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Received for review April 7, 1986. Revised manuscript received September 23, 1986. Accepted November 18, 1986.

Effects of Deamidation with Chymotrypsin at pH 10 on the Functional Properties of Proteins

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The effect of deamidation on the functional properties of proteins was investigated by proteolytic deamidation in an controlled condition. The emulsifying and foaming properties of egg white proteins (ovalbumin, lysozyme) and soy proteins (7S globulin, 11S globulin) were improved by the enzymatic deamidation with chymotrypsin at pH 10. About 20% of the asparaginyl or glutaminyl residues in these proteins was deamidated by the treatment with chymotrypsin without proteolysis or with a slight proteolysis. The surface hydrophobicity and the flexibility detected by digestion velocity of deamidated proteins increased, resulting in the improvement of the foaming and emulsifying properties. Thus, proteolytic deamidation was proposed to be a useful method for the improvement of functional properties of food proteins.

Various enzymatic and chemical modifications of food proteins have been attempted to improve the functional properties. These will offer the possibility for food application of unutilized protein sources, if the safety for food use is solved. We have noted and studied the deamidation of proteins as the most promising method to improve the solubility and surface properties (Matsudomi et al., 1982, 1985 a,b). The deamidation of proteins is expected to cause increases in the solubility and flexibility due to an increase in the negative charge of proteins.

The mild acid treatment to deamidate proteins was very effective to improve their functional properties. However, the mild acid treatment causes deamidation of asparaginyl and glutaminyl residues in proteins together with not only denaturation but also the cleavate of peptide bond. Therefore, a mild deamidation is desirable to investigate the effect of deamidation on the functional properties of food proteins.

In a previous paper (Kato et al., 1987), we reported that proteases have the deamidation activity of proteins in an alkaline pH region where proteolytic activity was at the minimum. Especially, ovalbumin and lysozyme were

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0021-8561/87/1435-0285$01.50/0 © 1987 American Chemical Society

deamidated in a state being scarcely subject to proteolysis by proteases. Thus, it has become feasible to investigate the effect of deamidation on the functional properties of proteins. Of various proteases, chymotrypsin may be suitable for this approch, because pronase has a considerable proteolytic activity even at pH 10, papain requires cysteine and ethylenediaminetetraacetate for deamidation, and trypsin has a low deamidation activity (Kato et al., 1987).

Therefore, the enzymatic deamidation with chymotrypsin was investigated in this paper for the improvement of the functional properties of food proteins.

MATERIALS AND METHODS

Materials. Ovalbumin was prepared from fresh egg white by crystallization with sodium sulfate and recrystallized five times (Kekwick and Cannan, 1936). Lysozyme was prepared from fresh egg white by a direct crystallization method and recrystallized five times (Alderton and Fevold, 1946). 7S and 11S globulin were prepared from soybean by the method of Thanh et al. (1975). Chymotrypsin was purchased from Miles Laboratories.

Enzymatic Deamidation with Chymotrypsin. A 10-mL sample of 0.5% protein solution was adjusted to pH 10.0 with 1 N NaOH, and then 0.5 mg of chymotrypsin was added. The reaction mixture was incubated at 20 °C for 2 h. The protein solution allowed to stand for 2 h at pH 10 and 20 °C was used as the standard to check the effect of alkalinity in the experiments on the deamidation and functional properties. The sample solution was immediately freeze-dried.

Measurement of Deamidation of Proteins. Sample dissolved in 5 mL of 3 N HCl was sealed in a 10-mL glass ampule and heated at 110 °C for 3 h to deamidate completely. The amount of ammonia released was determined by the method described in a previous paper (Kato et al., 1987).

Measurement of Proteolysis. After chymotrypsin reaction, 3 mL of 10% trichloroacetic acid was added to 3 mL of sample solution, and a filtrate was obtained. The amount of peptide and amino acid in the filtrate was estimated by measurement of absorbance at 280 nm. The extent of proteolysis was indicated by expressing the absorbance of the filtrate as a percentage of the absorbance of the untreated protein solution after correction for differences in dilution.

Surface Hydrophobicity (S_0). Surface hydrophobicity measurement was carried out using *cis*-parinaric acid as a fluorescence probe as reported by Kato and Nakai (1980). An ethanolic solution of *cis*-parinaric acid, 3.6×10^{-3} M, was purged with nitrogen, and equimolar butylated hydroxytoluene was added as an antioxidant. *cis*-Parinaric acid solution (10 μL) was added to 2 mL of protein solution in 0.02 M phosphate buffer, pH 6.0. The *cis*-parinaric acid–protein conjugate was excited at a wavelength of 325 nm, and the relative fluorescence intensity was measured at a wavelength of 420 nm in an Aminco-Bowman spectrophotofluorometer, Model J4-8962. The fluorescence intensity is expressed in arbitary units. The fluorescence of unit intensity was adjusted to 1.0 when 10 μL of *cis*-parinaric acid solution was added to 2 mL of 0.02 M phosphate buffer, pH 7.4, in the absence of protein. The initial slope (S_0) of fluorescence intensity vs. protein concentration was used as a measure of surface hydrophobicity.

Chymotrypsin Digestibility of Proteins. Chymotrypsin digestion was performed as follows: To 3 mL of 0.1% protein solution in 0.05 M phosphate buffer, pH 7.4, was added 200 μL of 0.1% chymotrypsin. The enzymatic reaction was carried out at 38 °C for a given times. After the chymotrypsin digestion, 3 mL of 10% aqueous trichloroacetic acid was added and then the precipitates were removed by filtration with filter paper (Toyo Roshi Ltd. No. 5B). The amount of peptide and amino acid in the filtrate was estimated by measurement of absorbance at 280 nm. The extent of digestion was indicated by expressing the absorbance of the filtrate as a percentage of that of the untreated protein solution after correcting for differences in dilution. The digestion velocity was taken as the initial slope of a plot of digesion percentage vs. time (Kato et al., 1985).

Table I. Deamidation and Proteolysis Percentages of Food Proteins by Treatment with Chymotrypsin at pH 10

proteins	deamidation, %	proteolysis, %
ovalbumin	20	2
lysozyme	21	0
7S globulin	24	5
11S globulin	19	8

Foaming Powder and Foam Stability. Foaming property was determined by measuring the conductivity of foams produced when air at a constant flow rate of 90 cm^3/min was introduced for 15 s into 5 mL of 0.1% protein solution in 0.1 M carbonate buffer, pH 9.5, in a vertical glass column (2.4 × 30 cm) with a glass filter at the bottom (Kato et al., 1983). The conductivity of foams was measured by an electrode that had a cell. The cell was fixed inside the glass column 1 cm apart and 2.4 cm above the filter and was connected to conductivity meter (Kyoto Electrics Industry Co., Model CM-07). The conductivity was recorded continuously on a chart recorder. Foaming power was defined as the maximum conductivity of foams produced after aeration. Foam stability was shown as the disappearance time of foams (Kato et al., 1983).

Emulsifying Activity. Emulsifying activity was determined by the method of Pearce and Kinsella (1978). To prepare emulsion, 1 mL of corn oil and 3 mL of protein solution in 0.1 M carbonate buffer, pH 9.5, were shaken together and homogenized in an Ultra Turrax (Hansen and Co.) at 12 000 rpm for 1 min at 20 °C. Emulsion (50 μL) was taken from the bottom of the container after different times and diluted with 5 mL of 0.1% SDS solution. The absorbance of diluted emulsions was then determined at 500 nm. Emulsifying activity was determined from the absorbance measured immediately after emulsion formation.

RESULTS AND DISCUSSION

Table I shows the extent of deamidation and proteolysis in the four proteins after treatment with chymotrypsin at pH 10 at 20 °C. About 20% asparaginyl or glutaminyl residues was deamidated. Proteolysis was slight. Soy proteins were more subject to proteolysis than lysozyme and ovalbumin. This was further confirmed by electrophoretic analysis, which has been reported elsewhere (Kato et al., 1987). As shown in a previous paper, no differences in the SDS electrophoretic patterns of ovalbumin and lysozyme were observed between native and deamidated proteins. This suggests that the majority of ovalbumin and lysozyme is not subject to proteolysis by the treatment with chymotrypsin at pH 10 at 20 °C. Thus, it has been possible to evaluate the effect of deamidation on the functional properties of ovalbumin and lysozyme. On the other hand, soy proteins are slightly subject to proteolysis in addition to deamidation. Therefore, changes in the functional properties of soy proteins may be due to the effect of not only deamidation but also peptide cleavage.

Table II shows the structural parameters of deamidated proteins. The surface hydrophobicity of proteins is a

Table II. Surface Hydrophobicity and Digestion Velocity of Deamidated Proteins

proteins	surface hydrophobicity (S_0)	digestion velocity, %/min
ovalbumin		
native	10	0.23
deamidated	20	0.34
lysozyme		
native	10	0.20
deaminated	52	0.31
7S globulin		
native	99	2.93
deamidated	183	3.53
11S globulin		
native	44	2.53
deamidated	153	4.70

Table III. Foaming and Emulsifying Properties of Deamidated Proteins

proteins	foaming power, μs/cm	foam stability, min	emulsifying act. (OD_{500})
ovalbumin			
native	82	2.0	0.220
deamidated	208	6.5	0.450
lysozyme			
native	54	1.0	0.150
deamidated	96	3.5	0.230
7S globulin			
native	1250	7.7	0.420
deamidated	2550	10.2	0.540
11S globulin			
native	1300	9.8	0.390
deamidated	2250	11.4	0.600

sensitive parameter for surface structure (Kato and Nakai, 1980), and the digestion velocity of proteins is a sensitive parameter for flexibility (Kato et al., 1985). Increases in the surface hydrophobicity and the digestion velocity were observed in deamidated proteins, as shown in Table I. An increase in the negative change due to deamidation may cause flexible conformation and may result in an increase in the surface hydrophobicity and digestion velocity of proteins.

The foaming and emulsifying properties of deamidated proteins were investigated to evaluate the effect of deamidation on functionality (Table III). It is well-known that the functional properties of proteins are improved by alkaline treatment. Therefore, the proteins allowed to stand for 2 h at pH 10 at 20 °C were used as the standard proteins to check the effect of alkaline conditions in the experiment on the functional properties. Figures 1 and 2 show the changes in the foaming and emulsifying properties of proteins by the treatment with free chymotrypsin at pH 10 at 20 °C. As shown in Figures 1 and 2, the foaming and emulsifying properties of proteins were increased by proteolytic deamidation. This improvement of functional properties may be dependent on increases in the surface hydrophobicity and the flexibility of proteins by deamidation. However, the effect of contaminant chymotrypsin on the functional properties should be considered during the measurement of foaming and emulsifying properties. Therefore, measurements of these functional properties were carried out in alkaline pH 9.5 where proteolysis was inhibited. As shown in Table II, the foaming power of deamidated proteins was increased about 2 times and the foam stability of deamidated ovalbumin and lysozyme was increased about 3 times, while that of deamidated soy proteins was less markedly increased. On the other hand, the emulsifying activity of deamidated

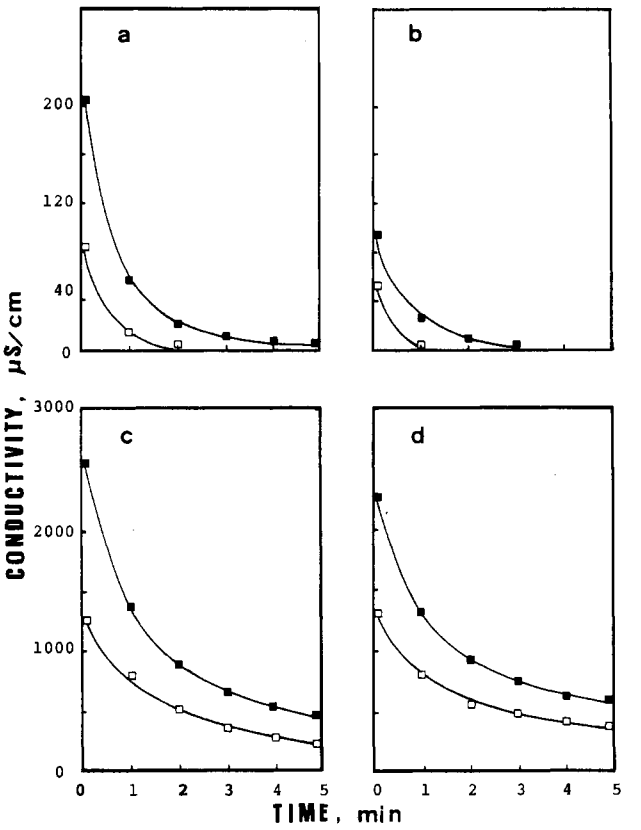

Figure 1. Changes in the conductivity of foams produced from control and deamidated proteins: a, ovalbumin; b, lysozyme; c, 7S globulin; d, 11S globulin. The control proteins (□) were subjected to pH 10 for 2 h at 20 °C in the absence of chymotrypsin. The deamidated proteins (■) were subjected to pH 10 for 2 h at 20 °C in the presence of chymotrypsin.

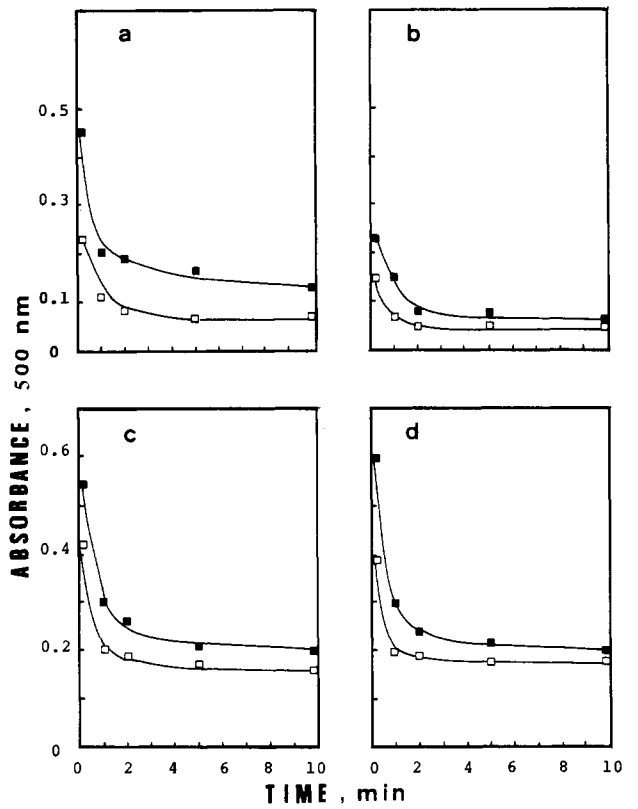

Figure 2. Changes in the turbidity of emulsions produced from control and deamidated proteins: a, ovalbumin; b, lysozyme; c, 7S globulin; d, 11S globulin. The control (□) and deamidated (■) proteins were the same as those in Figure 1.

ovalbumin was increased 2 times, while that of lysozyme and soy proteins was increased 1.5 times.

Thus, proteolytic deamidation by chymotrypsin can be used as useful method to improve the functional properties of proteins. Since deamidation of proteins causes an increase in solubility, this proteolytic deamidation may contribute to a larger effect for proteins poor in solubility. Alkaline treatment of proteins is generally used for food application to improve the functional proerties such as solubility and emulsifying and foaming properties. However, excessive alkaline treatment results in undesirable effects on food proteins such as lysinoalanine formation and racemization of amino acids. On the other hand, proteolytic deamidation in a mild alkaline condition is a favorable method for food application. It is more desirable for food application that immobilized proteases be used to deamidate proteins. Therefore, further studies are proceeding in our laboratory to promote the deamidation rate of proteins using various immobilized protease systems.

Registry No. Chymotrypsin, 9004-07-3; lysozyme, 9001-63-2.

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Received for review May 21, 1986. Revised manuscript received December 8, 1986. Accepted January 16, 1987.