Studies on Soybean Proteins

Part I. Water Dispersibility of Protein of Defatted Soybean Flour as a Criterion for Degree of Denaturation*

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Concerning the heat treatment of defatted soybean flour, the fitness of water dispersibility of protein as a criterion of the degree of denaturation has been investigated. Denaturation was measured by the change of susceptibility of the protein in water extract of the flour to protease action of *Aspergillus sojae* before and after heat treatment. It was found that the decrease of water dispersibility did not always run parallel with denaturation. The dispersibility method was applicable only to flours autoclaved for a definite period which varied according to temperature and the water content of the flour treated.

Discussion is made about the fitness of measuring the degree of denaturation by protease action.

INTRODUCTION

Shoyū (Japanese soy sauce) and miso are foods of daily necessity to the Japanese just like butter and cheese in Western countries. They have occupied a very important position in the food industries in this country. In particular, shoyū has been mass-produced on a large scale in modern industry.

Soybean and wheat are used as materials. They are autoclaved, inoculated with seed-koji (*Aspergilli*) and incubated for four days so as to grow the mold. The resulted mold koji is added with saline and incubated for approximately one year at room temperature. The soybean protein occupies about 70% of the protein in materials. The major component of protein in soybean is "glycinin". This protein is chiefly hydrolyzed by the proteases of *Aspergilli* during brewing time to give a good taste and flavour, and high nutritive value. Therefore in shoyū production it is essential to decompose the protein in materials easily and completely by the proteases in koji-mold. Since glycinin is a globular protein and

the globular proteins are, in general, hardly hydrolyzed in the native state, the soybean protein must be first denatured by some procedure. Hence the denaturation of soybean proteins is a most fundamental and important subject to be investigated.

Studies on the denaturation of soybean protein have been chiefly performed on the denaturation during the oil-extracting process. With regard to its criterion the measurement of water- or salt-dispersible nitrogen has been performed. Particularly, water dispersibility has been regarded as the best criterion¹⁾ for determining the degree of denaturation, because native soybean meal has a high dispersibility of nitrogenous constituents by water. This dispersibility is sensitively decreased by heat or practically by any other denaturing agent. The method of the water extraction has been investigated in detail by a number of workers.^{2,3,4)}

1) S. J. Circle, "Soybeans and Soybean Products, Vol. 1", Interscience Publishers, Inc., New York, 1950, p. 275.

2) A. K. Smith, S. J. Circle and G. H. Brother, *J. Am. Chem. Soc.*, **60**, 1316 (1938).

3) A. K. Smith and S. J. Circle, *Ind. Eng. Chem.*, **30**, 1414 (1938).

4) R. H. Nagel, H. C. Beckel and R. T. Milner, *Cereal Chem.*, **15**, 463 (1938).

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It is an unusual characteristic that a very high percentage of nitrogenous matter (more than 90%) can be extracted with water from soybean meal, which differentiates soybean meal protein from other seed meal proteins. Several explanations on the high water dispersibility of soybean protein have been offered such as the potassium phosphate theory (Osborn and Campbell; 1898 and Harvath; 1937), dialyzable salt theory (Ryndin; 1936), phosphatide theory (Nagel, Beckel, and Milner; 1938), and alkalinity theory (Smith, Schubert, and Belter; 1955). However, any satisfactory explanations have not yet been obtained.

In the meanwhile, it is generally known that the degree of coagulation of proteins is greatly influenced by various factors of environment, regardless of its denaturation state. Denaturation appears to be a quite different process occurring at the different rate from that of coagulation.^{5,6)} Accordingly, it must be fully investigated whether or not the degree of denaturation runs parallel with the decrease of water dispersibility of soybean meal protein. However any investigation on this problem has not yet been found in the literatures. In this paper the native protein in the water extract was measured by the change of the susceptibility to the protease action of *Asp. sojae* and the fitness of water dispersibility as a criterion of the degree of denaturation was investigated.

MATERIAL AND METHODS

Preparation of Oil-Free Soybean Flour. Shirahana soybean, the variety of late-ripening, grown in 1956 in Japan were used throughout this experimental work. Material was stored in the refrigerator after it was sun-dried. Fat-free soybean was prepared by dehulling, grinding followed by the screening through a 30-mesh, then removing the oil with *n*-hexan extraction at 35°C and air-dried. The fat-free flour had been kept in refrigerator less than two months to avoid denaturation

during storage. A typical fat-extracted flour sample showed the following percentage composition: moisture, 6.59; nitrogen, 8.559.

Heat Treatment of Soybean Protein. One gram of defatted soybean flour and a definite amount of water were mixed in a 25-ml Erlenmeyer flask and autoclaved.

Determination of Water-Dispersible Nitrogen. The single extraction method of Smith et al.³⁾ was modified as follows. Heat-treated soybean flour was transferred to a 500-ml polyethylene bottle and shaken mechanically for sixty minutes at 30°C with 200 ml water. The contents of the bottle were transferred to a 250-ml graduated flask, and the volume was adjusted to 250 ml by water. A portion was centrifuged at 2440 × g for six minutes. Total nitrogen was determined on a 20 ml-sample, the supernatant from centrifugation, by the semi-micro Kjeldahl method.

Enzyme Preparation.

(1) *Protease of Asp. sojae*: The alkaline protease was isolated from *Asp. sojae* by the method of Mizunuma et al.⁷⁾; the method included purification of the enzyme by ion exchange chromatography (IRC-50) of the water extract of a wheat bran koji. The resulted preparation contained less than 1% of original amylase activity and 2% of neutral protease, but no acidic protease.

(2) The crystalline protease of *Bac. subtilis* (Purchased from Nagase Co., Ltd.):

Determination of Protease Activity. Casein digesting activity was measured by a modification⁸⁾ of the Anson method⁹⁾. One ml of 2% milk casein (Hammarsten) solution was poured into a test tube containing 0.5 ml of the enzyme solution and 0.5 ml of 0.2 mol. phosphate buffer (pH 7.2) and the mixture was incubated at 30°C for ten minutes. Then 2 ml of 0.4 mol. trichloroacetic acid was added to the digest to stop the reaction and the resulted precipitate was removed by filtration after standing for twenty minutes at 30°C. Five ml of 0.4 mol. sodium carbonate and 1 ml of Folin's reagent¹⁰⁾ were successively added to 1 ml of the filtrate and it was kept at 35°C for twenty minutes. Optical density was measured using a EPO-B Type Hitachi Photometer with a 660 mμ filter against distilled water. The same experiment was carried out with a blank which was operated essentially in the same way as above except that the enzyme was added later. Optical density of the blank test was subtracted from that of

5) S. Mizushima and S. Akabori, "Chemistry of Proteins, Vol. 2" Kyoritsu Press, Japan, 1954, p. 549.

6) K. J. Laidler, "Introduction to the chemistry of Enzymes", McGraw-Hill Book Company, Inc., 1954, p. 145.

7) T. Mizunuma and N. Iguchi, This Bulletin, **22**, 35 (1958)

8) B. Hagihara, Ann. Rep. Soc. Osaka Univ., **2**, 35 (1954).

9) M. L. Anson, J. Gen. Physiol., **22**, 79 (1938).

10) O. Folin and V. Ciocalteu, J. Biol. Chem., **73**, 627 (1927).

the test run and converted into mg equivalent tyrosine liberated by enzyme action. One unit, $[PU]_{m.eq.tyr.}^{ens.30^\circ FR}$, represented the activity which liberated 1 mg equivalent of tyrosine in trichloroacetic acid-soluble fraction per minute under the above conditions.

Enzymatic Hydrolysis of Water-Extracted Soybean Protein. A mixture of 1 ml of substrate solution, 1 ml of enzyme solution, and 0.5 ml of 0.2 mol. phosphate buffer solution (pH 7.2) was incubated at 30°C for a definite time. One ml of 1.2 mol. trichloroacetic acid was added to stop the reaction and the filtration was done after complete formation of the precipitate. One ml of the filtrate was assayed for protein as described above. The value was expressed as the correspondent amount of tyrosine (γ) which represented the degree of hydrolysis.

RESULTS

I. Water Dispersibility of Soybean Flour Protein

(1) Effect of Time and Temperature an Autoclaving: To examine whether water dispersibility is adequate as a criterion of the degree of denaturation of soybean flour protein, the effect of time and temperature in autoclaving was investigated. Fig. 1 shows the effect of time and temperature in autoclaving on water dis-

FIG. 1. Effect of Time and Temperature on Water Dispersibility of Soybean Flour Protein Autoclaved under Addition of 100% Water.

—○— 100°C, —●— 110°C, —△— 120°C

persibility with soybean flour to which an equal amount of water was added. At 100°C, the extracted nitrogen gradually decreased up to one hundred and twenty minutes, when dispersibility reached its minimum. At 110°C or 120°C, minimum dispersibility occurred after a few minutes and afterwards extracted nitrogen increased. The higher the temperature, the larger was the increase of the dispersed nitrogen. These results showed the unfitness of water dispersibility as a criterion of denaturation because water dispersibility of the soybean protein increased with heating time after its minimum point was attained.

(2) Effect of Water Content: The water content of flour before autoclaving had great influence on water dispersibility as shown in Figs. 2 and 3. Fig. 2 shows

FIG. 2. Effect of Time and Water Content on Water Dispersibility of Soybean Flour Protein Autoclaved at 120°C.

—○— Without addition of water
—●— With addition of water

the water-dispersible nitrogen of the flour autoclaved at 120°C with and without the addition of an equal amount of water. The amount of water-dispersed nitrogen was larger in the flour added with water than in flour without water addition. In fact, denaturation occurred more easily in the former than in the latter, as described later.

After autoclaving the samples containing different levels of water at a definite temperature (100°C) for

FIG. 3. Effect of Water Content on Water Dispersibility of Soybean Flour Protein Autoclaved at 100°C for Thirty Minutes.

definite period (thirty minutes), the minimum of water-dispersed nitrogen was observed by the addition of 200% water as shown in Fig. 3.

II. Determination of Native protein in Soybean Water Extract

From the above results, it was recognized that water dispersibility is not a good criterion of denaturation, because denatured soybean flour protein was extracted without coagulation under a certain condition. On account of this the measurement of the native protein in water extract by protease action was examined.

The water extract of native flour was divided into two parts: the one was added with the proteases without heating the flour and the other was heated at 100°C for ten minutes before protease addition. The results are shown in Figs. 4 and 5. As shown the protein of the heated aqueous extract was digested much faster than that of the unheated one. In this case no precipitates were produced in the water extract of flour, as long as it was diluted below definite concentrations. Moreover, there was no substance inhibitory to protease action of *Aspergillus sojae* or *Bacillus subtilis* in the extract (Table I). Accordingly, it was reasonable to attribute this increased susceptibility of protein in the

FIG. 4. Course of Hydrolysis of Soybean Water Extract by *Asp. Sojae* Protease.

—○— Treated at 100°C for fifteen min.
—●— Not treated
Total nitrogen of substrate: 0.48 mg/cc

FIG. 5. Course of Hydrolysis of Soybean Water Extract by *Bac. Subtilis* Protease.

—○— Treated at 100°C for fifteen min.
—●— Not treated
Total mitrogen of substrate: 0.48 mg/cc

heated water extract to protease action to modification of the native protein itself. Thus, the following techniques were set up for determining the native protein in solution. If necessary, the solution was diluted, and then it was divided into two parts, one being unheated and the other heated at 100°C for ten minutes.

TABLE I. EFFECT OF SOYBEAN WATER EXTRACT ON PROTEASE ACTION

Method: Experimental conditions were the same as "Determination of Protease Activity" in the present paper. The value was expressed in γ tyrosine.

	Protease of <i>Asp. Sojae</i>		Protease of <i>Bac. Subtilis</i>
	Refined Alkaline Protease	Water Extract of Wheat Bran Koji	Crystal-line Protease
Control	24.8	23.8	33.1
(Control)+(Soybean Water Extract)	23.1	23.0	32.0

Each part was added simultaneously with the alkaline protease of *Asp. sojae* (total activity; $10[\text{PU}]_{\text{m.eq.tyr.}}^{\text{cas. } 30^\circ \text{FR}}$), and incubated for thirty minutes under the same conditions described in "Materials and Methods". The difference of value between the two parts represented the amount of native protein in solution. If measurement was made in samples of the definite concentrations of protein, this difference was proportional to the amount of the native protein in the sample as shown in Fig. 6. Figs. 7, 8, 9 shows the amount of

FIG. 6. Relationship between Amount of Native Protein in Solution and Value Obtained by this Method.

Substrate: Total nitrogen 0.4 mg/cc. The ratio of the unheated water extract to the heated (100°C, 10 min) water extract was varied as indicated.

native protein* in water extracts of soybean flours autoclaved under various conditions, by the application of this method.

By autoclaving at 100°C under the addition of 100% water to the flour, the amount of native protein in the water extract gradually decreased with heating time and reached zero after one hundred and twenty minutes,

* Strictly this value is not quantitative, because the protein concentration of the substrates was not regulated.

FIG. 7. Native Protein in Water Extract of Soybean Flour Autoclaved under Addition of 100% Water.

—△— 100°C —○— 110°C —×— 120°C

whereas at 110°C and 120°C, the native protein in the water extract disappeared after heating for several minutes (Fig. 7). The amount of native protein in the water extract always reached zero at the minimum of water-dispersible nitrogen (Fig. 1, Fig. 7). The longer the heating time beyond the minimum was, the more total dispersed nitrogen amounted. A part of this increased-nitrogen was originated in denatured protein.

It was very important to examine the effect of water content on denaturation and water dispersibility of proteins. The flour autoclaved under the addition of 100% water was larger in water-dispersed nitrogen than that under no addition. On the other hand, the

FIG. 8. Native Protein in Water Extract of Soybean Flour Autoclaved at 120°C.

—○— Without addition of water
—×— With addition of 100% water

native protein in water extract assayed by the enzymatic method already reached zero after heating for a few minutes in flour containing 100% water, while it required more than thirty minute's heating to be completely denaturated in the flour heat-treated without water addition (Fig. 8, Fig. 2). In Fig. 2 and Fig. 8 as well as in Fig. 1 and Fig. 7, the time when native protein disappeared coincided with the point at which the dispersed nitrogen reached its minimum. Fig. 9 shows

FIG. 9. Native Protein in Water Extract of Soybean Flour Autoclaved at 100°C for Thirty Minutes.

the effect of water addition to flour prior to autoclaving on the amount of native protein contained in the water extract. By autoclaving the flour contained twice as much water at 100°C for thirty minutes, the resulted water extract contained no native protein; the minimum of the water-dispersed nitrogen occurred under the same conditions. In spite of the abrupt increase of water-extracted nitrogen beyond the minimum point, native protein never existed in the extract. Also, in this case the native protein in water extract completely disappeared when water-dispersed nitrogen reached its minimum. It therefore appears that the higher the water content became, the more easily the flour protein is denatured.

DISCUSSION

The possibility of using protease for measurement of the degree of denaturation has been suggested by several workers. Linderstrøm-Lang et al.¹¹⁾ presented data indicating that native lactoglobulin might not be entirely attacked by enzyme and Haurowitz et al.¹²⁾ have

pointed out that the susceptibility of native fibrous proteins to the action of trypsin is attributed to the expanded configuration of their peptide chains rendering their peptide bonds accessible to the enzyme. Lineweaver et al.¹³⁾ and Rice et al.¹⁴⁾ tried to measure the degree of denaturation of human serum albumin or hemoglobin by means of papain susceptibility. Bernheim et al.¹⁵⁾ carried out measurements of the rates of tryptic hydrolysis of horse serum albumin and horse pseudoglobulin in an attempt to detect differences in the internal structure of some proteins, i.e., native, irreversibly denatured, and the so-called regenerated proteins. Recently, Okunuki et al.^{16,17)} have described a quantitative method for the determination of the degree of denaturation of enzyme proteins, using crystalline protease of *Bac. subtilis* which did not act on the native protein of enzymes and hydrolyzed the denatured enzyme protein quantitatively. These authors found that the inactivation of crystalline enzymes, such as Taka-amylase and *Bac. subtilis* amylase, is proportional to the degree of hydrolysis by the protease.

Globular proteins, in general, increase susceptibility to the protease after denaturation. This has been explained from various observations¹⁸⁾ as follows. The atomic groups serving as points of combination with active centers of the protease are located inside the molecule in native globular proteins and are inaccessible to the protease. Denaturation which apparently involves an unfolding of the closely packed

11) K. Linderstrøm-Lang, R. D. Hotchkiss and G. Johansen, *Nature*, **142**, 966 (1938).

12) F. Haurowitz, M. Tunca, P. Schwerin and V. Goksn, *J. Biol. Chem.*, **157**, 621 (1945).

13) H. Lineweaver and S. R. Hoover, *J. Biol. Chem.*, **137**, 325 (1941).

14) R. G. Rice, G. A. Ballou, P. D. Boyer, J. M. Luck and F. G. Lumi, *J. Biol. Chem.*, **158**, 609 (1946).

15) F. Bernheim, H. Neurath, and J. Q. Erickson, *J. Biol. Chem.*, **144**, 259 (1942).

16) K. Okunuki, B. Hagihara, H. Matsubara, T. Nakayama and M. Yanagida, *Symposia on Enzyme Chemistry (Japan)*, **9**, 1 (1954).

17) K. Okunuki, B. Hagihara, H. Matsubara and T. Nakayama, *J. Biochem. (Japan)*, **43**, 453 (1956).

18) F. Haurowitz, "Chemistry and Biology of Proteins", Academic Press, New York, 1950, p. 127.

peptide chains, renders these groups accessible to the protease. Therefore, the value of the increase of susceptibility of the protein to the protease should be considered to be that directly reflecting the expanded configuration of the molecule. The major proteins of soybean are of the globular type and increase susceptibility to protease as shown in Figs. 4 and 5. Therefore, the measurement of the native protein in soybean meals by using protease should be regarded to be the most suitable method.

The proteases suitable for the measurement of native protein in water extract of soybean meal or flour should have its optimum pH around the neutral pH and not be inhibited by any constituents in the water extract. It was found that both protease of *Aspergillus sojae* and *Bacillus subtilis* are suitable for the above conditions (Fig. 3, Fig. 4). Nevertheless, protease of *Aspergillus sojae* was used in this paper because protease of *Aspergillus sojae* had less activity toward native protein than that of *Bacillus subtilis* though both proteases hydrolyzed it gradually.

Inadequacy of water dispersibility as criterion of the degree of denaturation is shown in this paper. However, this method serves as a criterion for the flours autoclaved within the range where the minimum point is not reached, because in this range the amounts of the native protein in water extract run parallel to water dispersibility. It should be mentioned, however, that even within the above range the absolute amount of nitrogen dispersed by water after heat treatment varies significantly depending upon the temperature or water contents of flour. And, with regard to the samples which are treated at different conditions of temperature and water content of flour, the dispersed nitrogen does not always run parallel to the amount of the native protein. For this reason, the dispersibility method is not satisfactory for comparison of the degree of denaturation of these samples. That the denatured soybean protein is solu-

bilized by water content, may be explained in connection to the fact¹⁹⁾ that the coagulation of denatured proteins is inhibited by the existence of soluble protein.

Hitherto studies for denaturation of soybean proteins have been carried out in regard to heat denaturation during oil extraction and the water dispersibility method has been used as the only useful criterion for the degree of denaturation of soybean meal protein. Further, so far, the water dispersibility method has not been found to be inadequate. This is due to the following reason. During the oil-extracting process the treatment of samples has been carried out at a lower temperature and with a lower water content of flour. On account of this the measurement of denaturation resulted has only been performed upon samples which have not attained a minimum point of water dispersibility. Thus, water dispersibility method has been used as a criterion of denaturation without considerable contradiction being recognised.

Since the protein in native soybean flour is very soluble in water, the native protein in heat-treated flour is expected to be solubilized easily and completely by water. On account of this, the protease method described here is usable for measurement of the total amount of the native protein contained in soybean flour itself. Whether or not the native protein in heated flour is extracted as easily and completely as that in unheated protein will be described later.

SUMMARY

(1) In order to investigate the fitness of water-dispersible nitrogen of heat-treated soybean flour as a criterion of the degree of denaturation, a method for determining the amount of native protein in aqueous solution which was based on the increase of susceptibility to protease action of *Asp. sojae* prior and after heat treatment was established.

19) S. Mizushima and S. Akabori, "Chemistry of Proteins Vol. 2", Kyoritsu Press, Japan, p. 550.

(2) In the autoclaving of flour, there existed a minimum of water-dispersed nitrogen with regard to the heating time or the water content of flour. The higher the temperature of treatment or the water content of flour, the larger was the increase of dispersed nitrogen beyond the minimum.

(3) The higher the temperature or the water content of flour, the easier denaturation occurred. The native protein in the water extract of heated flour disappeared at the minimum point of water dispersibility.

(4) The water dispersibility method served as a criterion of denaturation for the flour heated within the range where a minimum point of water dispersibility was attained. Within this range amounts of water-dispersed

nitrogen ran parallel to the amount of the native protein content. However, this water dispersibility method is not suitable for comparison of the degree of denaturation of flours treated under different conditions.

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