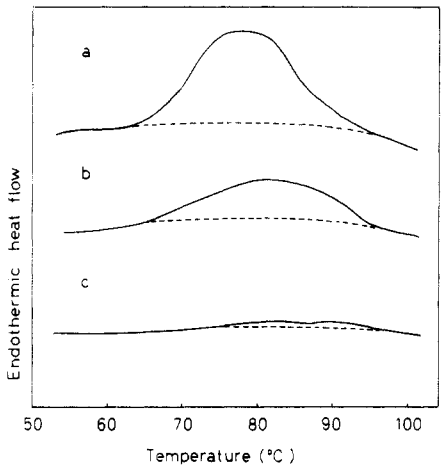

Figure 6. DSC patterns of native and heat-treated ovoinhibitors: (a) native ovoinhibitor; (b) insolubilized trypsin adsorbed fraction; (c) insolubilized trypsin unadsorbed fraction. Amounts of ovoinhibitor used for experiment were 3.43, 6.17, and 4.46 mg for native, insolubilized trypsin adsorbed fraction, and insolubilized trypsin unadsorbed fraction, respectively.

(Figure 6). The relative peak areas per protein weight of the absorbed and unabsorbed fractions on the insolubilized trypsin were 27% and 4%, respectively, of that of the native ovoinhibitor. Both the results of CD and DSC studies showed that the insolubilized trypsin absorbed fraction of heated ovoinhibitor was conformationally different from the native ovoinhibitor.

Similar results were obtained about partially heat-inactivated ovoinhibitor by using an insolubilised chymotrypsin column (data not shown). This seems to show that parts of the ovoinhibitor molecule having a chymotrypsin inhibitory activity behave the same way as those having a trypsin inhibitory activity. All these results suggest that heat treatment produces many kinds of denatured ovoinhibitor molecules of which domain structure is destroyed to a different degree.

ACKNOWLEDGMENT

We thank J. Yanagisawa for her skillful technical assistance.

Registry No. Trypsin inhibitor, 9035-81-8; chymotrypsin, 9004-07-3; ovoinhibitor, 62449-23-4.

LITERATURE CITED

Barrett, A. J. *Methods Enzymol.* **1981**, *80*, 771.
Beeley, J. G.; McCairns, E. *Biochim. Biophys. Acta* **1972**, *271*, 204.
Davis, J. G.; Zahnley, J. C.; Donovan, J. W. *Biochemistry* **1969**, *8*, 2044.
Deutsch, H. F.; Morton, J. I. *Arch. Biochem. Biophys.* **1961**, *93*, 654.
Laskowski, M., Jr.; Kato, I. *Annu. Rev. Biochem.* **1980**, *49*, 593.
Matsushima, K. *Nippon Nogei Kagaku Kaishi* **1958**, *32*, 211.
Osuga, D. T.; Feeney, R. E. In "Food proteins"; Whitaker, J. R.; Tannenbaum, S. R., Eds.; Avi Publishing Co.: Westport, CT, 1977; p 209.
Porath, J.; Aspberg, K.; Drevin, H.; Axen, R. *J. Chromatogr.* **1973**, *86*, 53.
Sato, Y.; Nakamura, R.; Yoshikawa, Y.; Takagi, K. *Nippon Nogei Kagaku Kaishi* **1960**, *34*, 1000.
Schwert, G. W.; Takenaka, Y. *Biochim. Biophys. Acta* **1955**, *16*, 570.
Sen, L. C.; Whitaker, J. R. *Arch. Biochem. Biophys.* **1973**, *158*, 623.
Waheed, A.; Salahuddin, A. *Biochem. J.* **1975**, *147*, 139.

Received for review December 20, 1982. Revised manuscript received December 13, 1983. Accepted February 27, 1984.

Improvement of Water Absorption of Soybean Protein by Treatment with Bromelain

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When 5 mL of 5% acid-precipitated soybean protein and 11S globulin were heated at 100 °C for 10 min and then treated with 0.5 mL of 5 mg/mL bromelain, the viscosity of the protein solutions gradually increased until the solutions coagulated after reaction times of 110 and 15 min, respectively. The coagula were soft gel and floated when shaken. The degradation product of 11S globulin treated with bromelain has a molecular weight of about 15 000. These fragments associated mainly through hydrophobic interaction and disulfide bonds. It was also observed through an electron microscope that the coagulation of 11S globulin formed a network structure accompanied by aggregation. The formation mechanism of the coagulum is believed to have begun when the protein strands formed by heat treatment were broken down and then varied strands were exposed to the surface and associated readily, until finally the network structure was formed. This enzymatic treatment improved water absorption of acid-precipitated protein and 11S globulin about 2–2.5 times that of the native ones.

Soybean protein isolates have high nutritional value and various functional properties. As a result, their consumption has increased steadily. In particular they have been used as an additive to meat and marine products because of their functional properties such as emulsifying, foaming, hydration, and other properties (Kato and Nakai, 1980; Nakai et al., 1980; Kato et al., 1981; Furukawa and Ohta, 1981; Voutsinas et al., 1983). Many researchers (Aoki et al., 1977; Hermansson, 1977; Yanagi et al., 1978; Kinsella, 1979; Furukawa and Ohta, 1981; Voutsinas et al., 1983) have tried to improve these functional properties by means of heat denaturation. But enzymatic modification

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0021-8561/84/1432-0486$01.50/0

has not been attempted often in spite of its effectiveness in unfolding proteins. Pour-El and Swenson (1976) reported that treatment with plant proteases resulted in the formation of a better gel than that with animal enzymes. Itoh (1975) found that plant proteases could coagulate soy milk, and Fuke and Matsuoka (1980) reported that heat-extracted soy milk was coagulated by treatment with ficin or bromelain. This study attempts to explain the coagulation mechanism of acid-precipitated soybean protein treated with bromelain and to find materials with good functions.

MATERIALS AND METHODS

Preparation of Acid-Precipitated Protein. Soybean meal defatted under low temperature was purchased from Yoshihara Seiyu Co. The protein from an aqueous extract of the defatted soybean meal was precipitated at pH 4.6, dialyzed against distilled water for 48 h and lyophilized.

Preparation of Crude 7S and 11S Globulin Fractions. Crude 7S and 11S globulin fractions were prepared by the methods of Thanh et al. (1975). Both fractions were dialyzed against distilled water and lyophilized.

Treatment with Bromelain. Stem bromelain was purchased from Wako Pure Chemical Industries, Ltd., Osaka [9.6×10^3 units/mg by the definition of Hagihara et al. (1958)]. The protein solution in a test tube (5 mL of 5% protein solution) was heated in a water bath at 100 °C for 10 min. Then the test tube was removed and cooled to 35 °C, 0.5 mL of bromelain solution was added, and the test tube was incubated at 35 °C.

Electrophoresis. The protein solution treated with bromelain was analyzed by polyacrylamide gel electrophoresis (PAGE) and SDS–PAGE following the methods of Davis (1964) and Laemmli (1970), respectively. Electrophoresis was carried out in 6.5 and 10% polyacrylamide gels according to the methods of Davis (1964) and Laemmli (1970), respectively. The gels were stained with amido black 10B and destained with 7% acetic acid.

High-Performance Liquid Chromatography (HPLC). Chromatography was performed with a Hitachi 638-30 liquid chromatograph. A TSK-Gel G3000SW column (65 cm × 7.5 mm) connected with a precolumn (10 cm × 7.5 mm) was used. The solvent used was 30 mM sodium phosphate buffer at pH 7.0 containing 0.1% SDS. The solvent flow was maintained at 0.4 mL/min, and the effluent was monitored with a Hitachi Variable-wavelength UV monitor at 215 nm.

Determination of Hydrophobic Region. The hydrophobic region was determined with 8-anilinonaphthalene-1-sulfonate (ANS) by the method of Takagi et al. (1979). ANS was obtained from Tokyo Kasei Kogyo Co. Concentration of the protein was 0.02% and concentration of ANS was 0.01 mM. The mixture of protein and ANS was left to stand at room temperature for 2 h. The relative value of fluorescence intensity was determined with a Hitachi fluorescence spectrophotometer MPF-4 with fluorescence excitation at 375 nm and reading of emission spectra at 475 nm.

Transmission Electron Microscope. One-millimeter cubes of gel were fixed in 2% glutaraldehyde at pH 7.2 and at 4 °C for 2 h, and after the glutaraldehyde fixation, they were fixed in 1% osmium tetraoxide in the same manner. The fixed samples were dehydrated through a series of increasing ethanol concentrations (50%, 70%, 80%, 90%, 95%, and absolute ethanol). The specimens were impregnated with propylene oxide/Epon 812 for 2 h, placed in pure Epon 812 solution for 2 h, transferred to fresh Epon 812, and polymerized at 45 °C for 24 h, and after that time the temperature was increased to 60 °C for an additional 12 h. Thin sections were stained with uranyl acetate and lead acetate and examined with a Hitachi H-700H electron microscope.

Table I. Coagulability[a] of Bromelain for Heated Acid-Precipitated Protein

enzyme concentration, mg/mL	−Cys	+Cys
1	>180	130
2	170	130
3	160	100
4	160	100
5	110	60
6	110	60
7	110	20
8	70	20

[a] Coagulability is expressed as the time (min) required for coagulation (the content does not run down when the tube is placed up side down) of 5 mL of protein solution with 0.5 mL of enzyme solution at 35 °C.

Water Absorption. Water absorption was determined by the method of Hutton and Campbell (1977). Protein samples of 0.5 g each were transferred to preweighed centrifuge tubes. Twenty milliliters of water was added to each and the samples were stirred at room temperature for 30 min. After centrifugation for 30 min at 1500*g*, the supernatant was decanted and the centrifuge tubes with residue were placed mouth down at an angle of 15–20° on paper towels in an air oven at 50 °C and allowed to drain and dry for 25 min. The samples were cooled in a desiccator for 30 min and weighed. The water absorption or hydration capacity was calculated as the difference between hydrated and original weight and expressed as a percentage of the original weight of the sample.

RESULTS

Coagulability of Bromelain for Acid-Precipitated Protein. Five milliliters of acid-precipitated protein was digested with 0.5 mL of bromelain solution of various concentrations at pH 7.0, 35 °C (Table I). When the protein solution preheated at 100 °C for 10 min was treated with bromelain, its viscosity and turbidity increased gradually, and it did not run down even when the tube was placed upside down. The protein solution that was not preheated produced precipitate when treated with bromelain. The coagulation of the preheated protein was accelerated in the presence of L-cysteine as shown in Table I. Since bromelain is a protease whose active site has a SH group, its proteolytic activity increases when L-cysteine is present. Coagulation was not observed when an inactivated enzyme was used. Therefore, it was proved that coagulation occurred as a result of proteolysis.

Coagulability of Bromelain for 7S and 11S Globulins. Acid precipitated protein consists mainly of 7S and 11S proteins. Crude 7S and 11S globulin fractions were both prepared and treated with bromelain but coagulation was observed only in the case of the 11S fraction. Thus, coagulation of acid-precipitated protein with bromelain was caused by the action of bromelain on 11S globulin. 7S and 11S globulins and their products treated with bromelain were analyzed by PAGE and SDS–PAGE (Figure 1). All of the subunits of 7S (α, α', and β) and 11S (acidic and basic subunits) were almost completely degraded by bromelain. Therefore, the above phenomenon was not caused by any difference in digestabilities of 7S and 11S globulins but by a difference in the characteristics of the proteins.

Process of Coagulation of 11S Globulin. Figure 2 shows SDS–PAGE of 11S protein treated with bromelain. 11S coagulated at a 15-min incubation time, and at that

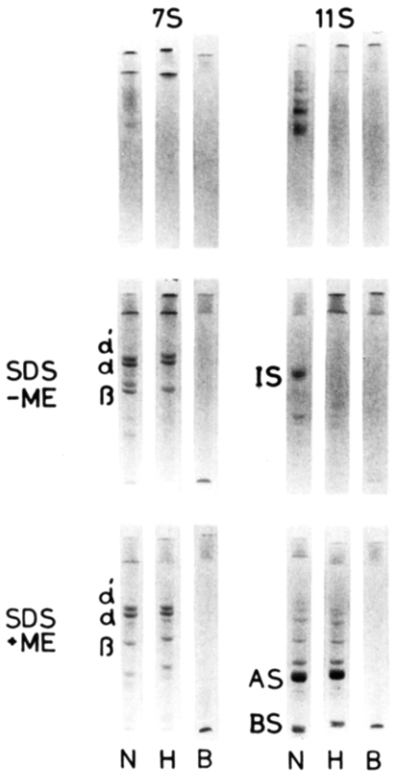

Figure 1. Analyses of 7S and 11S globulins treated with bromelain by PAGE and SDS–PAGE. Proteins were treated with bromelain in 30 mM phosphate buffer, pH 7.0. Aliquots were subjected to electrophoresis. N, native; H, heated; B, treated with bromelain. AS, acidic subunit; BS, basic subunit; IS, intermediary subunit.

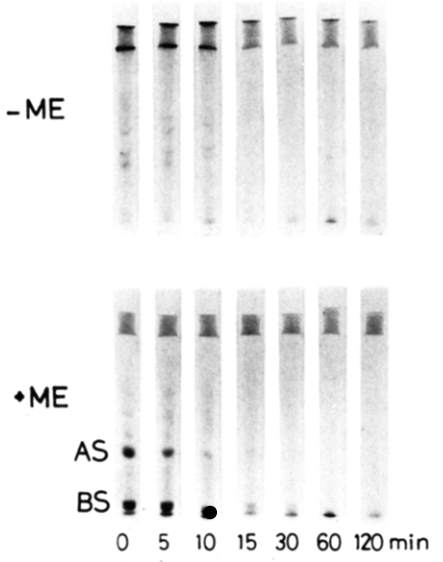

Figure 2. Time course for digestion of heated 11S globulin analyzed by SDS–PAGE. Heated 11S globulin was degraded under the same conditions as given for Figure 1.

time it was observed that both acidic and basic subunits were almost degraded when examined by SDS–PAGE with 2-mercaptoethanol. When no mercaptoethanol was used, there was an aggregate that could not migrate into the stacking gel, because the disulfide bonds could not be dissociated. The sizes of the hydrolyzed protein aggregates were anlayzed by HPLC (Figure 3). The aggregate produced by preheating (void volume) remained after 120 min of enzymatic treatment when analyzed with HPLC using a system without mercaptoethanol but disappeared in the mercaptoethanol system. This indicates that the aggregate

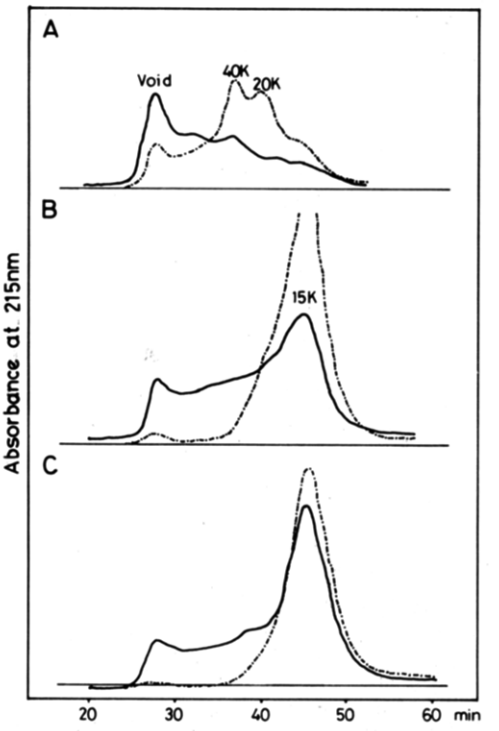

Figure 3. High-performance liquid chromatography of enzyme-treated 11S globulin. Operation conditions were as follows: column packed with TSK-Gel G3000SW; elution buffer of 30 mM phosphate buffer, pH 7.0, containing 0.1% SDS; flow rate of 0.4 mL/min. (A) Only heated; (B) reaction time, 10 min; (C) reaction time, 120 min. (-·-) With 2-mercaptoethanol; (—) without 2-mercaptoethanol. Molecular weight is shown in the figure.

Table II. Fluorescence Intensity[a] of 11S Globulin–ANS Complex

	fluorescence
native	1.00
heated	2.30
treated with bromelain	0.77

[a] Fluorescence intensity is represented as the relative value against that of native 11S globulin.

has disulfide bonds. The peaks of acid and basic subunits disappeared after reaction for 10 min, and a peak that corresponds to 15 000 molecular weight reappeared.

In addition, the hydrophobic region of 11S protein treated with bromelain was determined (Table II). The hydrophobic region increased with heating but decreased with enzymatic treatment. That is, the protein was denaturated by heating, and the basic subunits that have strong hydrophobicity might be exposed to the surface. Therefore, the amount of ANS binding to the protein increased. But the amount of ANS binding to the degradation products of 11S globulin (enzymatic treatment) decreased, because they associated through hydrophobic interaction in addition to disulfide bonds.

Network Structure of the Coagulum. Figure 4 shows micrographs of the network structure of 11S globulin treated with the enzyme. Unlike a heat-induced gel, fragments forming the network structure were broad, rough, and flossy. This coagulum was not self-supporting, and when shaken, it moved easily like yogurt. These properties are believed to have come from this network structure.

Water Absorption of the Coagulum of Acid-Precipitated and 11S Proteins. Water absorption of both coagula of acid-precipitated and 11S proteins was about 2 times more than that of the native ones (Table III). But

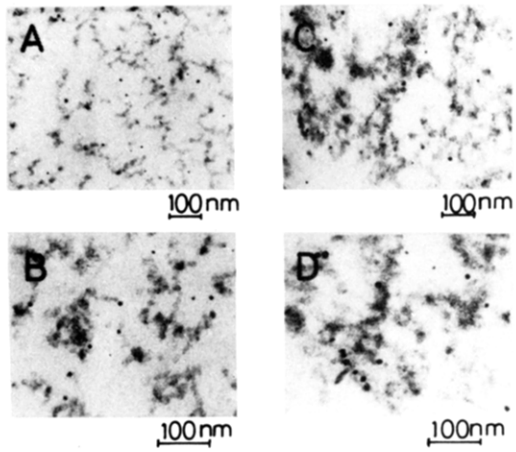

Figure 4. Transmission electron micrographs of heat-induced gel (A and B) and enzyme-induced coagulum (C and D) of 11S globulin. Magnification: 25000× (A and C), 40000× (B and D).

Table III. Water Absorption[a] of Enzyme-Treated Acid-Precipitated Protein and 11S Globulin

	APP	11S
native	224	272
heated	286	526
treated with bromelain	504	542

[a] Water absorption was calculated as the difference between hydrated weight and original dry weight and is expressed as a percentage of the original weight of the sample. APP: acid-precipitated protein.

no great change was found in hydration between heated 11S and enzymatically treated 11S globulin. When water was added to the lyophilized enzyme-treated product, a soft gel formed as before.

DISCUSSION

Stem bromelain has one reactive sulfhydryl group per molecule (Igunami and Murachi, 1963). The sulfhydryl group is essential for catalytic activity. The enzyme can be fully activated in the presence of cysteine. Table I shows that the coagulability of bromelain for acid-precipitated protein was also activated in the presence of cysteine. This indicates that coagulation was brought about through proteolysis.

Though bromelain hydrolyzed 7S and 11S globulins to the same extent, 7S globulin did not coagulate while 11S globulin coagulated after reacting with bromelain for only 15 min (Figure 1). The small fragments produced by enzymatic treatment migrated through the low-concentrated polyacrylamide gel, and the bands of proteolytic products of 7S were not seen in PAGE. On the other hand, in the case of 11S globulin treated with bromelain, there was an aggregate that could not migrate into the stacking gel, and this aggregate was seen in SDS–PAGE (without mercaptoethanol) and was degraded into small fragments as shown by SDS–PAGE (with mercaptoethanol). This aggregate resulted from disulfide bond interaction.

Bromelain prefers basic amino acyl residues, but the preference is not as strict as in the case of papain (Murachi and Neurath, 1964). The extent of hydrolysis of bromelain for 7S globulin seems to be the same as that for 11S globulin, as there is no remarkable difference in the contents of basic amino acyl residues of 7S and 11S globulins (Koshiyama, 1968; Catsimpoolas, 1971). 7S globulin is glycoprotein containing mannose and *N*-acetylglucosamine

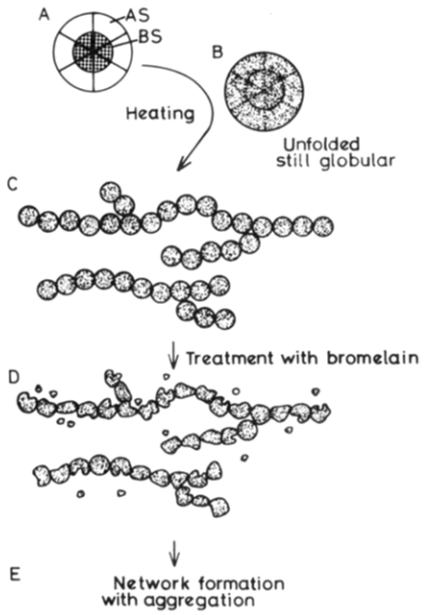

Figure 5. Coagulation mechanism of enzyme-treated 11S globulin.

(Koshiyama, 1967) and has only a small amount of disulfide bonds that take part in dissociation and association (Draper and Catsimpoolas, 1978). That is, 7S globulin is relatively hydrophylic, and being hydrolyzed by the enzyme after heat treatment, the digested fragments remained in the solution and did not associate again. On the other hand, 11S globulin has 22 mol of disulfide bonds and 2 mol of sulfhydryl groups per molecule (Draper and Catsimpoolas, 1978). Therefore, 11S globulin after heat denaturation was hydrolyzed by enzymatic treatment and the produced fragments associated again through disulfide bonds and exchange of sulfhydryl groups and disulfide bonds.

The time course of the treatment of 11S globulin with bromelain showed that the aggregate formed by heating at 100 °C for 10 min was degraded with time but a small amount remained after 120 min of reaction time (Figure 2, without mercaptoethanol). Basic subunits may be easily attacked by bromelain because they contain more basic amino acid residues than acidic subunits, but nevertheless, the former was degraded by the enzyme more slowly than the latter. When the protein coagulated after a 15-min treatment, the entire band of acidic subunits and most of the basic subunits disappeared, and a new band of protein having a lower molecular weight appeared (Figure 2, with mercaptoethanol). This fact shows that the basic subunits are surrounded by acidic subunits in native 11S globulin, and they are not easily attacked by bromelain in spite of heat denaturation (Kamata and Shibasaki, 1978).

The size of enzyme hydrolysis products of 11S globulin was about 15 000, and the products associated through disulfide bonds (Figure 3). Parts B and C in Figure 3 have similar patterns, and 10 min of treatment was necessary for coagulation to occur.

After heat treatment, the amount of ANS bound to 11S globulin was 2 times greater than that for the native one. The hydrophobic region was buried in the inner part of the molecule and ANS could not easily bind to it. This result agreed with the report of Takagi et al. (1979). The hydrophobic region of enzymatically treated 11S globulin after heat treatment was buried again and the amount of ANS bound to it decreased (Horiuchi et al., 1978).

From these results, the coagulation mechanism of 11S globulin by bromelain can be deduced as follows (Figure

5). Acidic subunits surround basic subunits in 11S globulin (A), and by heat treatment 11S globulin molecules are unfolded but still kept globular (B). Then they associate and form the aggregate whose molecular weight is 8 000 000 (C) (Nakamura et al., 1984). When the associate is treated with bromelain, its structure and characteristics change because cutting occurred in the strands or degradation products recombine (D). This association is believed to be due mainly to disulfide bond and hydrophobic interaction, because it dissociates into smaller fragments of 15 000 molecular weight in the presence of SDS and 2-mercaptoethanol. As shown in Figure 4, these strands form a network structure and also aggregates by getting entangled (E).

This coagulum with a soft gel appearance had good hydration. Water absorption of the enzyme-treated products of acid-precipitated protein improved about 2 times more than that of the native and heated ones. But there was no significant improvement in hydration of heated and enzymatically treated 11S globulin. Water absorption of the lyophilized coagulum of 11S globulin was less than that before lyophilization. Lyophilization seems to be the cause for this decrease in water absorption of the coagulum. However, the improvement of water absorption of acid-precipitated protein is seen to be advantageous for food materials.

Registry No. Bromelain, 37189-34-7; water, 7732-18-5.

LITERATURE CITED

Aoki, H.; Nagatomo, R.; Hotta, K.; Yamaguchi, H. *Nippon Shokuhin Kogyo Gakkaishi* **1977**, *24*, 618.
Catsimpoolas, N. *Int. J. Protein Res.* **1971**, *3*, 63.
Davis, B. J. *Ann. N.Y. Acad. Sci.* **1964**, *121*, 404.
Draper, M.; Catsimpoolas, N. *Cereal Chem.* **1978**, *55*, 16.
Fuke, Y.; Matsuoka, H. *Nippon Shokuhin Kogyo Gakkaishi* **1980**, *27*, 275.
Furukawa, T.; Ohta, S. *Nippon Shokuhin Kogyo Gakkaishi* **1981**, *28*, 451.
Hagihara, B.; Matsubara, H.; Nakai, M.; Okunuki, K. *J. Biochem. (Tokyo)* **1958**, *45*, 185.
Hermansson, A. M. *J. Food Technol.* **1977**, *12*, 177.
Horiuchi, T.; Fukushima, D.; Sugimoto, H.; Hattori, T. *Food Chem.* **1978**, *3*, 35.
Hutton, C. W.; Campbell, A. M. *J. Food Sci.* **1977**, *42*, 454.
Igunami, T.; Murachi, T. *Biochemistry* **1963**, *2*, 1439.
Itoh, H. *Daizu Kaihatsu* **1975**, *30*, 9.
Kamata, Y.; Shibasaki, K. *Agric. Biol. Chem.* **1978**, *42*, 4343.
Kato, A.; Nakai, S. *Biochim. Biophys. Acta* **1980**, *624*, 13.
Kato, A.; Tsutsui, N.; Matsudomi, N.; Kobayashi, K.; Nakai, S. *Agric. Biol. Chem.* **1981**, *45*, 2775.
Kinsella, J. E. *J. Am. Oil Chem. Soc.* **1979**, *56*, 242.
Koshiyama, I. *Agric. Biol. Chem.* **1967**, *31*, 87.
Koshiyama, I. *Cereal Chem.* **1968**, *45*, 394.
Laemmli, U. K. *Nature (London)* **1970**, *227*, 680.
Murachi, T.; Neurath, H. *J. Biol. Chem.* **1964**, *235*, 99.
Nakai, S.; Ho, L.; Helbig, N.; Kato, A.; Tung, M. A. *Can. Inst. Food Sci. Technol. J.* **1980**, *13*, 23.
Nakamura, T.; Utsumi, S.; Mori, T. *J.* Agric. *Food Chem.* **1984**, in press.
Pour-El, A.; Swenson, T. S. *Cereal Chem.* **1976**, *53*, 438.
Takagi, S.; Akashi, M.; Yasumatsu, K. *Nippon Shokuhin Kogyo Gakkaishi* **1979**, *26*, 133.
Thanh, V. H.; Okubo, K.; Shibasaki, K. *Plant Physiol.* **1975**, *56*, 19.
Voutsinas, L. P.; Cheung, E.; Nakai, S. *J. Food Sci.* **1983**, *48*, 26.
Yanagi, S.; Miyaguchi, H.; Saio, K.; Watanabe, T. *Cereal Chem.* **1978**, *55*, 157.

Received for review October 10, 1983. Accepted January 10, 1984.

Changes in Composition and Subunits in the Storage Proteins of Germinating Lupin Seeds

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The modifications occurring during 16 days of germination in weight, length, lipids, total nitrogen, water-extracted and salt-extracted nitrogen, and amount and protomer composition of water-soluble proteins and of each separate salt-extracted protein were studied in *Lupinus albus* seeds and seedlings. As germination proceeds, nitrogen shifts from the storage globulins to other compounds. The rate of breakdown differs for each globulin. The two major vicilins are degraded first and at different rates, indicating a structural diversity of the two proteins. Legumins follow. Conglutin γ undergoes no change over the period studied. Extensive interruption of the covalent continuity in the subunits of vicilins (globulins 4 and 6) likely makes the breakdown easier, while interpeptide disulfide bonds in legumins (globulins 8 and 9a) and in conglutin γ slow down proteolysis.

Storage proteins in seeds are mobilized during germination with resulting formation of amino acids and peptides, which serve mainly as sources of nitrogen and carbon skeleton in molecules of the developing seedling.

Proteolytic activity and breakdown of storage proteins during germination have been studied in various legume seeds: peanut (Bagley et al., 1963), pumpkin (Khokhlova, 1971; Lott and Vollmer, 1973), broad bean (Briarty et al., 1970; Lichtenfeld et al., 1979), pea (Basha and Beevers, 1975; Konopska, 1978, 1979), soybean (Catsimpoolas et al., 1968), and mung bean (Baumgartner and Chrispeels, 1978).

The mobilization of total storage proteins in lupin seeds of different species germinated in the dark and in the light was studied and proteolysis was found to occur earlier in seeds kept in the light than in those grown in the dark (Prus-Glowialki, 1975). Vicilin and legumin breakdown in lupin seeds was described by Morawiecka (1961). However no information is available on the behavior of the various proteins in each fraction and of their constituent protomers during the germination of the seed. Indeed, in previous work we separated the storage proteins from lupin seeds into six major and six minor oligomeric proteins (Duranti et al., 1981; Restani et al., 1981). It therefore appeared worthwhile to study the degradation rates of each

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0021-8561/84/1432-0490$01.50/0