AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON, D. C. 20523

BIBLIOGRAPHIC INPUT SHEET

FOR AID USE ONLY
BATCH #14

1. SUBJECT CLASSIFICATION

A. PRIMARY: Agriculture — AS10-0336-0000

B. SECONDARY: Food composition--Oil crops--Soybean

2. TITLE AND SUBTITLE

Enzymatic hydrolysis of alcohol-denatured soybean proteins

3. AUTHOR(S)

Fukushima,Danji

4. DOCUMENT DATE	5. NUMBER OF PAGES	6. ARC NUMBER
1969	14p.	ARC 664.33.F961e

7. REFERENCE ORGANIZATION NAME AND ADDRESS

Cornell

8. SUPPLEMENTARY NOTES *(Sponsoring Organization, Publishers, Availability)*

(In Cereal chemistry,v.46,p.405-418)

9. ABSTRACT

10. CONTROL NUMBER	11. PRICE OF DOCUMENT
PN-RAA-669	
12. DESCRIPTORS	**13. PROJECT NUMBER**
Digestion Enzymes Proteins Soybean	**14. CONTRACT NUMBER** CSD-1815 Res.
	15. TYPE OF DOCUMENT

AID 590-1 (4-74)

7

PA4

664.33
F961e

Enzymatic Hydrolysis of Alcohol-Denatured Soybean Proteins[1]

DANJI FUKUSHIMA[2], Central Research Institute, Kikkoman Shoyu Co., Noda-shi, Chiba-Ken, Japan

ABSTRACT

The effect of denaturation conditions on the enzymatic digestion of soybean proteins was studied. The maximum degree of hydrolysis was 5 to 10% higher in alcohol-denatured proteins as compared to water-denatured ones, although the initial speed of hydrolysis was slower in the former. During the denaturation treatments with both alcohol and water, and especially with water, some soybean flour oligosaccharides disappeared as the treatments continued. The maximum degree of hydrolysis also decreased with time of denaturation. However, it was ascertained that these oligosaccharides were not responsible for the decrease of maximum hydrolysis. Next, the effects of denaturation of soybean protein fractions on enzymatic digestibilities were examined and showed that the acid-precipitated fraction and whey fraction increased in their degree of maximum proteolysis after alcohol-denaturation, as compared with water-denaturation. The proteolytic behavior of the denatured proteins is discussed from the standpoint of the three-dimensional structure of soybean protein molecules.

Soybeans or defatted soybeans are widely used, not only as foods or feed, but also in the Orient as a material of soy sauce or miso. In the former, the soybean proteins are hydrolyzed by the proteinases of the gastrointestinal tract, and the degree of hydrolysis relates to their nutritive values, whereas in the latter they are hydrolyzed by the mold proteinases, and its degree relates to its yield or taste. Therefore, proteolytic hydrolysis is very important in soybean utilization.

Studies of the proteolytic hydrolysis of soybean proteins (1-4) indicated that native soybean proteins are resistant to hydrolysis by proteinases and that the initial velocities of proteolytic hydrolysis were proportional to the degree of structural disruption in the protein molecules. Others have also reported on the

---

[1] Presented at the 53rd Annual Meeting, Washington, D. C., April 1969.

[2] Address until August 31, 1970: Department of Food Science and Technology, New York State Agricultural Experiment Station, Cornell University, Geneva, N. Y. 14456.

relation between denaturing treatments of soybean proteins and their *in vitro* digestibilities (5-9). In these experiments the denaturing treatments involved variations in heat and moisture only.

The author reported previously that lower alcohols possess strong denaturing abilities toward soybean proteins and that they apparently act through mechanisms other than water (10). Therefore, the three-dimensional structure and enzymatic digestibilities should differ between alcohol- and water-denatured soybean proteins.

In this paper the enzymatic hydrolysis by various proteinases was compared between the soybean proteins denatured with alcohols and with water under the conditions for complete denaturation. The behavior of the enzymatic hydrolysis for the denatured proteins is discussed in terms of the three-dimensional structures of soybean protein molecules.

MATERIALS AND METHODS

Defatted soybean flour was prepared as described in the previous report (10).

For sugar-free soybean flour, the oligosaccharides contained in the defatted soybean flour were removed by extraction with saturated ammonium sulfate. The residue was dialyzed against distilled water and then freeze-dried. Total nitrogen was 11.00%.

Denaturation of soybean proteins with solvents was done by the method described in the previous report (10).

Enzymatic Hydrolysis of Denatured Soybean Proteins

Denatured soybean proteins were suspended in water and transferred quantitatively into digestion tubes. After addition of 10 ml. of a buffer solution, 10 ml. of an enzyme solution, and 1 ml. of toluol, the total volume was made up to 45 ml. by the further addition of water. The digestion tube was tightly stoppered and incubated in a 30°C. water bath with continuous gentle shaking, as described previously (2). After being shaken for the desired time, the digestion mixture was transferred to a volumetric flask, made up to 100 ml., heat-treated at 95° to 100°C. for 5 min., and filtered. At the same time, the blank test was performed in the same manner, except for inclusion of the protein sample. On these filtrates, trichloroacetic acid-soluble nitrogen and amino nitrogen were measured and corrected by the value of the blank.

The proteinase preparations used here were the crude proteinases and the purified alkaline proteinase from *Aspergillus sojae* (a yellow aspergillus), the crude acid proteinase from *A. saitoi* (a black aspergillus), the proteinase of *Bacillus subtilis*, the proteinase of *Streptomyces griseus*, pepsin, trypsin, and papain. For the crude proteinase preparation from *A. sojae*, the freeze-dried water extract from wheat bran koji of *A. sojae* KS was used. The refined alkaline proteinase preparation was made by the method of Mizunuma et al. (11). The crude acid proteinase preparation of *A. saitoi* was obtained by 70 vol.% ethanol precipitation from water extract of the wheat bran koji of *A. saitoi*. *B. subtilis* proteinase (Nagase) came from the Nagase Sangyo Co., Japan; the Kyoken Kagaku Co., Japan, supplied proteinase of *S. griseus* (Pronase); and Difco supplied pepsin, trypsin, and papain. Papain was activated with 0.1M KCN at pH 5.0 for 2 hr. at 40°C. before use.

The amounts of the proteinases used in each experiment were those giving a

hydrolysis of 80% for water-denatured soybean flour proteins (120°C., 60 min.). The total activities of each proteinase used are 160, 114, 1126, 66, 34, 164, 28, and 164 X 10^{-4} (PU) $^{30°FR}_{meq.\ tyr.}$ in *A. sojae* crude proteinases, *A. sojae* alkaline proteinase, *A. saitoi* crude proteinases, *B. subtilis* proteinase (Nagase), *S. griseus* proteinase (Pronase), pepsin, trypsin, and papain, respectively, when the activities were measured by the modified anson's casein method (1).

The buffer solutions used were sodium orthophosphate (mono-H) solution (final 0.1M-PO_4) in trypsin, the KCl-HCl buffer (final 0.01M - K^+) in pepsin, the citrate-HCl buffer (final 0.1M citrate) in *A. saitoi*, and the sodium orthophosphate (mono-H)-sodium orthophosphate(di-H) buffer (final 0.1M-PO_4) in other proteinases. The pH adjustment of the digestion mixtures in the case of pepsin and *A. saitoi* was made by addition of excess HCl to the buffer solutions. The digestion pH was 8.3, 1.7, 2.7, and 7.2 in trypsin, pepsin, *A. saitoi*, and other proteinases, respectively.

Trichloroacetic Acid(TCA)-Soluble Nitrogen

One volume of 1.2M TCA solution was added to 2 vols of the digestion filtrate described above, with vigorous shaking. After standing overnight at room temperature, the mixture was centrifuged at 12,000 times *g* for 20 min. The nitrogen contained in the supernatant was determined by the semimicro Kjeldahl method. Total of the TCA-soluble nitrogen produced by the enzyme was expressed as percent for the nitrogen contained in the sample.

Amino Nitrogen

Twenty milliliters of the digestion filtrate was transferred into a 200-ml. beaker and made at pH 8.5 with NaOH, followed by the addition of 10 ml. formaldehyde solution. The resultant mixture was titrated by 0.05N NaOH solution, until the pH reached 8.5 with the Model HM-5A pH meter of Toa Dempa Co., Japan. The total of the formol nitrogen produced by the enzyme was expressed as percent of total nitrogen of the sample or of the formol nitrogen of the acid hydrolysate (soybean flour:8N sulfuric acid = 1:6) of 24 hr. digestion at boiling point.

Sugar Analysis

Extraction of Sugars from Samples. The denatured sample corresponding to 4 g. before denaturation was treated for 1 min. in a blender with 40 ml. of 80 vol.% ethanol, transferred quantitatively to a 200 ml. flask, and refluxed for 3 hr. The resultant mixture was filtered hot, and the residue was washed with hot fresh ethanol of the same concentration. By this procedure, the sugars contained in the sample were extracted into ethanol solution. The further extraction was repeated twice. All the filtrates and washing solutions were combined and concentrated to one-fifth volume by heating under reduced pressure. A 10% lead acetate (neutral) solution was added to the concentrated solution, until no further precipitation occurred. The resulting precipitate was separated by centrifugation and washed with a little water. The supernatant and the wash water were combined together, 0.5 g. of calcium carbonate was added, and hydrogen sulfide gas was bubbled into it to remove lead. After the centrifugation, the supernatant was concentrated below 5°C. under vacuum, transferred to a 10-ml. volumetric flask, filled up to mark with water, mixed, and filtered. The filtrate was used as a preparation for paper chromatography.

Development and Identification of Sugars on Paper Chromatography. A portion (0.03 ml.) of the preparation described above was spotted on a Toyo No. 51 filter paper and developed by n-butanol-pyridine-water (60:40:30) (12) or ethyl acetate-pyridine-water (25:10:35) (13). These solvents were irrigated in the ascending direction with a triple or quadruple development technique. Separated sugars were detected and identified by the following reagents:

1. Aniline-diphenylamine-phosphate (12): 5 vols. of 4% solution of aniline in 95% ethanol, 5 vols. of 4% solution of diphenylamine in 95% ethanol, and 1 vol. of syrupy phosphoric acid were mixed before use, sprayed on dried chromatogram strips, and heated for 5 to 10 min. at 110° to 120°C. to allow the color to develop.

2. Aniline hydrogen phthalate (14): the reagent was prepared by adding aniline (0.93 g.) and phthalic acid (1.66 g.) to water-saturated n-butanol (100 ml.), sprayed on dried chromatogram strips, and heated for 5 to 10 min. at 110° to 120°C. to allow the color to develop.

3. Alpha-naphthol (15: 50 ml. of 1% (w./v.) solution of α-naphthol in ethanol and 5 ml. of phosphoric acid were mixed before use, sprayed on dried chromatogram strips, and heated for 10 min. at 50°C. to allow the color to develop.

Quantitative Determination of Sugars. The Dubois method (16) was modified as follows: Two sheets of Toyo No. 51 filter paper 24 by 40 cm. were prepared as described below. One of the sheets was used as a blank. Before a sample solution was placed on the paper, two lines were drawn lengthwise 4.5 cm. from edge of paper, and one line was drawn at 7 cm. from the foot. On the 7-cm. line of the latter section of the paper, 0.03 ml. of sample solution to be analyzed was applied precisely with a micro pipet in the form of five spots with equivalent intervals of 3 cm., and on the two strips 4.5 cm. from the edge, an amount enough for color development.

After the triple ascending development technique by n-butanol-pyridine-water (60:40:30) was applied to the spotted paper, the 4.5 cm. strips, which were the resulting strips, were cut off from the developed chromatogram, sprayed with the alpha-naphthol-phosphoric acid reagent, and heated for 5 min. at 90°C. With the aid of the treated-marking strips, appropriate sections were then cut from the unsprayed center portion of the chromatogram, and each section was transferred to petri dishes. The blank paper was cut up to correspond to the area and location of the sugars of the other paper. Distilled water (15 ml.) was added to each of the petri dishes, which were then covered, allowed to stand for 2 hr. with occasional shaking, and filtered through glass wool. The sugar concentration in the eluates was determined by the anthrone method of Dimler et al. (17) and corrected by that of the corresponding blank paper.

The anthrone method was as follows: The eluate was diluted so that the sugar content was in the range of 3 to 30 γ per ml. Three milliliters of the resultant diluted eluate was put into the test tube and cooled in an ice-water bath. Next, 6 ml. of the cooled anthrone reagent (prepared by dissolving 0.2 g. of anthrone in 100 ml. of concentrated sulfuric acid) was added to it gently. The contents were mixed thoroughly, heated for 10 min. (± 15 sec.) in a boiling-water bath, and cooled to a cold bath. The optical densities of the resulting solutions were measured at 620 mμ against a blank of water plus reagent which was heated at the same time. The concentrations of sugars were determined from their respective standard curves.

RESULTS

Enzymatic Hydrolysis of Alcohol- and Water-Denatured Soybean Proteins

The enzymatic hydrolysis of alcohol-denatured soybean proteins by various proteinases was investigated in detail and in comparison with water-denatured soybean proteins. Conditions for complete denaturation were used for substrate soybean flour proteins both in alcohol- and water-denaturations.

Figures 1, 2, and 3 show the time course of hydrolysis of these denatured soybean proteins by the various proteinases. According to these figures, the maximum hydrolysis of alcohol-denatured soybean proteins by various kinds of proteinases was always higher than that of water-denatured ones. This does not

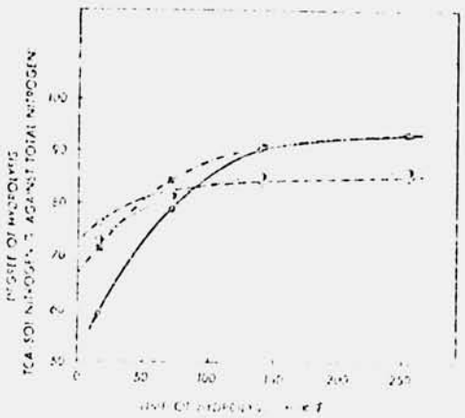


Fig. 1. Enzymatic hydrolysis of water- and alcohol-denatured soybean flour proteins by crude proteinases of *Aspergillus sojae* KS. Denaturation conditions: ○—90 vol.% ethanol, 90°C., 60 min.; ●—90 vol.% ethanol, 120°C., 60 min.; ◇—water, 90°C., 60 min.; ◆—water, 120°C., 60 min.

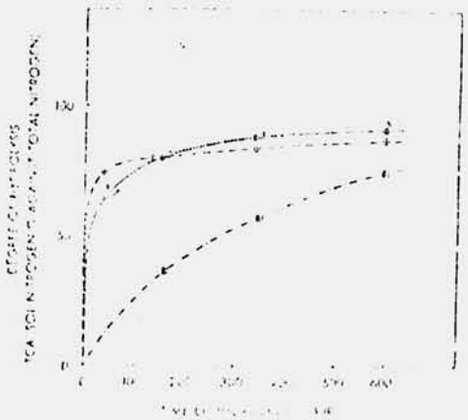


Fig. 2. Tryptic hydrolysis of water- and ethanol-denatured soybean flour proteins. Denaturation conditions: ○—90 vol.% ethanol, 90°C., 60 min.; ●—90 vol.% ethanol, 120°C., 60 min.; ◇—water, 90°C., 60 min.; ◆—water, 120°C., 60 min.

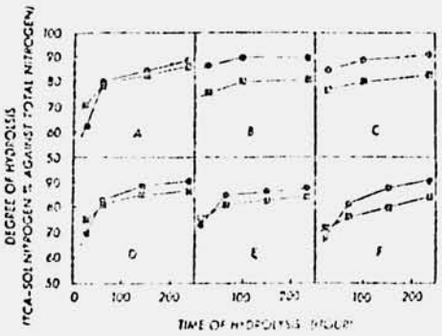
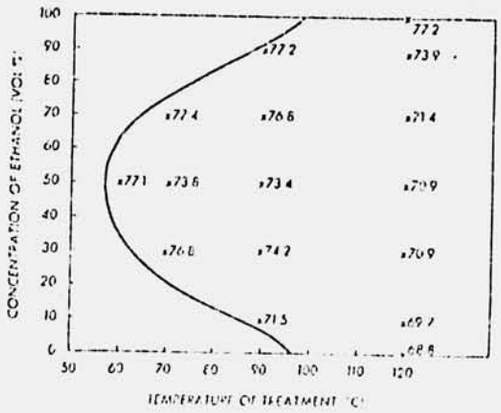


Fig. 3. Proteolytic hydrolysis of water- and ethanol-denatured soybean flour proteins by various proteinases. Denaturation conditions:—○— 90 vol.-% ethanol, 90°C., 60 min.; —□— water, 120°C., 60 min. Proteinases: A, alkaline proteinase of *A. sojae* KS; B, pepsin; C, papain; D, proteinase of *S. griseus* (Pronase); E, crude acid proteinases of *A. saitoi*; F, proteinase of *B. subtilis* (Nagase).

mean, however, that the velocity of hydrolysis is higher. The initial velocities of alcohol-denatured proteins are lower than water-denatured ones in most cases. In particular, the speed of tryptic hydrolysis of low-temperature alcohol-denatured proteins was exceptionally slow, and the hydrolysis did not reach a maximum within the time tested, although the incubation time was longer than that for other proteinases which easily attained a maximum value.

These results suggest possibilities for the utilization of alcohol-denatured soybean proteins for proteinase-digested products. Among these proteinases, those of *Aspergillus* spp. will be most important, because they have played an important role in food industries. It has been recognized that the maximum values of hydrolysis by the proteinases of *Aspergillus* spp. relate directly to the yield of soy sauce. Therefore, the next experiments were performed in detail on the effects of temperature, alcohol concentrations, and time on the maximum hydrolysis by proteinases of *Aspergillus* spp. Figure 4 shows comparison of the degrees of maximum hydrolysis by proteinases of *A. sojae* among the defatted soybean flours treated with ethanol under such conditions where the native proteins may disappear completely. The curve in this figure is a limit, on the right side of which the native proteins are not present, as described in the previous report (10). According to this figure, the values of maximum hydrolysis increased with the decrease of temperatures and the increase of the ethanol concentrations during the treatment. The largest values of the maximum hydrolysis by enzymes were obtained at the conditions which are in the belt along the upper half of the curve in Fig. 4. This indicates that the presence of alcohol during the treatments is necessary for the increase of the maximum enzymatic hydrolysis values. No difference between maximum hydrolysis values was observed when different alcohols were used (Table I). In either alcohol or water denaturation, the time of treatment had great influence on maximum hydrolysis, and its values decreased with increase of time (Fig. 5). It should be noted, however, that the alcohol-denatured proteins are always higher in the maximum hydrolysis values than the corresponding water-denatured ones, except when the denaturation time is extremely long.



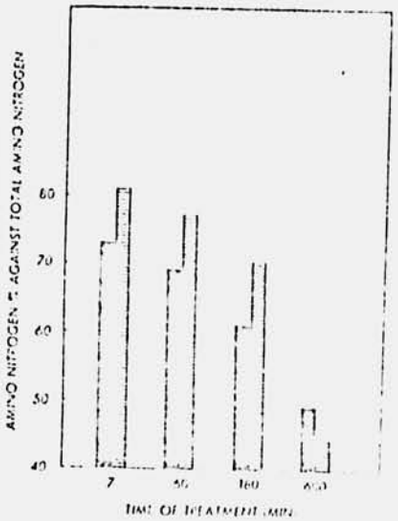


Fig. 4(left). Effect of temperature and ethanol concentration during denaturation on maximum hydrolysis values in the proteolysis of ethanol-denatured soybean flour proteins by crude proteinases of *A. sojae*. Numerals are the values shown in percent of amino nitrogen hydrolyzed by enzymes at 30°C. for 253 hr., against total amino nitrogen. Curve is explained in text.

Fig. 5(right). Effect of denaturation time on maximum enzymatic hydrolysis value of ethanol- and water-denatured soybean flour proteins by crude proteinases of *A. sojae* RS at 30°C. for 253 hr. [illegible] 90 vol.-% ethanol, 120°C.; [illegible] water, 120°C.

Reaction between Sugars and Proteins during Denaturing Treatment

Generally, it is known that the nonenzymatic browning reaction occurs when proteins are heated, cooked, or stored with reducing sugars and that the resulting proteins become resistant to enzymatic hydrolysis (18,19). Soybeans contain an abundance of carbohydrates which readily yield some reducing sugars under the

TABLE I. EFFECT OF TYPE OF ALCOHOL ON MAXIMUM HYDROLYSIS VALUES OF ALCOHOL-DENATURED SOYBEAN FLOUR PROTEINS

Conditions of Denaturation[a]			
Alcohol	Concentration	Temperature	Amino Nitrogen Hydrolyzed[b]
	vol.%	°C.	%[c]
Methanol	100	61	76.3
	90	56	75.7
Ethanol	100	100	76.9
	90	90	76.3
Isopropanol	95	120	75.3
	90	110	76.4

[a]Time of denaturation, 60 min.
[b]Hydrolyzed by crude proteinases of *A. sojae* RS at 30°C. for 253 hr.
[c]Percent against total amino nitrogen.

denaturing conditions (20). Hence, it is very important to ascertain whether the reaction between sugars and proteins during the denaturing treatment is responsible for the difference of the maximum enzymatic hydrolysis values between the alcohol- and water-denaturing proteins, or for decrease of the maximum hydrolysis values which are accompanied by elevation of temperature and increase of time during treatments with water or alcohols.

First, it was confirmed qualitatively from the preliminary experiment shown in Fig. 6 that considerable decomposition of the saccharides occurs during denaturation treatments of defatted soybean flour. The quantitative experiments by paper chromatography were subsequently carried out on the water- and alcohol-treated flour. The results were shown in Table II. Stachyose, raffinose, and sucrose decreased with time of denaturation, and reducing sugars appeared and increased with time in both water and alcohol. However, there existed a striking difference between the two in rate of disappearance of sugars and the accompanying appearance of new sugars, as is shown in this table. On the presumption that the formation of sugar-protein complexes may be responsible for the difference of maximum enzymatic hydrolysis values between water and alcohol treatment, the author proceeded to the following model experiment. A sugar-free soybean flour protein preparation, which possessed all the proteins contained in soybean flour but no mono- or oligosaccharides, was made by leaching the defatted soybean flour with a saturated ammonium sulfate solution. These sugar-free soybean protein preparations were mixed with mono- or oligosaccharides, heat-treated in water or alcohol, and then enzymatically hydrolyzed. The results are shown in Table III.

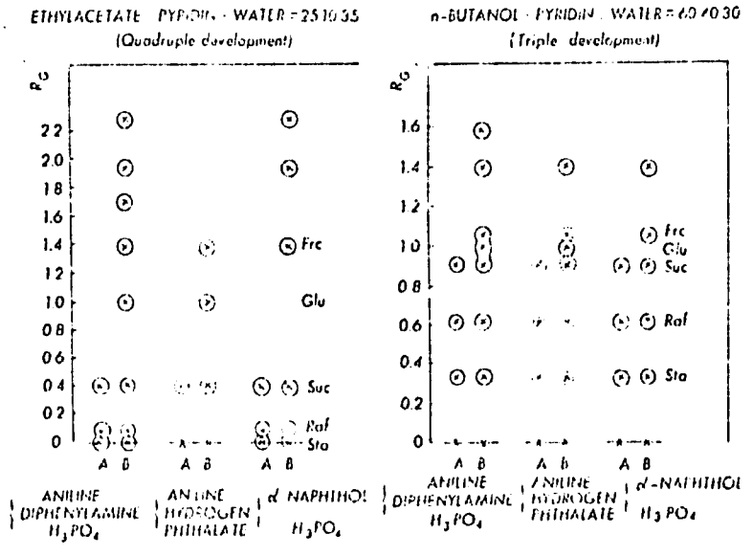


Fig. 6. Paper chromatogram of sugars contained in the heat-treated soybean flour. A, not treated; B, treated for 10 hr. at 120°C. with water.

TABLE II. CHANGE OF SUGARS IN DEFATTED SOYBEAN FLOUR DURING DENATURING TREATMENT WITH ETHANOL AND WITH WATER AT 120°

Denaturing Treatment		Sugars			
Solvent	Time min.	Stachyose %	Raffinose %	Sucrose %	Glucose Fructose %
None	None	4.45	1.40	6.53	0
Water	30	4.45	1.23	6.52	0.11
	180	3.35	1.11	4.85	0.52
	600	0.97	0.54	0.62	0.9[illegible]
Ethanol[a]	30	4.45	1.35	6.52	0
	180	3.73	1.34	5.39	0
	600	3.08	1.33	4.18	0.1[illegible]

[a]Concentration of 90 vol. %.

Contrary to the presumption described above, the results showed that the [illegible] soybean sugars such as sucrose, raffinose, and stachyose had no influence on maximum enzymatic hydrolysis value after denaturation either with water or with alcohol. On the other hand, all reducing sugars examined, which were not present in soybean flour before denaturation, had a remarkable decreasing effect on [illegible] hydrolysis after both water- and alcohol-denaturation. Particularly, it should be noted that the damaging effects of reducing sugars are much larger in alcohol [illegible] in water. For example, the maximum enzymatic hydrolysis value of sugar-free soybean proteins decreased by 23% in water-denaturation by the addition of arabinose, which has the strongest damage effect, whereas the ratio of the decrease in alcohol denaturation was 69%.

TABLE III. EFFECT OF ADDING SUGARS BEFORE DENATURING TREATMENT WITH ETHANOL OR WATER, TO SUGAR-FREE SOYBEAN FLOUR, ON MAXIMUM ENZYMATIC HYDROLYSIS VALUE BY CRUDE PROTEINASE OF A. SOJAE KS AT 30°C. FOR 232 HOURS

	Amino Nitrogen		TCA Soluble Nitrogen	
Sugar Added	Water[a] %[c]	Ethanol[b] %	Water[a] %	Ethanol[b] %
None	46.9	52.4	84.3	[illegible]
Sucrose	45.2	50.8	82.1	[illegible]
Raffinose	45.2	51.0	81.6	[illegible]
Stachyose	45.2	51.6	82.2	[illegible]
Mixed sugars[d]	45.3	50.3	[illegible]	[illegible]
Fructose	42.9	22.9	82.3	[illegible]
Glucose	40.4	2[illegible].1	85.1	[illegible]
Galactose	3[illegible].9	2[illegible]	76.6	[illegible]
Xylose	3[illegible].8	20.4	71.1	[illegible]
Arabinose	3[illegible].2	18.[illegible]	69.9	[illegible]

[a]Treated [illegible]

[b]Treated with [illegible]

[c]Value [illegible]

[d]Mixture of [illegible]

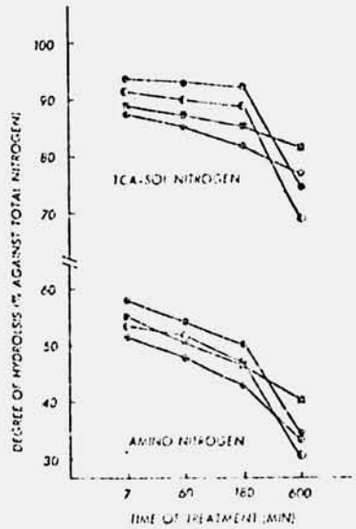


Fig. 7. Effect of denaturing time of sugar-free soybean proteins on maximum hydrolysis values by crude proteases of *A. sojae* IFS at 30°C. for 233 hr., compared with sugar-containing soybean proteins.—○— sugar-free soybean proteins treated with 90 vol.-% ethanol at 120°C.;—□— sugar-free soybean proteins treated with water at 120°C.;—△— defatted soybean flour proteins with 90 vol.-% ethanol at 120°C.;—●— defatted soybean flour proteins with water at 120°C.

There is other evidence that the sugars contained in soybeans are not responsible for the decrease of maximum hydrolysis values. Soybean flour proteins, free from oligosaccharides (Fig. 7), showed the decrease of maximum enzymatic hydrolysis values with the heating time, and the relation of the decrease to time was the same as for the soybean flour protein containing oligosaccharides. Further, similar curves were obtained on the acid-precipitable proteins, which lack whey and residue protein fractions (see Fig. 8) but are both oligosaccharide-free and polysaccharide-free.

Behavior of Soybean Protein Fractions

The soybean flour proteins were fractionated as shown in Fig. 8, and the resulting fractions were heat-treated with water or alcohol, followed by proteolytic hydrolysis by *Aspergillus* spp. (Table IV). Maximum enzymatic hydrolysis by alcohol denaturation was not observed in all fractions. However, with regard to both the acid-precipitable proteins and whey proteins, which comprise most whole soybean flour proteins, an increase in maximum hydrolysis value by alcohol was observed.

DISCUSSION

Consideration of the three-dimensional structure of soybean protein molecules and the difference of the mechanisms between alcohol- and water-denaturation were presented in the previous report (10). The differences of behavior observed here for enzymatic hydrolysis can be also explained reasonably from that of the three-dimensional structure of denatured soybean proteins, as described below.

One can consider the following four general matters as the factors relating to the

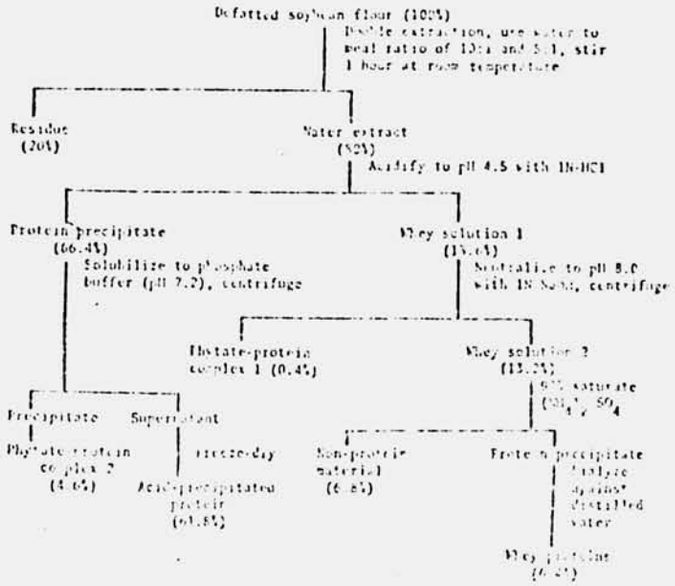


Fig. 8. Fractionation of defatted soybean flour proteins. The numerals in parentheses are percent of nitrogen against total nitrogen of defatted soybean flour.

degree of enzymatic hydrolysis of proteins; that is, (a) the substrate specificities of enzymes, (b) the amino acid sequence of the substrates, (c) the modification of the amino acid side-chains of the substrate, and (d) the three-dimensional structures of the whole molecules of the substrate proteins; (a) is characteristic of each enzyme, and (b) is characteristic of the substrate proteins, which cannot be changed by the denaturing treatments of the substrates. It is unlikely that the amino acid side-chain residues of proteins are destroyed or modified by denaturing treatments of proteins which are not accompanied by reducing sugars or other constituents. Therefore, the difference of the enzymatic hydrolyzabilities observed among the variously denatured soybean proteins should be ascribed to the three-dimensional structures of soybean protein molecules.

TABLE IV. MAXIMUM HYDROLYSIS VALUES OF SOYBEAN PROTEIN FRACTIONS DENATURED WITH ETHANOL AND WITH WATER BY L. A. SOJAE AT 30°C. FOR 333 HOURS

	Amino Nitrogen		TCA Soluble Nitrogen	
Protein Fraction	Water[a] %	Ethanol[b] %	Water[a] %	Ethanol[b] %
Residue	47.5	47.8	81.2	8[illegible].0
Whey protein	46.4	52.2	76.3	8[illegible].8
Acid precipitated protein	54.5	57.4	91.8	[illegible].2
Phytate-protein complex 2	[illegible]	24.0	72.[illegible]	4[illegible].4

[a]Treated with water at 120°C. for 60 min.

[b]Treated with 50 vol.% ethanol at 120°C. for 60 min.

[c]Values shown as percent against total nitrogen.

When the polypeptide chains of the protein molecules are loose and flexible, enzymatic hydrolysis will proceed very rapidly, because the active center of the enzymes can fit very easily to the active sites of the substrate proteins. When the polypeptide chains are not so loose, the speed of the hydrolysis will be slow, since a part of the active sites of the substrate will be inaccessible. In this case, however, the masked active sites may appear slowly with the progress of hydrolysis, and the hydrolysis then proceeds to a high degree of completion. On the other hand, when the chains are folded to form such rigid and compact structure, enzymatic hydrolysis will not occur.

As is discussed in detail in the previous report (see Fig. 7 in reference 10), alcohol in aqueous solution is able to destroy both the hydrophilic and hydrophobic regions of the protein molecules, whereas water destroyed only hydrophilic ones. Consequently, the very rigid parts contained in the native soybean protein molecules will be found to be decreased more in alcohol-denatured proteins than in water-denatured ones. It seems most probable that the higher maximum enzymatic hydrolysis values of alcohol-denatured soybean proteins should be ascribed to the greater disruption of the rigid parts of the protein molecules. On one hand, the facts of the marked decrease of maximum hydrolysis values with increase of time during denaturation both in alcohol and in water suggest secondary formation of rigid parts which may be caused by intermolecular or intramolecular interactions among the polypeptide chains unfolded during denaturation. In this case, it is presumed that the force responsible for secondary formation of the rigid parts is the hydrogen bonds in alcohol denaturation and the hydrophobic bonds in water-denaturation

In water-denaturation, however, it should be noted that formation of the very flexible parts in protein molecules also proceeds at the same time as formation of the very rigid parts described above. This change of the protein molecules should be reflected in the initial speed of enzymatic hydrolysis. From experiments done with water-extract of defatted soybeans (Fig. 9) and Figs. 1, 2, 5, and 7, it is unquestionable that there is a reverse relation between initial speed and maximum degree of hydrolysis in water denaturation. This indicates that the two changes on polypeptide chains occur at the same time during denaturing treatment with water.

The initial speed of tryptic hydrolysis of low-temperature, alcohol-denatured soybean proteins was exceptionally low. Since trypsin inhibitors were absent in this alcohol-denatured soybean flour and the same phenomenon was found with the acid-precipitated soybean proteins, this low rate of tryptic digestion can be ascribed to the three-dimensional structure of substrate proteins. Trypsin can split specifically only the peptide bonds in the C-terminal side of lysine and arginine residues in protein molecules. Therefore, the most plausible explanation is that in regions containing lysine and arginine the polypeptide chains of the low-temperature, alcohol-denatured proteins are rigidly folded. Since lysine and arginine are hydrophilic amino acids, most of them in the native soybean protein molecules may be located at the parts of the shell, which cannot be disrupted easily by alcohol but can be, by water. This is also supported by the fact that most of the lysine and arginine in mioglobin, hemoglobin, and lyzozyme molecules is located at the outside shell in the molecules (21-23). The fact that trypsin possesses the strongest ability to hydrolyze the water-denatured soybean proteins, compared with other proteinases investigated, is also very interesting in this connection. (See

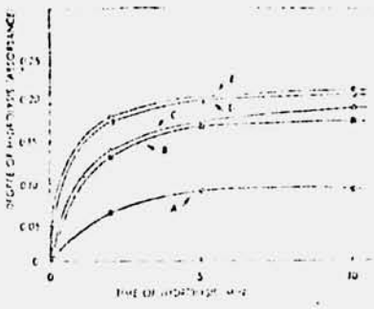


Fig. 9. Initial speed of enzymatic hydrolysis by crude proteinase of *A. sojae* KS. Water-extract of defatted soybean flour was used as substrate. Degree of hydrolysis is expressed as absorbance for the application of Folin's reagent. Method of hydrolysis is described in ref. 1 in detail. Conditions of heat-treatment: A, not treated; B, 15 min. at 100°C.; C, 10 min. at 115°C.; D, 300 min. at 120°C.; and E, 600 min. at 120°C.

"Materials and Methods." Trypsin can hydrolyze 80% or more of the water-denatured soybean proteins in the least activities which were measured for milk casein.)

Such a phenomenon with regard to trypsin indicates that there is no advantage in using alcohol-treated soybean meal for feeds or foods. For soy sauce manufacture, however, only the maximum hydrolysis values with meal proteinases are important, as described in "Results." In fact, the method of soy sauce brewing using the alcohol-treated soybean meal has been established in Japan and has made a great contribution to the increase of their yield (24).

Acknowledgment

The author wishes to express his sincere thanks to Drs. Masatoshi Mogi, Tomoyoshi Yokotsuka, and Koya Mogi for their support and to Mr. Akira Arai and Mr. Masaru Tomizawa for technical assistance.

Literature Cited

1. FUKUSHIMA, D. Studies on soybean proteins. I. Water dispersibility of protein of defatted soybean flour as a criterion for degree of denaturation. Bull. Agr. Chem. Soc. Japan (now Agr. Biol. Chem.) 23: 7-14 (1959).
2. FUKUSHIMA, D. Studies on soybean proteins. II. A new method for quantitative determination of the degree of denaturation of protein in soybean flour. Bull. Agr. Chem. Soc. Japan (now Agr. Biol. Chem.) 23: 15-21 (1959).
3. FUKUSHIMA, D. Internal structure of soybean protein molecule (11S protein) in aqueous solution. J. Biochem. (Tokyo) 57: 822-823 (1965).
4. FUKUSHIMA, D. Internal structure of 7S and 11S globulin molecules in soybean proteins. Cereal Chem. 45: 203-225 (1968).
5. EVANS, R. J. Hydrolysis of soybean oil meal proteins by some proteolytic enzymes. Arch. Biochem. Biophys. 11: 15-21 (1946).
6. HOU, H. C., HERSHEY, H. W., and FUKUSHIMA, C. A. Influence of heating on liberation of certain amino acids from whole soybeans. Proc. Soc. Exptl. Biol. Med. 70: 316-319 (1949).
7. RIESEN, W. H., CLANDININ, D. R., FUKUSHIMA, C. A., and CRAVENS, W. W. Effect of the methionine content of soybean, peanut, and cottonseed oil meals. J. Nutr. Chem. 117: 143-150 (1967).
8. EVANS, R. J., and BUTTS, H. A. Studies on heat inactivation of lysine in soybean oil meal. J. Biol. Chem. 178: 543-550 (1949).
9. EVANS, R. J., and BUTTS, H. A. Studies on the heat inactivation of methionine in soybean oil meal. J. Biol. Chem. 178: 543-548 (1949).

10. FUKUSHIMA, D. Denaturation of soybean proteins with organic solvents. Cereal Chem. 46: 156-163 (1969).
11. MIZUNUMA, T., and IGUCHI, N. Purification of alkaline proteinase by ion-exchange resin. Bull. Agr. Chem. Soc., Japan (now Agr. Biol. Chem.) 22: 35-38 (1958).
12. GIRI, K. V., and NIGAM, V. N. Circular paper chromatography. VIII. Separation, identification and quantitative estimation of sugars and oligosaccharides. J. Indian Inst. Sci. 36: 49-63 (1954).
13. MCFARREN, E. F., BRAND, K., and RUTKOWSKI, H. R. Quantitative determination of sugars on filter paper chromatograms by direct photometry. Anal. Chem. 23: 1146-1149 (1951).
14. PARTRIDGE, S. M. Aniline hydrogen phthalate as a spraying reagent for chromatography of sugars. Nature 164: 443 (1949).
15. RACHINSKII, V. V., and KNYAZYATOVA, E. I. Sugar analysis by paper-partition chromatography. Doklady Akad. Nauk SSSR 85: 1119-1122 (1952).
16. DUBOIS, M., GILLES, K. A., HAMILTON, J. K., REBERS, P. A., and SMITH, F. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28: 350-356 (1956).
17. DIMLER, R. J., SCHAEFFR, W. C., WISE, C. S., and RIST, C. E. Quantitative-paper chromatography of D-glucose and its oligosaccharides. Anal. Chem. 24: 1411-1414 (1952).
18. LOWRY, J. R., and THIESSEN, R. Studies of the impairment of proteins heated with carbohydrates. II. *In vitro* digestion studies. Arch. Biochem. Biophys. 25: 148-156 (1950).
19. FOLK, J. E. The influence of the lysine-glucose reaction on enzymatic digestion. Arch. Biochem. Biophys. 64: 6-18 (1956).
20. DAUBERT, B. F. Other constituents of the soybean: Carbohydrates. *In* Soybean and soybean products, ed. by K. S. Markley; vol. I, pp. 371-379. Interscience: New York (1950).
21. KENDREW, J. C., WATSON, H. C., STRANDBERG, B. E., DICKERSON, R. E., PHILLIPS, D. C., and SHORE, V. C. A partial determination by X-ray methods and its correlation with chemical data. Nature 190: 666-670 (1961).
22. PERUTZ, M. F., ROSSMANN, M. G., CULLIS, A. F., MUIRHEAD, H., WILL, G., and NORTH, A. C. T. Structure of hemoglobin, a three-dimensional Fourier synthesis at 5.5 A resolution, obtained by X-ray analysis. Nature 185: 416-422 (1960).
23. PHILLIPS, D. C. The three-dimensional structure of an enzyme molecule. Scientific American, pp. 78-90 (Nov. 1966).
24. FUKUSHIMA, D. Method for treatment of soybean proteins. U.S. Pat. No. 3,176,802 (1965).

[Received September 17, 1968.]

Reprinted from CEREAL CHEMISTRY, July 1969, Vol. 46, No. 4