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Extraction of Soybean Oil from Single Cells

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Single cells prepared from autoclaved soybeans and cellulase treatment of the cells were effective in digesting the cell walls of and extracting the oil from soybeans. The first cell wall of the soybean single cell was completely removed using cellulases; the thin and transparent second cell wall of the cell was swollen. Oil in the cell formed spherical or hemispherical oil drops, and oil leaking from the oil bodies was observed. The oil was almost retained within the second cell wall. Water-extractable substances were obtained at ∼>60% of the weight. Flotation of oil drops by centrifugation was easily done. Ambient *n*-hexane extraction was also possible; however, residual oil remained in the oil bodies. Protease or peptidase digested the structure of the oil bodies; however, separation of the oil and the hydrolysates was impossible. The oil from the oil bodies was obtained effectively (>85%) by pressing the single cells and/or cellulase-treated single cells.

KEYWORDS: Soybean oil; oil extraction; cellulase; single cell

INTRODUCTION

The purpose of this paper is to clarify the possibilities, effects, or limits of extraction of soybean oil, protein, and saccharides using the preparation of single cells and enzymes on a laboratory scale. Most of the conventional studies on enzyme-assisted extraction of soybean oil involved pretreatment and assistance before grinding of the soybeans or solvent extraction. We thought that investigation of the possibility and the actual effect of a method using single cells would be useful for determinating the limit of oil extraction without grinding or a new soybean processing.

Soybean oil is widely used and is an important foodstuff, accounting for ∼30% of the oil production from seeds and fruits (*1*). Soybeans contain only 20% oil, and the extraction is generally done by using a solvent such as hexane on a commercial scale. However, milder methods are needed for human consumption and environmental protection.

Fullbrook (*2*) reported the use of enzymes for processing oilseeds, and the use of enzymes to obtain much higher oil or protein extracts has been studied during the past two decades (*3*, *4*). However, in the case of soybeans, this is very difficult due to their low oil and high protein contents. Therefore, many researchers have been trying to perform efficient soybean oil extraction. Smith et al. (*5*) reported an extraction using a powerful mechanical expeller and pretreatment using enzymes; however, the extraction has not actually been done yet. Rosenthal et al. (*6*) studied the efficient extraction of protein and oil from soybeans using enzymes and reported that proteases were effective. Dominguez et al. (*7*) reported enzyme-assisted soybean extraction by hexane and reported ∼5% improvement by the use of cellulase.

A soybean is a cell assembly with a hard shell of the cell wall, and the oil exists in the oil bodies in the cell (*8*−*10*). Therefore, breaking of all the cells is needed to obtain the oil (*11*), and cellulase, hemicellulase, and protease can generally assist in the extraction recovery. However, after the cell wall has been broken, the soybean protein and the oil simultaneously seep out and form an emulsion of oil and water that is difficult to isolate.

We have attempted digestion using cellulase for raw or boiled soybeans and have observed that the digestion of the cell wall had little or no effect. Considering these facts and reports, we investigated an effective extraction method for soybeans without cell breakage. As a result, we found that single cells of soybeans were easily prepared by autoclaving, the first cell wall was easily removed by cellulase, and the oil was almost retained in the single cell, shrouded by the second cell wall. There are few reports on oil extraction using the single cells and enzymes. Actual actions of enzymes or the change in soybeans have been little reported, although the information is useful to show not only the possibility of a new strategy for the extraction of soybean oil but also the use or processing of soybeans.

MATERIALS AND METHODS

Soybeans. Typical Japanese soybeans (*Glycine max* L.), a Tsurunoko, cultivated in Hokkaido, Japan, were commercially obtained. The *n*-hexane-extractable oil content of the starting raw soybeans using a Soxhlet apparatus was estimated to be 17.6%, and the dry weight loss was 5.7%. The starting soybeans were cracked into four parts and dehulled.

Enzymes. Cellulase, 1000 units/mL, carboxymethyl cellulose hydrolase activity, was a kind gift from Daiwa Kasei Co., Ltd., Osaka,

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Japan. Hemicellulase, hemicellulase Amano 90, xylanase activity 0.20 unit/mg, and peptidase from *Aspergillus oryzae* for food, peptidase activity 70 units/g, were gifts from Amano Pharmacy Co., Ltd., Nagoya, Japan. Protopectinase, 350 units/mL, was a kind gift from Kurabo Co., Ltd., Osaka, Japan. Trypsin from lyophilized pancreas powder, 12400 units/mg of protein, was purchased from Sigma Chemical Co. St. Louis, MO.

Analysis. The amount of sugars or cellulolytic hydrolysis was estimated as glucose according to the Nelson−Somogyi method (*12*), or the total sugars were analyzed by using the phenol−sulfuric acid method (*13*). Proteins were estimated as serum albumin according to the Lowry method (*14*). The amounts of peptides or proteolytic hydrolysis were estimated as the amount of tyrosine using Folin−Ciocalteu's phenol reagent. The amount of hydrolysis was calculated from a standard curve of colorimetric values and known concentrations of glucose, serum albumin, and tyrosine. The water-soluble substances were estimated as the freeze-dried weight.

Soybean Treatment and Preparation of Single Cells. The cracked soybeans were dipped in 5 parts of water at 4 °C overnight. The dipped soybeans were filtered through paper, 5 parts of water was again added, and the mixture was then autoclaved at 121 °C for 10 min. After filtration using paper, the autoclaved soybeans were crushed into a paste with a spatula in water or enzyme reaction buffers. The buffer systems used were cellulase, 0.1 M acetate buffer, pH 5.0, hemicellulase, 0.1 M phosphate buffer, pH 6.5, protopectinase, 0.1 M phosphate buffer, pH 8.0, peptidase, 0.1 M Na_2CO_3−NaOH, pH 9.0, and trypsin, 0.1 M Tris-HCl, pH 7.5.

Cellulolytic Enzymatic Treatment. Cellulolytic enzymes were added to 1 mL of the soy paste (50% w/v) described above and kept at 40 °C for 18−24 h with or without stirring. The reaction mixture was centrifuged, 3000 rpm for 10 min, and the supernatant was analyzed.

Oil Content of Single Soybean Cells. The whole oil content of the single soybean cells was estimated as follows: The single soybean cells were freeze-dried, and the *n*-hexane-extractable oil using a Soxhlet apparatus was weighed. The oil weight was measured using an electric balance, type ER182A, A&D Co., Ltd., Tokyo, Japan (minimum weight, 0.1 mg; standard deviation of measurement values, 5.57×10^{-5} g).

Oil Flotation and Extraction. One milliliter of the reaction mixture of the enzymatically treated or untreated soybean paste was filled to 5 mL with water, mixed, and then centrifuged at 3000 rpm for 10 min. The oil extraction from the floating oil was quietly done by twice adding 2.5 mL of *n*-hexane.

Quantification of Oil Content in Enzyme Treatment. The *n*-hexane-extractable oil content of a solution was estimated as follows: The filtrate was extracted with *n*-hexane, and the *n*-hexane was evaporated in a tared 10 mL flask, in vacuo at 40 °C; the weight of the residual oil was then determined.

Ambient Oil Extraction from Single Cells and Raw or Boiled Soybeans. The oil was obtained by vortexing 2.5 mL of *n*-hexane for 5 min. The *n*-hexane layer was recovered and evaporated in vacuo, and then the extracted oil was weighed.

Cellulase Treatment for Raw and Boiled Soybeans. The effect of cellulase on raw soybeans and boiled soybeans was investigated as follows: Raw soybeans (0.2 g) were dipped into 0.75 mL of 0.1 M acetate buffer in a test tube, and boiled soybeans were prepared from the raw soybeans that had been boiled for 10 min in boiling water. Cellulase (20 units) was added, and then the enzymatic treatment was done at 40 °C for 15 h. The soybean cells were sliced and observed by light microscopy. Oil extraction from the soybeans was done in the same manner described above, and the extracted oil was weighed.

Proteolytic Enzyme Treatment. Treatment by the proteolytic enzymes was done as follows: After treatment by the cellulase, the reaction mixture was centrifuged at 3000 rpm for 10 min, the buffer was changed to the proteolytic enzyme buffer, and the proteolytic enzyme was added. The reaction mixture was kept at 30 °C for 18 h, and the free oil was floated by centrifugation.

Pressure Extraction of Soybean Oil. A sample of single cells or the single cells after cellulase treatment was previously dried at 40 °C, and oil recovery was attempted. Oil seepage was done with a pressure jack, model P-16B, capacity = 700 kg/cm^2, Riken Seiki, Co., Ltd., Tokyo, Japan. An anvil was placed in the pressure jack, and two pieces of filter paper, 55 mm in diameter, no. 2 of Toyo Roshi Kaisha Co., Ltd., Tokyo, Japan, were placed on the anvil. The sample of treated or untreated soybeans was put on the two pieces of filter paper, and then two pieces of the same filter paper were overlaid on the sample. The pressure conditions were 150 kg/cm^2 for 5 min. The seeped oil was absorbed on the filter papers. After removal of the pressed soybeans, the absorbed oil was extracted with *n*-hexane, and the *n*-hexane was then evaporated in previously tared Erlenmeyer flasks in vacuo at 40 °C. The extracted oil was then weighed. A blank test was done in the same manner.

Microscopic Observation. The soybean samples were observed under a light microscope equipped with a microscopic digital camera, Olympus BH-21 and Olympus DP-II, Olympus Co., Ltd., Tokyo, Japan. Polysaccharides in the samples were stained by PAS stain and 0.5% of periodic acid for 5 min and washed with water; Schiff reagent was added for 15 min, followed by washing with 1% sodium hydrogen sulfite in 0.05 M HCl and washing with water. The protein of the sample was stained with acrolein−Schiff reagent, 0.5% of acrolein ethanol for 20 min, and washed with 95% ethanol for 5 min (three times); the same procedure was done for PAS staining. The oil in the sample was stained using Sudan stain, 50% ethanol for 2 min, 1% of Sudan III in 70% ethanol at 37 °C for 1 h, and washed with 50% ethanol for 1 min.

RESULTS AND DISCUSSION

Preparation of Single Cells of Soybeans. We investigated the pretreatment of cellulolytic enzyme digestion and found that the autoclaved soybeans were easily dispersed as single cells; the single cells were obtained while retaining the soybean oil in the cells. Light microscopic photos of the treated and stained protein, saccharides, and oil of soybean cells are shown in panels a, b, and c of **Figure 1**, respectively. The autoclave treatment was very effective for the single cell dispersion. Most of the cells dispersed as singles; however, the single cells were not fragmented. The surface of the cell was recognized to be a nearly translucent envelope. Generally, adhesive substances between the cells were well-known such as glycine or hydroxyproline-rich protein or galacturonic polysaccharides (*8*). Autoclaving would solubilize and remove these adhesives between the cells of the soybeans. The internal organization containing the oil bodies was well-stained purple-red (**Figure 1a**), the cell wall was stained in red and covered like a capsule (**Figure 1b**), and the oil present in the cells was stained orange (**Figure 1c**). The oil was detected both in the oil bodies and as free oil. The free oil was found in the cells, especially in the center part of the soybean, and various drops of oil were observed; however, the autoclaving treatment itself did not produce this free oil. **Table 1** shows the results of water and autoclaving extraction in a series of soybean treatments. The cool water dissolved proteins, sugars, and polysaccharides and turned a muddy white color, but the loss of oil was very low. The autoclave treatment also solubilized and extracted many proteins, sugars, and polysaccharides, and the solution turned a muddy yellowish color. These total water extraction weights were >33%; however, oil loss was not detected. The autoclaved soybean cells were softened and easily dispersed as single cells while retaining their shape and the soybean oil (>98%).

Effects of Cellulolytic Enzymes. The results of digestion using cellulase, hemicellulase, and protopectinase for the single cells are summarized in **Table 2**, and the microscopic figures are shown in **Figure 2a−c**. The most effective enzyme to digest the first cell wall was cellulase, and the form change of the single cells was drastic. The first cell wall was completely removed, and the second cell wall was swollen. Furthermore, the free oil formed a hemispherical or spherical shape (**Figure**

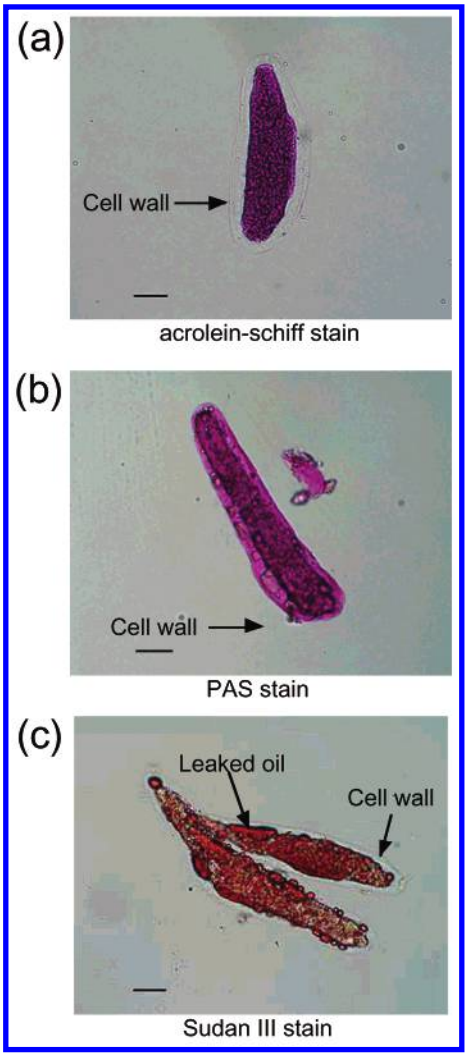

Figure 1. Light microscopy of stained single cells of soybeans: (a) the center of the cell was stained purple-red by acrolein–Schiff stain and was enclosed with the cell wall; (b) carbohydrates of the cell wall were stained red by PAS stain; (c) oil was stained orange by Sudan III. Black bar represents 10 μm length.

Table 1. Ratio of Extracted Substances from Soybeans by a Series of Procedures[a]

	solubilized substances (%)			
procedure	protein	total sugar[b]	oil[b]	water extract[c]
water extraction	1.9 ± 0.40	2.8 ± 0.51	ND[d]	10.3 ± 0.77
autoclave treatment	5.4 ± 1.85	6.1 ± 1.85	ND	23.5 ± 2.32

[a] Sample: $n = 5$; results are means ± SD. [b] Protein and total sugar value (percent) was calculated from the analysis. [c] The value was calculated on the basis of the starting weight of the soybeans. [d] Not detected.

2a). The free oil was easily floated and was obtained by destruction of the second cell wall with stirring by a magnetic bar, and the composition ratio of the fatty acid was the same as that of the residual oil in the oil body (data are not shown). However, the oil was well held within the second cell wall with no stirring (**Figure 2a**). The oil loss without mechanical stirring was only 1.6%. The ambient *n*-hexane extraction yield for the cellulase-treated cells was the highest. On the other hand, hemicellulase, mainly xylanase containing small amounts of amylase and cellulase, showed weak degradation. The released total sugars were more than with the cellulase treatment; however, the *n*-hexane extraction achieved only 35.4%. The cell

Table 2. Results of Digestion by Cellulolytic Enzymes and Oil Recovery by *n*-Hexane Extraction for Single Cells[a]

enzyme	used enzyme (units)	total sugar (mg)	protein (mg)	oil recovery[b] (%)
none	0	11000 ± 250	17900 ± 450	<1
cellulase	10	11300 ± 730	8200 ± 650	75.3 ± 13.5
hemicellulase	9	23900 ± 5700	15200 ± 850	35.4 ± 3.7
protopectinase	7	6200 ± 250	22000 ± 1240	1.7 ± 0.3

[a] Reaction conditions: soy paste solution (50% w/w) was incubated with each enzyme, kept at 40 °C, for 18 h. Samples: $n = 5$; results are means ± SD. Oil extraction was done with *n*-hexane by mixing under room temperature for 5 min. [b] Oil recovery was calculated on the basis of the raw soybean content (17.6% w/w).

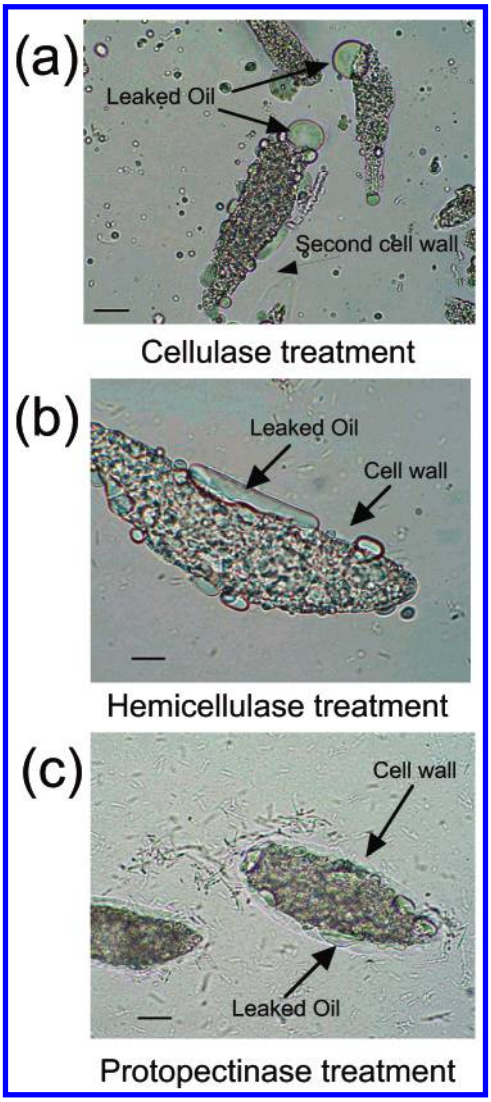

Figure 2. Light microscopy of single cells treated with cellulolytic enzymes: (a) cell treated with cellulase; (b) cell treated with hemicellulase; (c) cell treated with protopectinase. Black bar represents 10 μm length (a) and (c), and 5 μm length (b).

wall clearly remained during the hemicellulase use, so that the free oil in the cells did not form a sphere (**Figure 2b**). The effect of the protopectinase was low, the cell form did not change, and the oil was not extracted (**Figure 2c**). These results indicated that the cellulose component of the cell wall was very important for maintaining a rigid shape and also for holding the oil. Clearly, the cellulose component of the cell also hindered

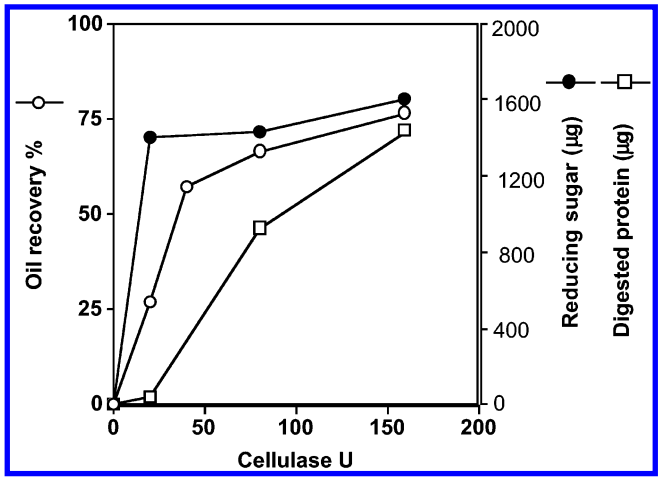

Figure 3. Single-cell paste (1 mL, 50% w/v) and the cellulase (20–160 units) were reacted, and the relationship between the amount of used cellulase and reducing sugar, the proteolytic hydrolysate, and the oil recovery by centrifugation was evaluated.

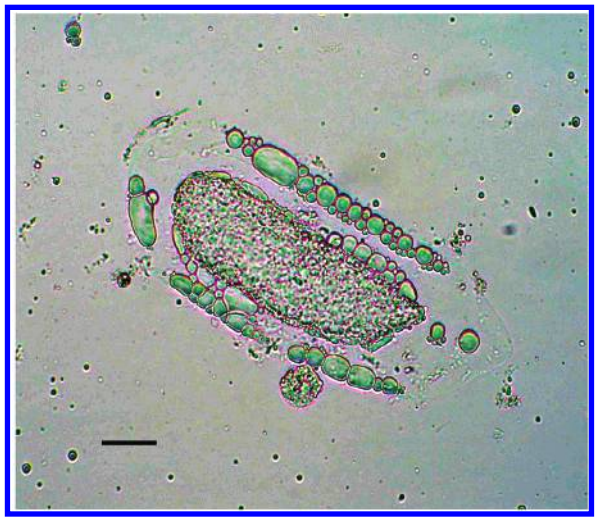

Figure 4. Light microscopy of leaked oil in the second cell wall. The single cell was treated with cellulase and was pressed using finger pressure. Conditions of enzymatic digestion: 20 units/single cells g; 40 °C for 18 h. Black bar represents 10 μm length.

the *n*-hexane extraction. Light microscopy observations of the cells after *n*-hexane extraction also supported the wet extraction yield; the oil in the cells and the cell wall were clearly detected.

Effect of Cellulase. Figure 3 shows the effect of the amounts of cellulase on the single soybean cells to obtain the oil. Reducing sugars and digested proteolytic hydrolysates were released and increased. The amount of the reducing sugars was not in proportion to the added amounts of cellulase. Cellulase at 10−20 units/g of single cells was sufficient to digest the cell wall. Proteolytic hydrolysates increased with the addition of cellulase. The thin and swollen second cell walls were broken by stirring, and then the floating soybean oil also increased. Static cellulase treatment gave the whole second cell walls and the oils (**Figure 4**). The increasing proteolytic hydrolysates were considered to be the result of residual protease contaminants. The contaminated proteases would be effective in the digestion of the oil bodies. From the microscopic investigation, oil leaking from inside the oil bodies was observed (**Figure 5**). The proteases would partially digest the oil bodies, and this action was considered to assist the oil leakage. Cellulase treatment of the single cells gave ∼28% weight loss as digested cell walls, protein, and sugar in the results. The total loss of weight from

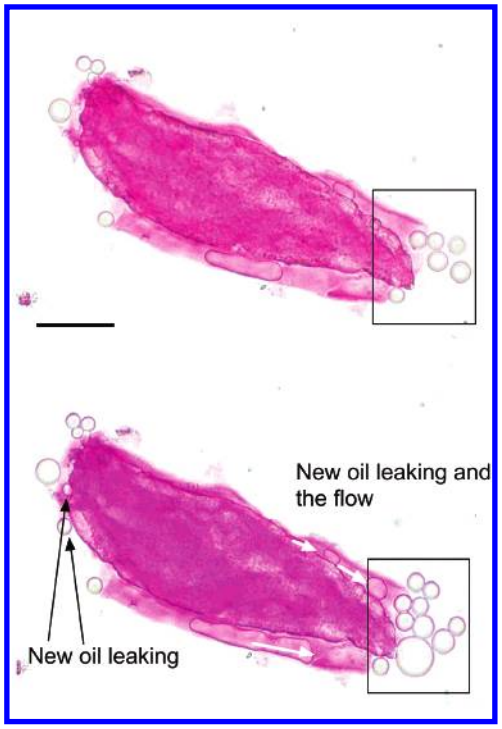

Figure 5. Light microscopy of leaking oil from oil bodies. Black arrows indicate leaking oil from the oil body and the flow. White arrows indicate the flow of leaking oil drops. Newly leaked oil drops are shown within the frame. Black bar represents 10 μm length.

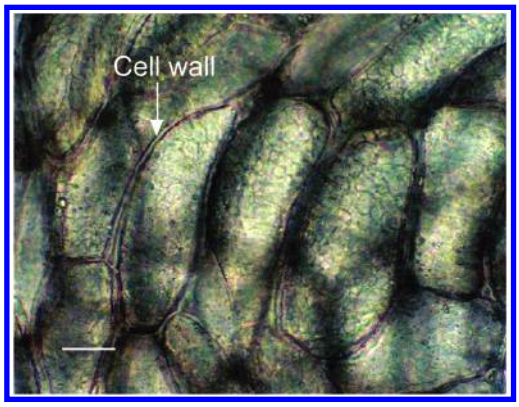

Figure 6. Light microscopy of slice of boiled soybeans treated with cellulase. White arrows indicate the cell wall and oil in the cell. White bar represents 10 μm length.

the starting initial dehulled raw soybeans was >60%, whereas the oil loss was only 1.6%. This result showed that the major part of the extractable and digestible materials in the soybeans was dissolved except the leaked free oil and the oil in the oil bodies.

We carried out the single-cell formation by autoclaving and then found that the cellulase treatment was effective for removing the first cell wall of the single cell. Many enzymes, especially cellulolytic enzymes, undergoing assisted soybean oil extraction have been studied. The general understanding is that soybean oil is contained in each cell, and the cell is mainly composed of cellulose and hemicellulose. Therefore, cellulolytic enzymes such as cellullase or hemicellulase were usually used in the first step of digestion to remove the first cell wall. However, the same cellulase could not digest the cells of raw or boiled soybeans as shown in **Figure 6**; cells were still tightly bonded to each other, and the cell wall and oil bodies were clearly observed. Ambient *n*-hexane extraction of the soybean

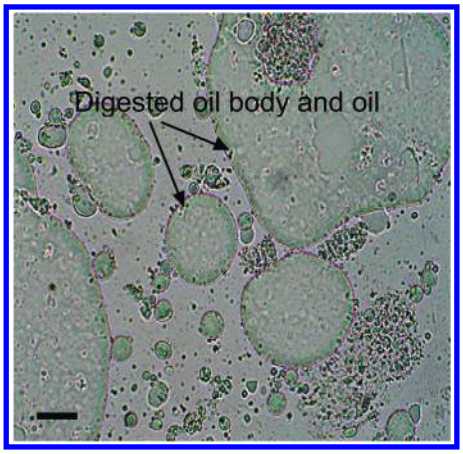

Figure 7. Light microscopy of oil and hydrolysates of floating layer from single cells of soybeans that were treated with peptidase digestion. Black bar represents 10 μm length.

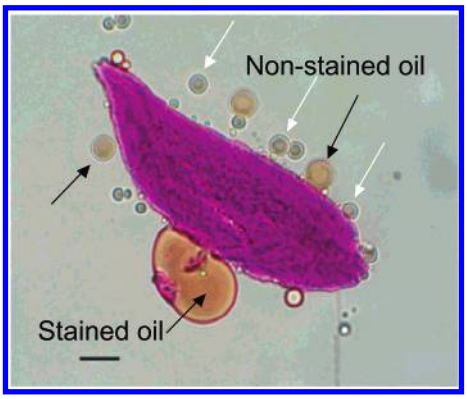

Figure 8. Light microscopy of the stained oil and nonstained oil newly leaking from the oil bodies due to finger pressure. Black arrows indicate stained oil and white arrows indicate nonstained oil which newly leaked from the oil bodies. Black bar represents 10 μm length.

oil was not detectable. The cell is a simple collection of cellulose capsules, but the cells are organized and coated by adhesive substances (*8*). The penetration of the enzymes is weak for the nonsingle cells, and the surface area is very low (*15*). On the other hand, our prepared single cells have a wide surface area, and the adhesive substance around the cell was removed. The cellulase would more easily digest the cell wall than the gathered or organized cells. The second cell walls were not at all digested by the enzymes tested in this experiment. Components of the second cell wall should be clarified, and an enzyme that can digest the second cell wall should be studied for more effective digestion.

Protease, Peptidase, and Trypsin Treatment. Proteolytic enzymatic treatment was considered to be effective for extracting the oil from the seeds. Therefore, we performed an additional treatment for the cellulase-treated soybean cells using several proteases and peptidases for food processing and then investigated the oil recovery and observed the results under a light microscope. Our best result was the use of peptidase from *A. oryzae*. The oil bodies were united, and fluid oil in the oil bodies was observed. A microscopic figure of the floating layer is shown in **Figure 7**. The flotation by centrifugation gave only a floating layer of pale brown hydolysates, and a yellowish oil was observed. The recovery of the oil was calculated to be 30−45% on the basis of the Soxhlet extraction of the raw soybeans with *n*-hexane. The soybean oil remained in the cells. **Figure 8** shows a visualization of this result; the stained oil was observed as an orange fluid, and the unstained oil was newly seeped out under microscopy by finger pressing. This means that residual oil was still present in the oil bodies.

Tzen et al. (*16*) reported that fusion of the purified oil bodies from sesame seeds was caused by trypsin treatment. We also tried to fuse the oil bodies; however, our trypsin treatment partially digested the oil bodies in the cell, but fusion of the oil bodies was not clearly observed. Probably, this would be why our obtained oil bodies were not purified as pure oil bodies; that is, proteins and/or saccharides which constitute complexes of the oil bodies would remain. These results indicated that oil leaking from the oil bodies was aided by proteolysis, but perfect leaking was difficult. Therefore, greater destruction of the structured or crude oil bodies is principally needed to extract more oil. Pressure oil extraction would be considered as a possible and effective method of obtaining oil from the oil bodies.

Pressure Extraction of Soybean Oil. Pressure oil extraction was investigated for cellulase-treated soybean single cells, non-cellulase-treated soybean single cells, and the raw soybeans. The optimum pressing condition was 150 kg/cm^2 for 5 min. The results of the recovery yield from these soybean samples were estimated to be 85.4 (SD = 3.09%), 85.4 (SD = 4.07)%, and 55.6 (SD = 9.3)%, respectively. Both the soybean single cells and the cellulase-treated single cells gave similar good extraction recoveries of the oil. This result suggested that the minimum condition for effective oil extraction was the removal of the adhesive materials of the cells and the preparation of single cells. Adhesive materials between cells could form a kind of cushion. Consequently, cellulolytic enzyme digestion was helpful but not essential.

In conclusion, we showed the possibility of effective digestion of soybeans and a non-solvent oil extraction by the preparation of the single cells of the soybeans. Cellulase was very effective in removing the first cell wall of the single cells but not the second cell wall. Oil leakage from the oil body was found; however, isolation of the oil itself from the digested oil body was difficult. Considering these results, digestion of the second cell wall and the structured oil bodies should be the next keys to study. We are now investigating the components of the residual second cell wall and the method of digestion. Today, soybean oil is generally extracted first with *n*-hexane, and then soybean protein is extracted from the defatted soybeans. We showed a backward method that used water extraction while retaining oil in the cells. Generally, defatted soybeans or okara, soybean residues, are recognized as hardly solubilized materials (*17*) because they are composed of high levels of fibers. However, our method using single cells and enzymatic digestion should be able to solve this problem.

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