 for 5 min by jack, and the broken DBCSS was observed by light microscopy. The surface of DBCSS was broken into the squamation. Black bar represents 5 μm.

Table 1. Composition of Body Complex^a

DBCSS	total sugars (%)	total uronic acid (%)	reducing sugar (%)	total protein (%)
from raw soybean	3.25 ± 0.07	0.79 ± 0.01	0.35 ± 0.02	76.93 ± 0.91
from soybean meal	5.87 ± 0.17	2.33 ± 0.05	0.41 ± 0.03	75.93 ± 0.95

^{a} DBCSS was solubilized with 1 M NaOH at 100 °C for 20 min and neutralized with 1 M HCl. The supernatant was analyzed for total sugar, total uronic acid, reducing sugar, and protein. Each value is expressed as percent by weight of the DBCSS. Samples: $n = 4$.

Table 2. Neutral Sugar of Body Complex^a

neutral sugar	mol %	neutral sugar	mol %
rhamnose	6.9	mannose	30.8
fucose	11.3	galactose	14.5
arabinose	9.7	glucose	12.7
xylose	14.2		

^{a} Sample of DBCSS was hydrolyzed by 2 N HCl at 100 °C for 2 h. The neutral sugar was analyzed according to the alditol–acetate method using GC. Each value is expressed as mole percent.

from the dehulled soybean seed were less than that from the soybean meal. **Table 2** shows the sugar analysis of the DBCSS. Mannose was rich in the hydrolysis.

Screening of Protease for Digestion of DBCSS. Most of the examined proteases could partially digest the DBCSS; the DBCSS was not completely digested. For example, trypsin could partially digest the DBCSS, with protein and sugar being simultaneously released, but the DBCSS was not completely digested (data not shown). A protease of Prolaser, an alkaline protease from *Bacillus* sp., could almost digest the DBCSS. The shell of DBCSS (SDBCSS) completely disappeared.
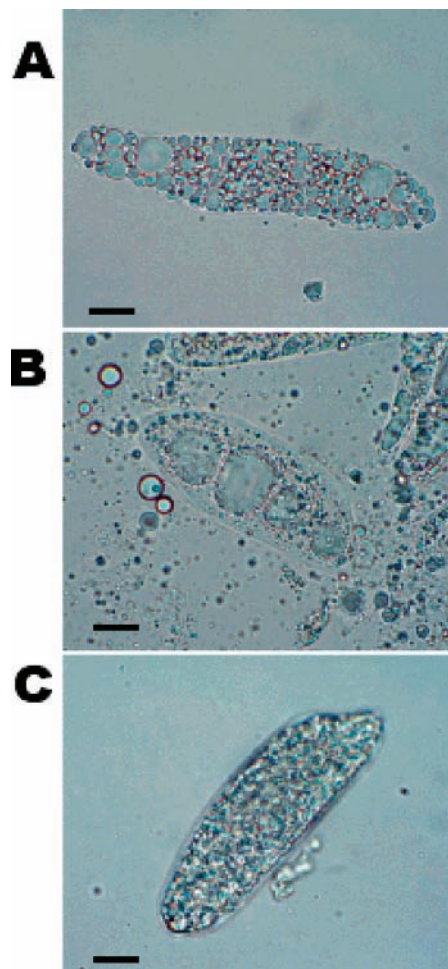

Figure 3. Enzymatic digestion of BCSS in the single cells. The single cells of soybean seed were prepared by autoclaving. The single cells dispersed in 0.1 M Na$_2$CO$_3$ buffer, pH 10, with or without Prolaser (1%) were incubated at 40 °C overnight: (**A**) BCSS of single cell of soybean was digested; (**B***)* finger-pressed single cell (big oil drops were detected); (**C**) blank test of single cell of soybean. Black bar represents 5 μm.

Enzymatic Digestion of BCSS in the Single Cells of Soybean Seed. The selected alkaline protease, Prolaser, could also digest BCSS in the single cells of soybean. **Figure 3** shows the results. The BCSS of the cell was digested and caused a collapse of the shape. The primary and secondary cell walls were not digested and retained their shape. The other examined proteases produced on a partial breaking of the BCSS shape in the cells. The oil drops were collected during the digestion. The digested single cells formed four to five big oil drops by finger pressing during the microscopy observation (**Figure 3B**). Each big drop was separately found in the cell. The barrier of the cell was not enough to investigate. In the blank test, the BCSS of the cells was not digested, and the shape was not changed (**Figure 3C**).

Enzymatic Digestion of BCSS. The DBCSS (1%) was dispersed in 0.1 M Na$_2$CO$_3$ buffer, pH 10.0, and 0.01% of Prolaser was added. The enzymatic digestion was carried out at 40 °C for 16 h with stirring. **Figure 4** shows the time course of the digestion, and **Figure 5** shows the microscopic observation of the DBCSS during this time. The digested protein and sugar were simultaneously released (**Figure 4**). Each piece of the squamate structures was gradually digested (**Figure 5A**−**C**). The blank test without the enzyme also showed a loss in weight, but the SDBCSS was clearly left (**Figure 5D**−**F**). A frame structure of DBCSS was observed as a resistant edifice
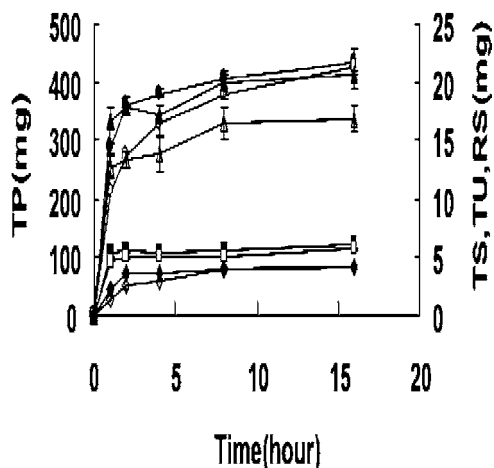
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Figure 4. Time course of enzymatic digestion of DBCSS. DBCSS (1%) was dispersed in 0.1 M Na$_2$CO$_3$ buffer, pH 10, and the mixture was stirred at 1200 rpm and 40 °C for 16 h with or without Prolaser (0.01%). A part of the reaction mixture was removed, and the mixture was centrifuged. The supernatant was analyzed for composition; the residue was washed, freeze-dried, and weighed: (○) total protein (TP) (blank); (●) protein (test); (△) total sugar (TS) (blank); (▲) total sugar (test); (□) total uronic acid (TU) (blank); (■) total uronic acid (test); (◇) reducing sugar (RS) (blank); (◆) reducing sugar (test).
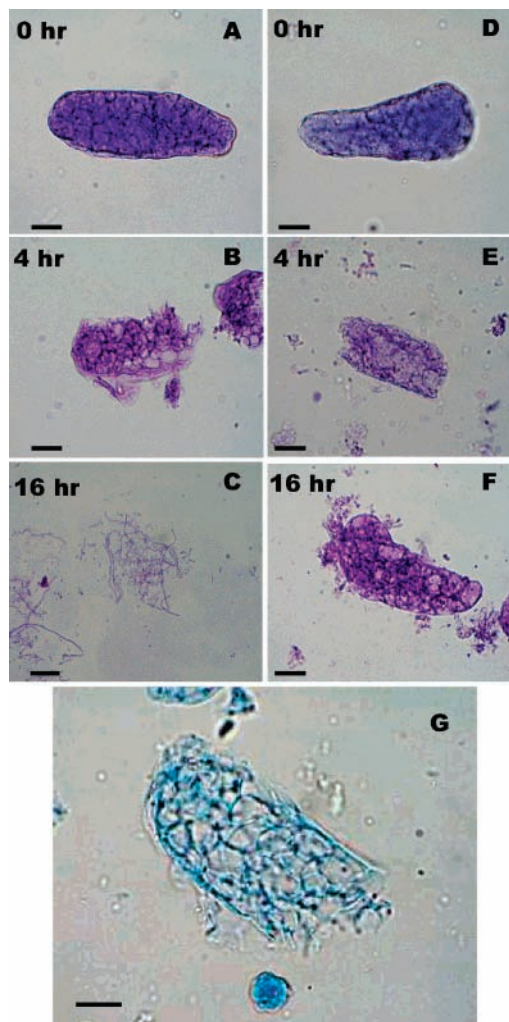

Figure 5. Time course and photographs of the digesting DBCSS. DBCSS (1%) and Prolaser (0.01%) were mixed in a 500 mL beaker at 40 °C overnight. The reaction mixture was the same as in **Figure 4**. The sequential samplings were done, and the residue was centrifuged (2500 rpm, 5 min) and collected. The centrifuged DBCSS was stained by toluidine blue O (**A**–**F**) and CBB (**G**) and then observed by light microscopy. **A**–**C** show results of 0, 4, and 16 h with the enzyme, and **D**–**F** show results of 0, 4, and 16 h without the enzyme, respectively. **G** shows the magnified enzymatic digestion of DBCSS. The thin part of DBCSS was first digested, and the frame line was left. The frame was finally digested in 16 h. Black bar represents 5 μm.

(**Figure 5G**). DBCSS was gradually digested and almost completely digested in 16 h (**Figure 5C**). The sodium carbonate buffer dissolving substance was found to be an internal protein of the DBCSS. The DBCSS was digested with a yield of >99.6%.

Native-PAGE of the DBCSS and SDBCSS. Figure 6 shows native-PAGE of the alkali-dissolved DBCSS and SDBCSS. A broad protein band was detected in the extraction of sodium carbonate buffer (0.1 M, pH 10) from DBCSS. A broad sugar band was also detected by PAS staining. DBCSS were easily soluble in 0.1 M Na$_2$CO$_3$ buffer, pH 10, but the shell of the DBCSS (SDBCSS) was not soluble. The residual SDBCSS was dissolved by boiling in 1 M NaOH. The bands of protein were detected near the cathode position.

SDS-PAGE of the DBCSS and SDBCSS. Figure 7 shows the SDS-PAGE of the alkali-dissolved DBCSS and SDBCSS. Two main bands of low molecular protein (DBCSS-LP; ∼5 kDa), medium molecular protein (∼32 kDa), and a trace of high molecular protein (∼85 kDa) were detected in the alkaline extraction from DBCSS. The broad tailing band of carbohydrates was also found by PAS stain. Two bands of low molecular protein were also detected in the alkaline extraction from SDBCSS (SDBCSS-LP), which were similar to DBCSS-LP. Both the DBCSS-LP and SDBCSS-LP gave a trace of sugar recognized by the PAS stain.

HPLC Analysis of Alkaline-Solubilized DBCSS and SD-BCSS. Figure 8 shows the elution profiles of the solubilized DBCSS and SDBCSS. DBCSS gave three signals of (UV-1, -2, and -3) and SDBCSS gave two signals (UV-1 and -3). The molecular weight of the UV-1 was estimated as 32 kDa and those of UV-2 and -3 were estimated as <1 kDa by the retention time. The elution profiles were fundamentally similar to each other. The freeze-dried sample of the DBCSS was not well soluble below pH 8, but the sample of the SDBCSS was easily soluble. The UV-1 and -2 of DBCSS were decreased and the UV-3 was increased by boiling in 1 M NaOH. The RI signals and the UV signals overlapped each other (data were not shown).

Composition of SDBCSS. The SDBCSS was dissolved by 1 M NaOH with boiling for 20 min. The main component was

protein (105.6 ± 1.4%), and a small of sugar (1.7 ± 0.03%) was found. The SDBCSS mostly consisted of low molecular weight protein and formed a water-insoluble organ.

Amino Acid Analysis of SDBCSS. Table 3 shows the amino acid analysis of the SDBCSS. Hydrophobic amino acids such as Ala, Gly, Leu, and Val were abundant and Glx, Pro, Ser, and Thr were poor in the hydrolysis compared with that of soybean. This result agreed with the paper of Fischer et al. (*1*). SDBCSS was hardly dissolved by hot water or digested by many kinds of proteases, and SDBCSS was generally an indigestible residue.

Solubilization of SDBCSS. SDBCSS was not soluble by guanidine hydrochloride and 8 M urea, but was dissolved by 1 M NaOH (100 °C for 20 min or room temperature for 60 h) or 1% SDS−mercaptoethanol (100 °C for 24 h). The solubilized protein from the SDS treatment showed as a broadened protein of ∼3 kDa in the SDS-PAGE (**Figure 9**). The SDBCSS was hardly soluble and a hard one. The Prolaser protease easily
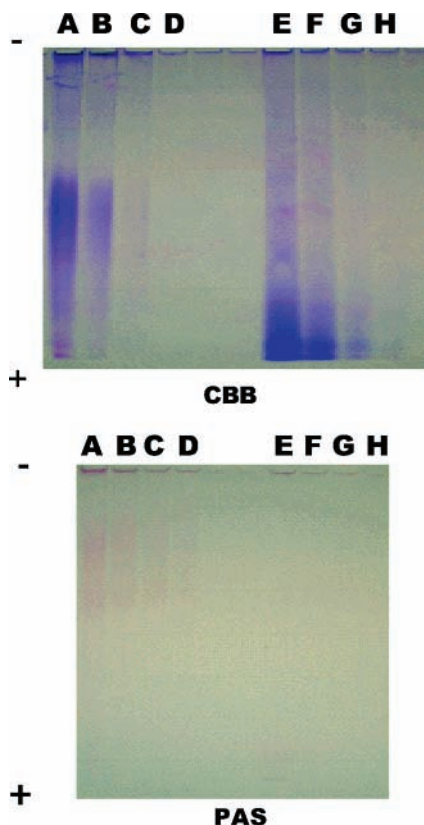
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Figure 6. Native-PAGE of alkali-solubilized DBCSS. DBCSS (1%) was dissolved in 1 M NaOH at 100 °C for 20 min, the supernatant was collected and neutralized by 1 M HCl, and the solution was dialyzed overnight. The freeze-dried sample (1%) was analyzed on SDS-PAGE (5–20% gel) with 20 mA for 75 min. Staining was done using CBB and PAS: (lane A) sample size = 20 μL; (lane B) sample size = 10 μL; (lane C) sample size = 5 μL; (lane D) sample size = 2.5 μL; (lane E) sample size = 20 μL; (lane F) sample size = 10 μL; (lane G) sample size = 5 μL; (lane H) sample size = 2.5 μL.

digested SDBCSS. A trace amount of a low protein signal using the HPLC analysis was detected in the water extraction of SDBCSS during water boiling for 15 min (data not shown). The component of SDBCSS would be a kind of conglutinate aggregation of the 3 kDa protein.

High Digestion of Soybean Seed and Defatted Soybean Meal. The results of the alkaline protease digestion of DBCSS and SDBCSS indicate that the soybean seed could be finally solubilized. **Table 4** shows the results of the sequential enzymatic digestion of the soybean seed and the defatted soybean meal. Both digestions achieved >99.5%. In the case of the dehulled soybean seed, each differential weight was considered as nearly equal to the weight of the extractable protein and carbohydrate, the primary cell wall, the secondary cell wall, soybean oil, BCSS, and DBCSS. DBCSS makes up at least ∼27% of the soybean. The defatted soybean was more extractable than the soybean seed. The destruction of the cells would be preferable for digestion and extraction. There may be a reason for breaking the cells by segmentation.

Final Organ Residue. The final residues were investigated by microscopy observation. The alkaline treatment (1 M NaOH) left a paired-hexagonal organ (**Figure 10A**), and the protease digestion also left the same one (**Figure 10B**) and a thin filamentous organ (**Figure 5C**). A small amount of the stained composition was observed around the paired-hexagonal organ in the alkaline protease digestion (**Figure 10B**). The paired-hexagonal organ would be an organ between the cells. Micros-
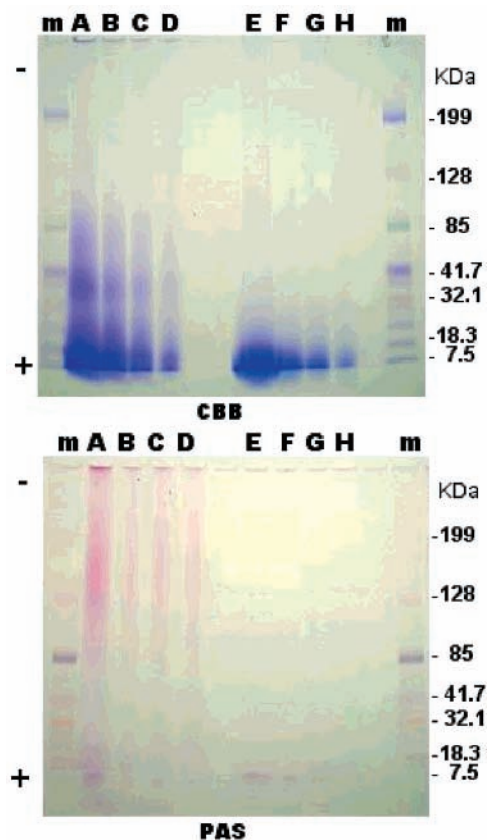

Figure 7. SDS-PAGE of alkali-solubilized DBCSS. DBCSS (1%) was dissolved in 1 M NaOH at 100 °C for 20 min, the supernatant was collected and neutralized by 1 M HCl, and the solution was dialyzed overnight. The freeze-dried sample (1%) was treated with 1% SDS and mercaptoethanol and analyzed on SDS-PAGE (5–20% gel) with 20 mA for 75 min. Staining was with CBB and PAS: (lane A) sample size = 20 μL; (lane B) sample size = 10 μL; (lane C) sample size = 5 μL; (lane D) sample size = 2.5 μL; (lane E) sample size = 20 μL; (lane F) sample size = 10 μL; (lane G) sample size = 5 μL; (lane H) sample size 2.5 = μL; (lane m) standard markers.

copy observation showed that a piece of the paired-hexagonal organ was found between the soybean cells or in the cells of the enzymatic digestion (**Figure 10C**). A small amount of filamentous organ was also found; it could be a binding for each cell.

DISCUSSION

The complex of protein and oil bodies was isolated, and the characteristics and the composition were investigated. A cell wall digestion was done by the formation of single cells by autoclaving and stirring (*6*, *7*) and the cellulase and pectinase treatment (*7*), and then the oil and protein body complex (BCSS) in the cells could be isolated from the soybean seed. The DBCSS consisted of an alkali-extractable protein and carbohydrates and the insoluble shell of DBCSS (SDBCSS). The obtained BCSS consisted of proteins (76.53 ± 0.91%) and sugar (3.25 ± 0.07%), which was rich in mannose (**Tables 1** and **2**). According to the study of Fischer et al. (*1*), the residual carbohydrates after a heat and enzymatic treatment was done were rich in glucose from cellulose. Their results are not the same as our results. In our studies, the cellulose of the cell wall was well digested by single-cell formation and cellulases. The result of the analysis of the amino acids of the SDBCSS was similar to their result. It was rich in Gly, Ala, Leu, and Val, which are hydrophobic
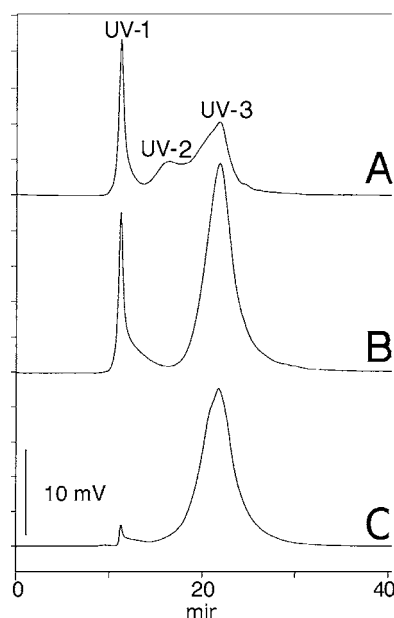
Figure 8. HPLC analysis of the extraction of DBCSS, SDBCSS, and the alkali-boiling treatment of the extraction of DBCSS. The samples were prepared by alkaline-boiling extraction, neutralized with 1 M HCl, dialyzed, and freeze-dried. The alkaline-boiling extraction from DBCSS was done by 0.1 M sodium carbonate buffer, pH 10, boiling for 20 min, and the extraction of SDBCSS was done by 1 M NaOH, boiling for 20 min. The samples of the freeze-dried extraction were analyzed by HPLC. Each elution profile of the extracted sample is indicated by abbreviations: (**A**) extraction of DBCSS; (**B**) extraction of SDBCSS; (**C**) alkali treatment (boiling in 1 M NaOH) extraction of DBCSS. The absorbance signals at UV 280 nm are shown as UV-1, -2, -3. Sample concentration, 0.5%; sample size, 10 μL; column, TSK-Gel G3000SWXL (7.8 $\times$ 300 mm) (Toso, Tokyo, Japan); eluent, 0.1 M phosphate buffer, 0.2 M NaCl, pH 7.0; flow rate, 1 mL/min.

Table 3. Amino Acid Analysis of the Dissolved SDBCSS

amino acid	SDBCSS[a]	Fischer et al.[b]	av[c]
Ala	9.3	8.4	5.96
Arg	3.4	3.1	5.73
Asx	11.3	10.2	10.96
Cys	0.1	0.1	1.0
Glx	11.6	10.5	16.88
Gly	11.1	10.0	7.0
His	3.1	2.8	2.21
Ile	4.8	4.3	4.74
Leu	12.4	11.2	8.12
Lys	6.9	6.3	5.44
Met	1.9	1.7	1.38
Phe	5.3	4.8	4.40
Pro	3.6	3.3	6.42
Ser	1.9	1.7	6.47
Thr	1.4	1.3	4.17
Trp			0.83
Tyl	3.8	3.5	2.95
Val	8.2	7.4	5.37

[a] SDBCSS was hydrolyzed with 6 N HCl for 22 h at 100 °C (expressed as mol %). [b,c] Data from Fischer et al. (*1*) (expressed as mol %).

amino acids compared with the amino acid analysis value of the average soybean (*1*). However, a bias of a specific amino acid was not found (**Table 3**). The indigestible proteinous residues of the soybean meal reported by Fischer et al. could be a part of DBCSS.

DBCSS can be extracted and/or digested; most of the soybean seed or soybean meal will be digested. The food-processing
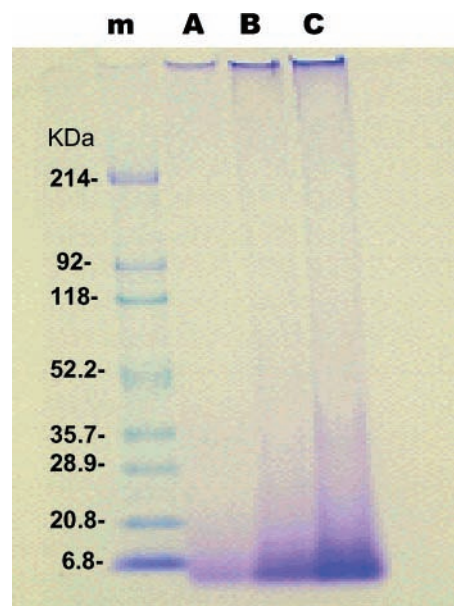

Figure 9. SDS-PAGE of SDS–mercaptoethanol-solubilized SDBCSS. SDBCSS (1%) was dissolved by SDS–mercaptoethanol at 100 °C for 24 h. The supernatant was treated with 80% ethanol, and the precipitate was washed with 80% ethanol. The precipitate was resolved in 10 mM phosphate buffer, pH 8.0, and was applied on a column of TOSO-HW50 equilibrated with the same buffer. The collected protein peak was collected and freeze-dried, and the sample solution (1%, 010 mM phosphate buffer, pH 8.0) was applied on SDS-PAGE (10–20% gel) in the Tris–tricine buffer system. Staining was with CBB and PAS: (lane A) sample size = 5 μL; (lane B) sample size = 10 μL; (lane C) sample size = 20 μl: (lane m) standard markers.

Table 4. Enzymatic High Digestion of Soybean Meal and Dehulled Soybean Seed[a]

	residual wt %	
procedure	soybean meal	soybean
autoclaving	35.6 ± 0.66	64.4 ± 0.54
cellulase treatment (enzymatic digestion of primary cell wall)	25.6 ± 1.60	55.8 ± 4.32
pectinase treatment (enzymatic digestion of secondary cell wall)	13.1 ± 1.00	48.0 ± 1.42
protease treatment (enzymatic digestion of DBCSS)	0.7 ± 0.10	<0.5

	differential wt %	
extracts and organ[b]	soybean meal	soybean
soluble extract (protein, carbohydrates)	65	36
oil[c]		20
primary cell wall (cellulose)	10	9
secondary cell wall (uronic acid, hemicellulose, protein)	12	8
body complex (protein)	13	27

[a] Digestion of soybean meal, dehulled soybean seed was carried out. Samples: $n = 4$; results means ± SD. Soybean meal and dehulled soybean seed (5 g) and 25 mL of water were mixed and then treated and digested by a series of procedures. The samples were centrifuged, and the composition of each supernatant was analyzed. Each of the residues was collected by centrifugation and washed with water, and the residue's freeze-dried weight was measured. [b] Each value was estimated by the differential weight of the residue of the sequential procedures. Values were terminated after the decimal point. [c] Oil content was estimated by weight of extraction using the Soxhlet method.

enzymes were screened for digesting the DBCSS, and an effective protease could be selected. Although some digestions using the examined proteases were recognized, only the Prolaser
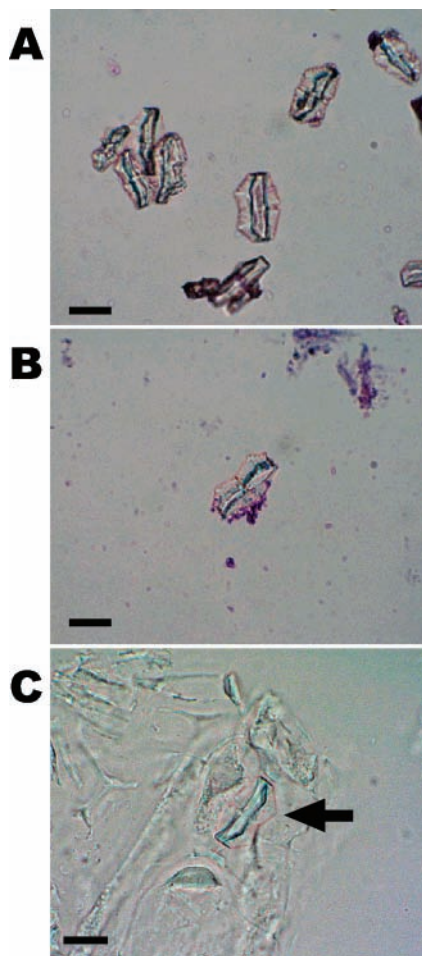
Figure 10. Final residue of the enzymatic digestion of soybean seed. SDBCSS was dissolved in 1 M NaOH or Prolaser (1%). The residues were observed by light microscopy: (**A**) residue of SDBCSS treated with boiling 1 M NaOH for 20 min; (**B**) residue of the enzymatic digestion of SDBCSS; (**C**) hexagonal organ found in the soybean cell. The soybean milk residues were autoclaved, cellulase and pectinase digestions were carried out, and the soybean cells in the enzymatic digestion (*15*) were observed. The residue was stained with toluidine blue O, and a small amount of the residual SDBCSS was detected (**A**, **B**). The arrow indicates the hexagonal organ in the cell (**C**). Black bar represents 5 μm.

of the alkaline protease could provide a significant digestion of the BCSS and SDBCSS. The BCSS preparation was established, and another screening to digest or study the BCSS would be possible. While this enzyme digestion was being examined, it was found that the protein in the BCSS could be easily extracted by sodium carbonate buffer (pH 10). This protein and the carbohydrates could be the same as the obtained high molecular weight protein after pressing the cells (*15*). The alkali-soluble weight of the DBCSS was 81.1 ± 0.25%, and it was found that the protein and carbohydrates were not a direct complex but would be indirectly combined by the native- and SDS-PAGE (**Figures 6** and **7**). The protein and oil bodies, or some organs consisting of proteins and carbohydrates, would be filled in BCSS. Further details for the allocation, the structure, and the components are now under study. The oil body is well-known to be in the BCSS and to consist of oleosin (24 and 16 kDa) and reported to give two isomers in the SDS-PAGE (*16−18*). The pattern of the two bands of DBCSS-LP in SDS-PAGE was similar to the pattern of the oleosin (*19*), but the molecular weight was not the same (*16−19*). Alkali treatment could denature the oleosin of oil bodies. The outside SDBCSS was

an insoluble protein using the sodium carbonate buffer, and its shell shape was left (**Figure 5**). The SDBCSS was not dissolved by even 8 M urea and 6 M guanidine hydrochloride, but was gradually dissolved by the SDS−mercaptoethanol boiling treatment, and gave a uniform low molecular weight protein (∼3 kDa). SDBCSS would be an agglutinate complex made of the protein. The inner contents of DBCSS and SDBCSS were easily digested by the selected alkaline protease. When the selected alkaline protease also acted on the single cells of soybean from the soybean seed, a digestion occurred in the cells. An oil body and BCSS in the cells were then clearly digested (**Figure 3**), and the primary and secondary cell walls remained. Four or five big oil drops were found in the cells as a result. The oil drops never fused into one by finger pressing. These results suggested that four or five spaces existed in the BCSS (**Figure 3**). The further structure should be studied in detail by observation such as scanning electron microscopy.

The advanced enzymatic high digestion of the soybean and the defatted soybean was done, and the digestion was almost completely achieved. As the result of doing these series of treatments for the defatted soybean and the soybean seed, both digestion yields were achieved at >99.5%. The differential weight of each treatment and digestion was the weight of the extractable or digestible substances; they included the water-soluble proteins and carbohydrates, the primary cell wall, the secondary cell wall, the soybean oil, BCSS, and SDBCSS. The knowledge of each organ weight would be useful for studying the food processing of soybean (**Table 4**). As for the defatted soybean, the soybean oil was already removed, the cells were destroyed by pressing, and it was shown that an extraction occurred much more readily than the extraction and enzyme digestion in the soybean seed. That is, a mechanical, physical, and/or chemical treatment would denature the organ (*1*). The denaturing is essential to digestion (*1*).

Principal organs in the soybean seed and the extraction and the digestion have been investigated (*6, 7, 15*). We finally achieved a high digestion of soybean using heat−humidity (autoclaving) and effective and selective combinations of enzymes. Another combination of enzymes for digestion can also be considered, and a better selection of the best combination is possible. Our results of detecting and obtaining the main organs of the soybean would be useful in screening for enzyme digestion, food processing, and studies of the components of the organs of the soybean and/or the localization of food functions. Furthermore, our method would be applicable to other agroproducts.

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