# Deamidation of Food Proteins by Protease in Alkaline pH

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A new method for the deamidation of proteins using protease in a controlled condition is described and proposed as a desirable alternative for food protein processing. A considerable amount of ammonia was detected by the protease treatment of proteins in carbonate buffer, pH 10. Deamidation activity was investigated in detail by use of ovalbumin substrate, with which deamidation was scarcely observed in the nonenzymatic control. The optimal pH of deamidation was 10, and the optimal temperature was 20 °C. The deamidation of proteins linearly increased in proportion to protease concentration. The deamidation of ovalbumin and lysozyme was about 20% with papain, pronase, and chymotrypsin at pH 10.0, while their proteolysis was scarcely observed. It was suggested from these results that deamidated proteins were obtained without proteolysis, or with slight proteolysis, by proteases in a controlled condition.

Deamidation of proteins has been usually a peripheral part of other studies in protein chemistry. However, deamidation of glutaminyl and asparaginyl residues in proteins may cause changes in the biological activity and physicochemical properties because of an increase in protein flexibility due to changes in charge balance. Robinson (1974) proposed that deamidation of proteins plays a central role as a molecular timer of biological events because an interesting correlation was found between the overall amide contents of proteins and their in vivo turnover rates. Since functional properties of food proteins have been reported to be improved by deamidation (Matsudomi et al., 1982), deamidation of proteins is an interesting problem for food research. Thus, much attention has been recently directed to the deamidation of proteins.

Deamidation of proteins is caused by mild acid treatment. However, the enzymatic deamidation of proteins is desirable to prepare deamidated proteins, because mild acid treatment of proteins causes not only denaturation but also cleavage of peptide bonds (Han et al., 1983). Transglutaminase, an enzyme that catalyzes the replacement of amide groups of glutamine residues, can also catalyze their hydrolysis, that is, deamidation (Mycek and Waelsch, 1960). However, the cross-links of proteins also occur simultaneously by transglutaminase activity, through the replacement of protein amide groups by the $\alpha$-amino or $\epsilon$-amino groups of proteins. Sung et al. (1983) reported that the partial deamidation of soy protein might be involved in the covalent incorporation of *N*-acetylhomocysteine by a papain-catalyzed acylation reaction. However, they did not call attention to the result because of a peripheral problem for them. Since papain has been known to catalyze transamidation and transesterification reactions in an alkaline pH region that is clearly different from that required for peptide hydrolysis, it may be potentially possible for papain to deamidate glutaminyl and asparaginyl residues in proteins. Nevertheless, there is little information on the deamidation of proteins by proteases. There is the renewed possibility of using proteases as catalysts in the deamidation of proteins, if the proteins undergo deamidation reaction by proteases without peptide hydrolysis in a specifically controlled condition.

This paper describes the deamidation of proteins by various proteases under controlled conditions.

## MATERIALS AND METHODS

Ovalbumin was prepared from fresh egg white by a sodium sulfate procedure 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). $\alpha$- and $\kappa$-casein were prepared from fresh milk by the method of Zittle and Custer (1963). Soybean 7S and 11S globulin were prepared by the method of Than et al. (1975). Trypsin (from bovine pancreas) was purchased from Sigma Chemical Co. $\alpha$-Chymotrypsin was purchased from Miles Laboratories. Pronase was purchased from Kaken Kagaku Co. A papain preparation (Merk Japan Co.) was purified by the method of Kimmel and Smith (1954).

**Direct Measurement of Ammonia Released during Protease Reaction.** The amounts of ammonia released from samples were determined by the microdiffusion method of Conway and O'Malley (1942). A 1-mL aliquot of 2% boric acid solution containing methyl red and bromocresol green was put into the central chamber of a microdiffusion unit. A 4-mL aliquot of 0.5% sample protein solution in 0.05 M carbonate buffer, pH 10.0, was put into the outer chamber of the microdiffusion unit, and then 0.1 mL of 0.2% protease solution was separately put into the outer chamber without mixing with the protein solution. The cover of the microdiffusion unit was immediately closed by use of arabia gum to prevent $NH_3$ from leaking outside, and then the protein solution and the protease solution in the outer chamber were mixed at 30 °C for 36 h to liberate ammonia gas.

**Deamidation by Papain.** A 10-mL aliquot of 0.5% protein solution containing 1 mM EDTA and 1 mM cysteine was adjusted to pH 10.0 with 1 N NaOH, and then 0.5 mg of papain was added. The reaction mixture was incubated at 20 °C for a given time. Samples were withdrawn at given times, readjusted to pH 7.0 with 1 N HCl, and immediately held at 90 °C for 5 min to inactivate papain. Sample solutions were dialyzed against deionized water at 5 °C for 48 h to remove ammonia and finally freeze-dried.

**Deamidation by Trypsin, Chymotrypsin, and Pronase.** A 10-mL aliquot of 0.5% protein solution was adjusted to pH 10.0 with 1 N NaOH, and then 0.5 mg of protease was added. The reaction mixture was incubated at 20 °C for 2 h. Samples were readjusted to pH 7.0 with 1 N HCl and immediately held at 90 °C for 5 min to inactivate protease. Sample solutions were dialyzed against deionized water at 5 °C for 48 h and finally freeze-dried.

**Measurement of Deamidation Percentages in Protein.** The deamidation percentage of protein was measured as follows. 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 from the samples was determined by the microdiffusion method of Conway and O'Malley (1942). A 1-mL

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0021-8561/87/1435-0224$01.50/0 

**Table I. Amounts of Ammonia Released from Proteins by Chymotrypsin and Papain Reaction at pH 10**

| protein | released ammonia, μg/20 mg protein: protein substr[a] | protein + chymotrypsin | protein + papain |
|---|---|---|---|
| ovalbumin | 1.70 | 12.75 | 10.20 |
| lysozyme | 7.65 | 33.15 | 22.95 |
| 7S globulin | 8.50 | 30.60 | 30.60 |
| 11S globulin | 10.20 | 35.70 | 29.75 |
| α-casein | 22.95 | 47.60 | 41.65 |
| κ-casein | 44.20 | 80.75 | 74.80 |

[a] Protein solution in 0.05 M carbonate buffer, pH 10.0, was allowed to stand for 36 h at 30 °C as standard.

aliquot of 2% boric acid solution, containing methyl red and bromocresol green, was put into the central chamber of a microdiffusion unit; 2 mL of 2.9 N NaOH and 3 mL of saturated potassium metaborate were used to liberate $NH_3$ gas in the outer chamber. A 2-mL aliquot of sample solution was put into the outer chamber of the microdiffusion unit without mixing with the alkali solution previously put in the chamber. The cover of the microdiffusion unit was immediately closed by use of arabia gum to prevent ammonia gas from leaking outside, and then the alkali solution and the sample solution in the outer chamber were mixed to liberate ammonia gas from the sample. The unit was allowed to stand for 36 h at 30 °C, and then the ammonia absorbed in the 2% boric acid solution in the central chamber was titrated with $^1/_{200}$ N $H_2SO_4$. The deamidation percentages of samples were determined from the titrated values of ammonia as follows:

$$\text{deamidation \%} = \frac{\text{titr value pronase-treated protein}}{\text{titr value native protein}} \times 100$$

The absolute values of ammonia released from ovalbumin, lysozyme, 7S globulin, and κ-casein were 115, 190, 170, and 187 μg/g, respectively, when 1% deamidation occurred in the proteins.

**Measurement of Proteolysis.** After protease reaction, 3 mL of 10% trichloroacetic acid was added to 3 mL of sample solution and filtrated. The amount of peptides and amino acids in the filtrate was estimated by measurement of absorbance at 280 nm. The extent of proteolysis was indicated as the digestion percentage of total protein.

**SDS–Slab Polyacrylamide Gel Electrophoresis.** A polyacrylamide gel was prepared by the method of Laemmli (1970). The 12.5% gel was set in a slab (50 × 80 × 1 mm). Electrophoresis was done for 4 h at 5 mA at room temperature. After electrophoresis, the gel was stained with 0.25% Coomassie Brilliant Blue R 250–acetic acid–methanol (5:1:5 by volume) to locate the protein bands.

## RESULTS AND DISCUSSION

**Direct Measurement of Ammonia Released during Protease Reaction.** Table I shows the amounts of ammonia released from various protein substrates by protease reaction at pH 10.0 in the microdiffusion unit. Since it took 36 h to absorb completely ammonia into the absorbent (2% boric acid solution) in the central chamber, the microdiffusion unit was allowed to stand for 36 h at 30 °C. Although a considerable amount of ammonia was released from the proteins (except albumin) without protease reaction due to the reaction at alkaline pH for a long time, a more significant amount of ammonia was released from the proteins by the protease reaction. Thus, it was suggested that proteases have deamidation activity. However, this direct measurement method has some disadvantages for the measurement of ammonia release from proteins. The main disadvantage is the nonenzymatic release of ammonia from proteins due to reaction in alkaline pH for a long time. Another disadvantage is the difficulty in following exactly the enzymatic reaction, because ammonia gas escapes when the cover of the microdiffusion unit is opened to stop the protease reaction. In addition, ammonia released by deamidation cannot be completely outgassed under the employed condition, pH 10. In order to liberate completely the ammonia gas, it was necessary to incubate in a saturated potassium metaborate solution, pH 11.3. However, since a significant amount of ammonia was released from proteins without protease reaction at pH 11.3, the amounts of ammonia released by protease could not be estimated exactly. To modify these disadvantages, the amide-type ammonia remaining in proteins was measured after protease reaction.

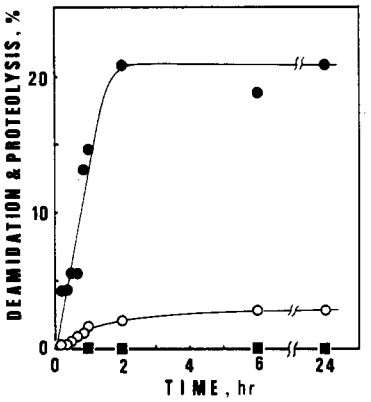


**Figure 1.** Time course of deamidation and proteolysis in ovalbumin by papain at pH 10. Key: ●, deamidation; ○, proteolysis; ■, deamidation of ovalbumin solution at pH 10 in the absence of papain.

**Measurement of Amide-Type Ammonia Remaining in Proteins after Protease Reaction.** The deamidation percentages of proteins were determined by measuring the amide-type ammonia remaining in proteins before and after protease reaction at alkaline pH.

Figure 1 shows the time course of deamidation in ovalbumin by reaction with papain at pH 10 at 20 °C. The deamidation of ovalbumin gradually proceeded and reached a maximum after 2 h. No deamidation occurred in the standard condition without papain at pH 10 at 20 °C. The absolute value of ammonia released from native ovalbumin was 11 500 μg/g of protein, when 100% deamidation occurred in 3 N HCl. On the other hand, that of protease-treated ovalbumin was 9090 μg/g of protein. Thus, about 20% of the amides were deamidated in protease-treated ovalbumin.

The pH dependency of deamidation in ovalbumin by papain is shown in Figure 2. The enzymatic reaction was carried out at 20 °C for 2 h. The optimal pH was about 10, where proteolysis was minimal.

Figure 3 shows the effect of temperature on the deamidation in ovalbumin by papain at pH 10.0 for 2 h. The optimal temperature was 20 °C for deamidation activity.

Table II shows the deamidation and proteolysis percentages of various proteins after reaction with papain at pH 10 for 2 h at 20 °C. Ovalbumin, lysozyme, and 7S globulin were commonly deamidated without or with slight proteolysis.

Table III shows the effect of SH blocking in papain on the deamidation and proteolysis of proteins to investigate the deamidation active center of papain. Deamidation activity was completely inhibited by masking the SH group in papain with *N*-ethylmaleimide and in the absence of

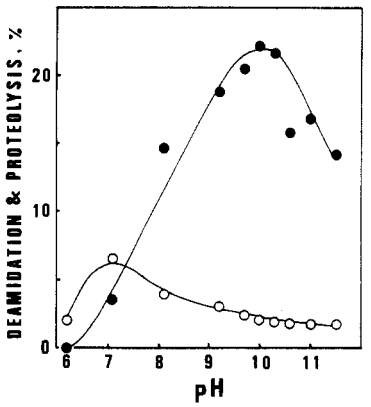


**Figure 2.** Effects of pH on deamidation and proteolysis in ovalbumin by papain. Key: ●, deamidation; ○, proteolysis.

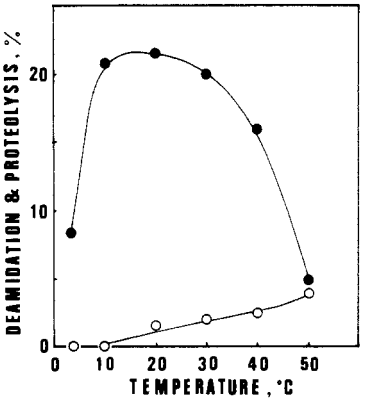


**Figure 3.** Effects of temperature on deamidation and proteolysis in ovalbumin by papain. Key: ●, deamidation; ○, proteolysis.

**Table II. Deamidation and Proteolysis of Various Proteins by Papain at pH 10.0[a]**

| protein | deamid, % | proteol, % |
|---|---|---|
| ovalbumin | 21.0 | 2.0 |
| lysozyme | 21.2 | 0 |
| 7S globulin | 27.1 | 5.1 |

[a] The amounts of ammonia released from ovalbumin, lysozyme, and 7S globulin were 115, 190, and 170 μg/g, respectively, when 1% deamidation occurred.

**Table III. Effects of SH Blocking in Papain on Deamidation and Proteolysis**

| papain | substrate | deamid, % | proteol, % |
|---|---|---|---|
| control | ovalbumin | 21.0 | 2.0 |
| | κ-casein | 35.0 | 47.0 |
| treated with *N*-ethylmaleimide | ovalbumin | 0 | 0 |
| | κ-casein | 0 | 0 |
| in the absence of cysteine and EDTA | ovalbumin | 0 | 0 |
| | κ-casein | 0 | 5.5 |

cysteine and ethylenediaminetetraacetic acid. This suggests that the sulfhydryl group of papain may be involved in the active center of deamidation. Therefore, papain is likely to catalyze the deamidation reaction in the same active site as proteolysis. The deamidation of proteins may certainly occur by protease, not by a contaminant enzyme such as deamidase.

Deamidation of proteins by proteases in a controlled condition was further investigated with pronase, chymotrypsin, and trypsin. The pH dependency of deamidation in ovalbumin by the proteases is shown in Figure 4. Pronase, chymotrypsin, and trypsin also had deamidation activity on proteins at pH 10.0 at 20 °C, where proteolytic activity was little observed. Pronase and chymotrypsin

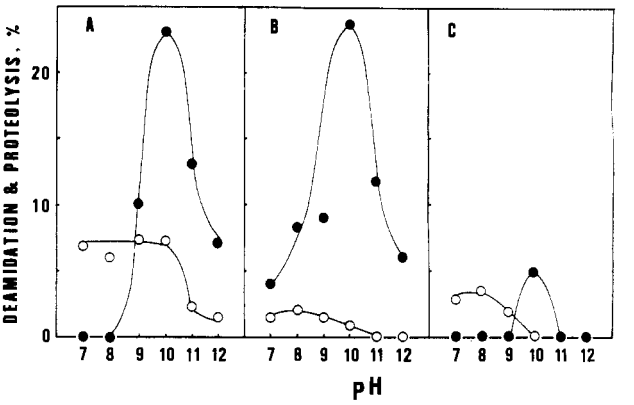


**Figure 4.** Effects of pH on deamidation and proteolysis in ovalbumin by chymotrypsin, pronase, and trypsin. Key: A, pronase; B, chymotrypsin; C, trypsin; ●, deamidation; ○, proteolysis.

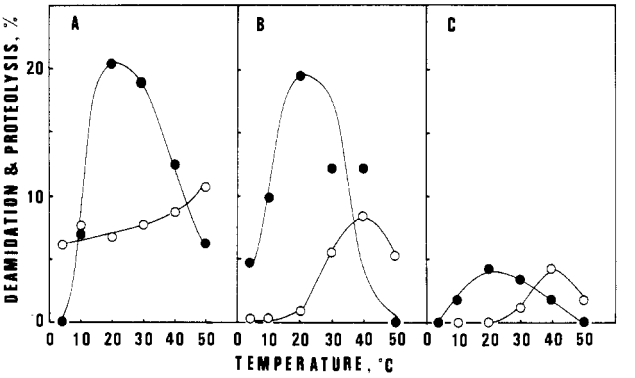


**Figure 5.** Effects of temperature on deamidation and proteolysis in ovalbumin by chymotrypsin, pronase, and trypsin. Key: A, pronase; B, chymotrypsin; C, trypsin; ●, deamidation; ○, proteolysis.

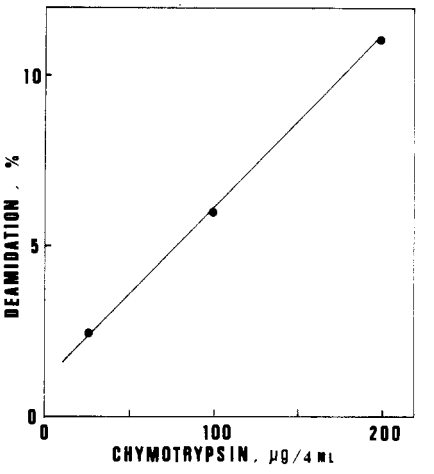


**Figure 6.** Dependence of the deamidation of protein on the protease concentration. Chymotrypsin was added to 4 mL of 0.5% 7S globulin solution, pH 10, and reacted for 60 min at 20 °C.

showed the same extent of deamidation activity as papain, whereas the deamidation activity of trypsin was much lower than papain. Figure 5 shows the temperature dependency of deamidation in ovalbumin by proteases at pH 10.0. The optimal temperature for these proteases was commonly 20 °C for deamidation, as seen with papain, while that for the proteolytic activity was 40 °C.

Figure 6 shows the dependence of the deamidation of protein on the protease concentration. The deamidation of protein linearly increased in proportion to the concentration of chymotrypsin.

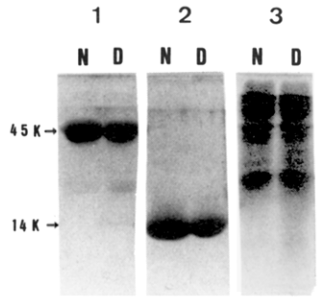


**Figure 7.** SDS–polyacrylamide gel electrophoresis of deamidated proteins by protease. Chymotrypsin was added to protein solutions, pH 10, and reacted for 2 h at 20 °C. Key: 1, ovalbumin; 2, lysozyme; 3, 7S globulin; N, native protein; D, deamidated protein.

Proteolysis was evaluated by the measurement of small peptides or amino acids in the filtrates in the presence of 5% trichloroacetic acid. The large peptides resulting from the cleavage of a small number of peptide bonds are not detected by this method. Therefore, this was checked by SDS–polyacrylamide gel electrophoresis. As shown in Figure 7, no differences in electrophoretic patterns were observed between native and deamidated proteins. That is, large peptides resulting from limited hydrolysis were not observed in electrophoretic patterns of proteins.

Thus, proteases have been shown to have deamidation activity at pH 10.0 at 20 °C, where proteolytic activity was suppressed. Careful analyses were carried out to avoid deamidation artifactually occurring during the experiments. The pH was adjusted without using buffers to avoid the effect of anion on deamidation, because Gilbert et al. (1949) reported that the deamidation rate depended on the type of anion in the buffer. As shown in Figure 1, no artifactual deamidation was observed in the condition used in the experiments. However, when carbonate buffer was used in the direct measurement of ammonia (Table I) to keep pH 10, a considerable amount of ammonia was released from the proteins. It is interesting that soy protein and casein are more extensively deamidated than ovalbumin in alkaline conditions. Carbonate buffer may promote nonenzymatic deamidation of proteins. However, long incubation at alkaline pH is required for effective deamidation. This treatment results in undesirable effects on food proteins such as lysinolanine formation. On the other hand, protease treatment results in more effective deamidation under mild conditions.

Transglutaminase can also catalyze the amide hydrolysis of proteins (Mycek and Waelsch, 1960). However, the cross-links of proteins also occur simultaneously with transglutaminase through the replacement of protein amide groups by the amino groups of proteins. On the other hand, proteases hydrolyze the amide groups of proteins without cross-link formation. In addition, proteases can be readily obtained in quantity. Although the deamidation of proteins is partially caused by proteases, this deamidation reaction can be utilized to convert or improve the functional properties of proteins. Thus, investigation is in progress in our laboratory. We have reported that the solubility and emulsifying properties of gluten are improved by deamidation with chymotrypsin (Matsudomi et al., 1986).

In conclusion, proteins can be deamidated by proteases at an alkaline pH without proteolysis, or with slight proteolysis. Therefore, the deamidation of proteins using proteases in an controlled condition may be a useful method for the improvement of functional properties of food proteins.

**Registry No.** Protease, 9001-92-7; papain, 9001-73-4; pronase, 9036-06-0; chymotrypsin, 9004-07-3; lysozyme, 9001-63-2; trypsin, 9002-07-7.

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Received for review November 25, 1985. Revised manuscript received May 1, 1986. Accepted October 1, 1986.