Action of Protein-Glutaminase on α-Lactalbumin in the Native and Molten Globule States

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The action of a novel protein-glutaminase from microorganisms on α-lactalbumin was investigated. When α-lactalbumin in the native state was incubated with protein-glutaminase, the deamidation proceeded gradually, i.e., the deamidation degree increased to 20% and 55% after 4 and 24 h, respectively. The transformation of α-lactalbumin from the native state to the molten globule state caused an increase in the rate of the enzyme-catalyzed deamidation, particularly in the early stage. The deamidation degree for the molten globule state reached 61% after 4 h, followed by a gradual increase to 66% after 24 h. CD spectral analyses of deamidated α-lactablumin revealed that the stability of the tertiary structure of α-lactablumin was closely related to the degree of deamidation, whereas the secondary structure was not affected by deamidation. Glutamine residues in α-lactalbumin to be modified by protein-glutaminase were identified as Gln[39], [43], [54], and [65]. Conformational characteristics of the amino acid sequence around these glutamine residues are discussed.

Keywords: *α-Lactalbumin; protein-glutaminase; molten globule state; deamidation*

INTRODUCTION

Deamidation is one of the most promising methods for improving the functional properties of food proteins (*1*). The exposure of carboxyl groups causes an increase in negative charge and hydration, thereby improving solubility. The surface properties are also improved by an increase in flexibility due to enhanced electrostatic repulsion. Chemical methods have been applied to deamidate proteins, especially plant seed proteins, which are rich in glutamine and asparagine residues. The mild acid treatment of wheat gliadin gave soluble preparations with significantly improved emulsifying and foaming properties (*2*). Oil seed proteins, such as soy and sesame proteins, could be also deamidated by heating at 100 °C using anions as catalysts, resulting in substantial increases in solubility, foam expansion, and emulsion capacity, etc. (*3*). However, the cleavage of peptide bonds, which leads to the loss of macromolecular characteristics of proteins, cannot be avoided by chemical treatments.

Deamidation by enzymatic reaction is more preferable for food systems, because the modification is selective and safe. The possibility of applying proteases as deamidating agents for the improvement of protein functional properties still remains an unsolved problem, although it was suggested that several proteases caused the deamidation of proteins (*4*). Transglutaminase can catalyze deamidation when water acts as an acyl acceptor (*5*). However, cross-linking of proteins predominantly occurred by transglutaminase activity through the replacement of the protein amide group by the α-amino or ϵ-amino groups of proteins. Peptidoglutaminase from *Bacillus circulans* could catalyze the deamidation of glutamine within the short peptide chain, but the activity of this enzyme toward casein and whey proteins was highly limited (*6*). Therefore, a new enzyme has been required that catalyzes the deamidation of proteins without other side reactions.

Recently, Yamaguchi and Yokoe (*7*) found a new enzyme from a bacterium (*Chryseobacterium proteolyticum*) which catalyzes the deamidation of proteins and show no transglutaminase activity, or cross-linking reaction. The enzyme is a monomer consisting of 185 amino acids with a pI of 10.0 and calculated molecular weight of 19860 (*8*). The primary sequence had no obvious homology to any published sequence. The targets of this enzyme were glutaminyl residues in short peptides or proteins, but not asparaginyl residues or free glutamines. The enzyme was therefore named protein-glutaminase. Yamaguchi et al. (*8*) also showed that caseins were the best substrates for protein-glutaminase, whereas globular proteins such as milk whey proteins were less susceptible to the enzymatic reaction.

α-Lactalbumin (α-LA) is one of the major components of milk whey proteins, and the improvement of its functional properties is highly required for the expansion of the use of whey proteins as a food stuff. α-LA is a compact globular protein (MW: 14200) and information on its primary structure is available. Furthermore, it is well-known that α-LA takes on the partially denatured structure which is called the molten globule state (*9*, *10*). Therefore, α-LA may be suitable for use as a substrate of protein-glutaminase for the purpose

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of studying the effects of primary sequence and conformation of the substrate on the enzymatic activity.

In the present paper, we investigated the action of protein-glutaminase on α-LA in the native and molten globule states. The extent of deamidation and the conformational changes after the deamidation in the two states were compared. The deamidation sites in α-LA were also determined.

MATERIALS AND METHODS

Materials. Protein-glutaminase derived from *Chryseobacterium proteolyticum* strain 9670 was purified by the method of Yamaguchi et al. (*8*). The purified enzyme was judged to be homogeneous by SDS−PAGE (*8*). Specific activity was 33.7 U/mg. The standard method for protein-glutaminase assay using benzyloxycarbonyl (Cbz)−Gln−Gly (Peptide Laboratory, Osaka, Japan) as a substrate and the definition of enzyme unit were described previously (*7*). A safety evaluation of the protein-glutaminase preparation, with regard to regulatory aspects, is ongoing. The bovine α-LA (catalog no. L-5385, type I) was purchase from Sigma (St. Louis, MO). *Staphylococcus aureus* V8 protease and carboxypeptidase Y were purchased from Wako Chemical (Osaka, Japan) and Oriental Yeast (Osaka, Japan), respectively. Other chemicals were purchased from Wako Chemical and Nakarai Tesque (Kyoto, Japan) as analytical reagent grade.

Deamidation of α-Lactalbumin by Protein-Glutaminase. Deamidation of α-LA was carried out in 20 mM Tris−HCl buffer (pH 7.0) containing α-LA (10 mg/mL) and protein-glutaminase (1.82 μg/mL). When α-LA in the molten globule state was used as a substrate, 5 mM EDTA was added to the reaction mixture (*11*, *12*). The enzymatic reaction was conducted at 42 °C for various periods of time (0 to 24 h). Amounts of ammonia released from deamidated glutamine residues were determined according to a method described in the following section. Degree of deamidation was expressed as the ratio of deamidated residues to total glutamine residues. Number of total glutamine residues in α-LA was assumed to be six according to previous data of primary sequence (*13*).

Determination of Ammonia. After incubation with the enzyme, aliquots of the reaction mixture (500 μL) were placed in microcentrifuge tubes, followed by addition of 50 μL of 12% TCA to stop the enzymatic reaction. After the filled tubes were centrifuged at 10 °C and 12000 rpm for 10 min, the released ammonia in the supernatant (100 μL) was determined. The total ammonia was measured by an NADH−glutamate dehydrogenase method with an ammonia determination kit according to the manufacturer's instruction (Boehringer, Mannheim, German).

Two-Dimensional Gel Electrophoresis. First-dimensional gel electrophoresis was carried out using an instrument for isoelectric focusing, protein IEF cell (Bio-Rad, Hercules, CA), according to the method of Choe and Lee (*14*). IPG Ready Strip pH 3−6 (Bio-Rad) was applied. Second-dimensional electrophoresis (SDS−PAGE) was conducted by the procedure of Laemmli (*15*) with a 15% acrylamide gel. The gel was stained with Coomassie brilliant blue R-250.

Measurement of Circular Dichroism. After incubation of α-LA with protein-glutaminase for 4 or 24 h, 10 mM *N*-ethylmaleimide was added to the reaction mixtures to stop the enzymatic reaction, because cysteine residues are suggested to be involved in the protein-glutaminase activity (*8*). The reaction mixtures were then diluted with 20 mM Tris−HCl buffer (pH 7) containing 2 mM EDTA or 2 mM $CaCl_2$. The final protein concentration was 0.04 wt %. The measurements of far- and near-UV circular dichroism (CD) were conducted at 42 °C using a spectropolarimeter (Jasco, J-720) equipped with a data processor (model DP-501). The far-UV spectra were measured using a cuvette with a 0.1-cm light path over the wavelength range 200−260 nm. The near-UV spectra were measured using a cuvette with a 1.0-cm light path over the wavelength range from 260 to 320 nm. The CD data were expressed as mean residue ellipticity (degree cm^2/dmol).

Reduction of Intact Disulfide Bonds. Disruption of disulfide bonds and carboxymethylation of generated sulfhydryl groups in nonmodified and deaminated α-LAs were carried out by the following procedure: incubation of α-LA (1 mg/mL) with 20 mM Tris−HCl buffer (pH 7) including 10 mM EDTA, 8 M urea, and 30 mM 2-mercapethanol at 25 °C for 4 h, followed by an addition of 50 mM iodoacetamide and an incubation at 25 °C for 2 h in the dark. After incubation, the solution was dialyzed against 0.01 N acetic acid during 48 h and then freeze-dried.

Digestion with V8 Protease. Freeze-dried protein (10 mg) was solubilized in 0.5 mL of 6 M urea solution. After incubation at 37 °C for 30 min, the protein solution was diluted twice by 0.5 mL of 0.1 M ammonium bicarbonate buffer (pH 7.6), followed by the addition of *Staphylococcus aureus* V8 protease (enzyme/substrate, 1:50, w/w). The reaction mixture was kept at 37 °C for 24 h. To inactivate the enzyme, the reaction mixture was heated at 100 °C for 5 min followed by the addition of 0.1% TFA.

HPLC Analysis. The peptide mixtures obtained by V8 protease digestion of α-LA were separated by HPLC on a YMC-pack protein-RP column (dimension, 250 × 4.6 mm; particle size, 5 μm). Elution of peptides from the column was carried out by applying a two-step linear gradient of acetonitrile in 0.1% TFA. First, a linear gradient of between 100% solvent A (0.1% TFA) and 38.6% solvent A/61.4% solvent B (0.1% TFA in 70% acetonitrile, v/v) was applied over a period of 43 min. Then, the second linear gradient between 38.6% solvent A/61.4% solvent B and 100% solvent B was applied from 43 to 97 min. Elution of peptides was monitored by measuring UV absorption (215 nm).

Determination of N-terminal Amino Acid Sequence. The peptides separated by RP-HPLC and α-LA molecules were applied to a protein sequencer (492 Procise, Applied Biosystems) that was equipped with highly sensitive detection for phenylthiohydantoin (PTH)-amino acids. PTH derivatives produced at each cycle were identified by using the programmable absorbance detector (785A, Applied Biosystems) with reference to standard derivatives of amino acids.

C-terminal Amino Acid Sequence Analysis. C-terminal analysis of α-LA molecules was performed by the method of Klemm (*16*). α-LA (50 nM) was dissolved in 0.1 M sodium phosphate buffer (pH 7) containing 0.5% SDS and 25 nM L-norleucine as internal standard, and the mixture was preincubated at 37 °C for 2 h. After addition of carboxypeptidase Y (0.5 nM), the reaction mixture was incubated at 37 °C for various periods of time (0 to 2 h). Each aliquot taken from the reaction mixture was diluted 10 times with sodium citrate buffer (pH 2.2). The liberated amino acids were separated from the protein by using a centrifugal filter device (Millipore Corp., Bedford, MA).

Amino Acid Analysis. The amino acids, which had been liberated from the C-terminal of α-LA according to the procedure of the previous section, were directly analyzed with an amino acid analyzer (L-8500A, Hitachi). For peptides separated by HPLC, the hydrolysis was carried out according to the following method. The separated peptide was placed in a reaction vial, oxygen was removed by purging with nitrogen gas three times, and the peptide was hydrolyzed with 6 N HCl containing 0.1% phenol at 110 °C for 24 h in an electrical heater (work station, Waters). The hydrolysates were analyzed using the amino acid analyzer.

RESULTS

Deamidation of α-Lactalbumin in the Native and Molten Globule States. The action of protein-glutaminase was investigated in the native and the molten globule states of α-LA. The degree of deamidation was determined by the measurement of total

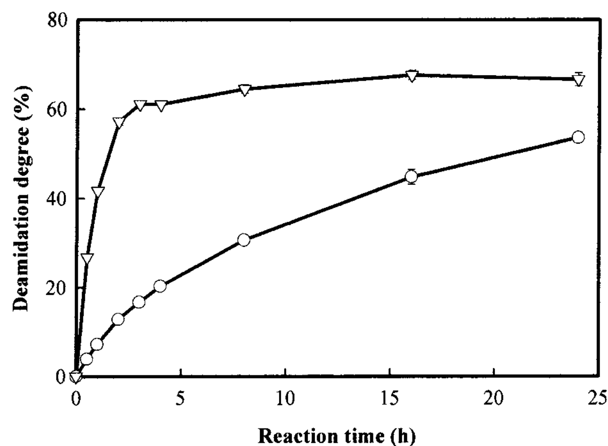

Figure 1. Time-dependent increase of the degree of deamidation of α-lactalbumin induced by incubation with protein-glutaminase. (○), α-lactalbumin in the native state; (▽), α-lactalbumin in the molten globule state.

ammonia released during the enzymatic reaction (Figure 1). The molten globule state was produced by EDTA treatment of α-LA as described above in Materials and Methods. The degree of liberated total ammonia from α-LA increased with reaction time, irrespective of whether α-LA was in the native or the molten globule state. For the native state, the degree of deamidation slowly increased to 20 and 55% after 4 and 24 h incubation time, respectively. On the other hand, the degree of deamidation for the molten globule state reached about 61% after 4 h, followed by a gradual increase of the degree to 66% after 24 h incubation time. Only trace amounts of ammonia were released after 24 h incubation of α-LA without the enzyme, irrespective of whether α-LA was in the native or the molten globule state (data not shown). The isoelectric point value of α-LA decreased as a result of deamidation from 4.71 (nonmodified) to 4.37 (highly deaminated (66%)).

The results (Figure 1) show that α-LA in the molten globule state is more rapidly attacked by the enzyme. Increased accessibility of glutamine residues to the enzyme induced by the transformation from the native to the molten globule states may affect the enzyme-catalyzed deamidation rate, particularly in the initial stage. It should be noted, however, that protein-glutaminase could act on α-LA even in the native state during long incubation (Figure 1). It is thought that protein-glutaminase can act on these glutamine residues, which are occasionally more exposed on the molecular surface during the fluctuation of side chains of α-LA even in the native state. Once the first glutamine residue is deamidated, the newly formed carboxyl group enhances the unfolding of the protein via electrostatic repulsion, thereby increasing the possibility of modification of glutamine residues by the enzyme.

Results of two-dimensional electrophoresis showed no cross-linking of α-LA after incubation with protein glutaminase, suggesting no transglutaminase activity of protein glutamine (data not shown). We have already shown that protein-glutaminase lacked transglutaminase activity in terms of hydroxamate-formation between benzyloxycarbonyl−Gln−Gly and hydroxylamine, or monodansyl cadaverine incorporation into casein (*8*).

Changes in CD Spectra of α-Lactalbumin by Deamidation. To investigate the effects of deamidation on the conformation of α-LA, CD spectra of nonmodified and deamidated molecules were compared. For α-LA that had been deamidated in the native state, two samples were chosen for CD spectral measurements, i.e., deamidated ones for 4 and 24 h corresponding to 20% and 55% degrees of deamidation, respectively, as shown in Figure 1. On the other hand, only the spectra of α-LA incubated with protein-glutamidase for 24 h (degree of 66% demidation) were measured, when the deamidation had been carried out in the molten globule state. As described in Materials and Methods, two buffer systems were employed for the measurements (i.e., EDTA and $CaCl_2$) to assess the contribution of Ca^{2+}-binding to the conformational stability of nonmodified and deamidated α-LA.

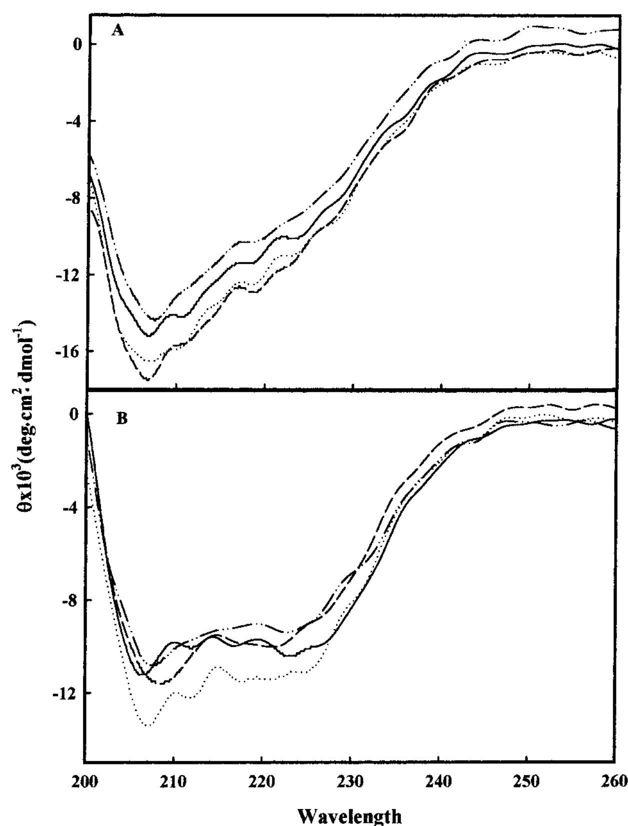

Figure 2. Far-UV circular dichroism spectra of α-lactalbumin solutions (0.04 wt %) at 42 °C. α-Lactalbumin was dissolved in Tris−HCl buffer (20 mM, pH 7.0) containing 2 mM EDTA (A) or $CaCl_2$ (B). (—), Nonmodified α-lactalbumin; (− ··), α-lactalbumin deamidated for 4 h in the native state; (···) α-lactalbumin deamidated for 24 h in the native state; (− −), α-lactalbumin deamidated for 24 h in the molten globule state.

Figure 2 shows far-UV CD spectra of nonmodified and modified α-LA. Panels A and B indicate the results of measurements with EDTA and $CaCl_2$ buffer systems, respectively. The spectra of nonmodified α-LA shown in A and B were very similar to those published earlier (*11*, *17*, *18*). Despite the small difference between the CD properties of nonmodified α-LA in EDTA and $CaCl_2$ buffer systems, both spectra reflect a high content of secondary structure. The deamidation did not induce a large alteration of the far-UV CD spectra of α-LA, although slight increases of negative intensity were observed for the spectra of the molecules with high degrees of deamidation such as 55% and 66%. Therefore, it is clear that CD spectra of deamidated α-LA have a high content in backbone secondary structure.

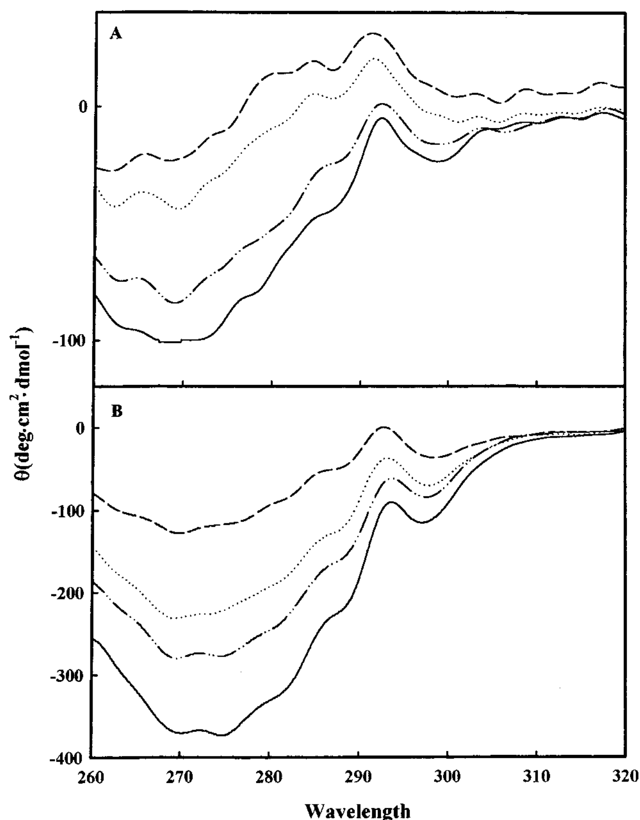

Figure 3. Near-UV circular dichroism spectra of α-lactalbumin solutions (0.04 wt %) at 42 °C. α-Lactalbumin was dissolved in Tris−HCl buffer (20 mM, pH 7.0) containing 2 mM EDTA (A) or $CaCl_2$ (B). (—), Nonmodified α-lactalbumin; (−··), α-lactalbumin deamidated for 4 h in the native state; (···), α-lactalbumin deamidated for 24 h in the native state; (− −), α-lactalbumin deamidated for 24 h in the molten globule state.

Figure 3 shows the near-UV CD spectra. For nonmodified α-LA, the spectrum in the EDTA buffer system (A) is drastically reduced in intensity as compared to that in the $CaCl_2$ buffer system (B) (note the difference in scale between the panels of the figure). A similar spectral change of α-LA induced by EDTA treatment was reported previously (*11*, *19*), suggesting the unfolding of tertiary structure to a large extent. The reduction in intensity of the CD spectra was observed for the deamidated α-LA in both the buffer systems. The degree of intensity reduction was closely related to the extent of deamidation. For instance, when α-LA in the native state was deamidated with the enzyme for 4 h, the degree of deamidation was just 20%, producing a reduction of spectral intensity to approximately three-fourths of that for the nonmodified one in the $CaCl_2$ buffer system (B). However, when α-LA was highly deamidated (66%) by the incubation of the protein in the molten globule state for 24 h, the spectral intensity was reduced to one-fourth. Similar changes according to the degree of deamidation were also seen in the EDTA buffer system (A). These results may be the first clearly showing that the deamidation of protein can alter the tertiary structure without touching the secondary structure.

It is worthwhile to note that near-UV CD spectrum of highly deamidated (66%) α-LA in the $CaCl_2$ buffer system (Figure 3B) resembles that of nonmodified α-LA in the EDTA buffer system (Figure 3A), indicating the similar environment of nonpolar side chains; in other words, unfolding of tertiary structure to a large extent. This result means that highly deaminated (66%) α-LA takes on the molten globule-like form even in the present of Ca^{2+}. Normally, Ca^{2+} stabilizes the tertiary structure of α-LA. However, $CaCl_2$ addition could not stabilize or restore the nativelike tertiary structure of deamidated α-LA. In this case, electrostatic repulsion of newly formed carboxyl groups in α-LA molecule by protein-glutaminase may be strong enough to compensate the stabilizing effect of Ca^{2+}. The alternative explanation for the absence of effect of Ca^{2+} addition is that deamidation affects the binding of Ca^{2+} to α-LA by distorting the Ca^{2+} binding loop in the molecule (*13*, *20*), which leads to the thermal instability of α-LA via the loss of Ca^{2+}-binding ability. A result of our preliminary experiment using Ca^{2+}-binding fluorescence dye, Quin-2, revealed that the Ca^{2+}-binding ability of α-LA is slightly, but significantly, decreased by deamidation (unpublished data). Further study is needed to clarify the relationship between Ca^{2+}-binding ability and conformational stability of deamidated α-LA.

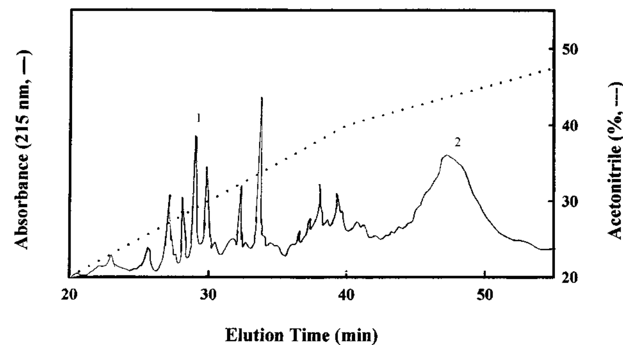

Figure 4. Reversed-phase HPLC separation of peptides derived from digestion of deaminated α-lactalbumin. α-Lactalbumin was digested by V8 protease. Peptides were eluted with a two-step linear gradient of acetonitrile (dotted line) and detected by measuring UV absorption (continuous line). Major peaks (marked by numbers) were collected and identified.

Identification of Glutamine Residues of α-Lactalbumin to be Attacked by Protein-Glutaminase. To know the site-specificity of protein-glutaminase, identification of modified glutamine residues in α-LA was carried out. From the results shown in Figure 1, it was thought that all possible reactive sites were deamidated by incubation of α-LA in the molten globule state with the enzyme for 24 h. Therefore, α-LA, which had been deamidated under this condition, was chosen as a sample for analysis. Deamidated α-LA was digested by V8 protease after the disruption of their intact disulfide bonds. The resultant peptides were separated using RP-HPLC. The elution pattern of the sample is shown in Figure 4. The main peaks were collected, and the N-terminal sequence of peptides in the fractions was analyzed. N-and C-terminal sequence of the whole α-LA molecule was also analyzed.

The process of identification of glutamine residues modified by protein-glutaminase is summarized in Table 1. Peak 1 from deamidated α-LA (Figure 4) included a peptide starting at Trp[26]. The peptide of peak 1 was identified as Trp[26]−Val ·····Thr−**Glu[39]**−Ala−Ile−Val−**Glu[43]** from the results of N-terminal amino acid sequencing and amino acid composition. These results showed the changes from glutamine to glutamic acid residues at positions 39 and 43. **Gln[43]** in the original sequence may be cleaved by V8 protease acting on newly formed **Glu[43]**. On the other hand, the reason for no

Table 1. Process of Identification of Glutamine Residues Deamidated by Protein-glutaminase in α-Lactalbumin

sample[a]	method of determination	sequence	identification
peak 1	N-terminal amino acid sequence	**W^{26}V- - -T*E*^{39}AIV*E*^{43}**	$Q^{39} \rightarrow E^{39}$
	analysis of amino acid composition	(original sequence[b] W^{26}V- - -T*Q*^{39}AIV*Q*^{43})	$Q^{43} \rightarrow E^{43}$
peak 2	N-terminal amino acid sequence	**I^{55}N- - -D*E*^{65}**	$Q^{54} \rightarrow E^{54}$
		(original sequence [b]- - -*Q*^{54}I^{55}N- - -D*Q*^{65}N- - -)	$Q^{65} \rightarrow E^{65}$
whole molecule	N-terminal amino acid sequence	**E*Q*^{2}L- - -**	Q^{2} (no modification)
whole molecule	C-terminal amino acid sequence (carboxypeptidase Y method)	**D*Q*^{117}WLCEKL^{123}**	Q^{117} (no modification)

[a] Peaks 1 and 2 were isolated from V8 protease digests of deamidated α-LA by HPLC as shown in Figure 4. [b] Primary sequence around glutamine residues in nonmodified α-LA. Existence of glutamine residues in original sequence was confirmed by applying the same analysis procedure to non-modified α-LA.

cleavage of the peptide bond between new **Glu[39]** and Ala[40] is not clear.

The peptide of peak 2 in Figure 4 started at Ile[55]. It was thought that the original sequence proceeding **Gln[54]** was deleted by V8 protease. This indicates the modification of **Gln[54]** to **Glu[54]**, leading to the emergence of a new reactive site for V8 protease. Unfortunately, we could not find the cleaved peptide, i.e., Tyr[50]−Gly−Ler−Phe−**Glu[54]**, in the other fractions in Figure 4, but it is reasonable to conclude that **Gln[54]** could be modified to **Glu[54]** by protein-glutaminase. The amino acid sequence analysis of the peptide in peak 2 showed that **Gln[65]** was changed to **Glu[65]**.

No modifications of Gln[2] and Gln[117] were confirmed by analyses of the N-terminal amino acid sequence and C-terminal sequence, respectively, of the whole molecule of deamidated α-LA. Therefore, it was shown that only 4 glutamine residues, that is **Gln[39]**, **Gln[43]**, **Gln[54]**, and **Gln[65]**, can be attacked by protein-glutaminase, although α-LA has 6 glutamines. The ratio of reactive to total glutamine residues is in good agreement with the degree of deamidation (66%) of deamidated α-LA demonstrated in Figure 1.

DISCUSSION

The results reported here showed that 4 out of 6 glutamine residues, **Gln[39]**, **Gln[43]**, **Gln[54]**, and **Gln[65]**, in α-LA were modified by protein-glutaminase. These glutamine residues were modified by protein-glutaminase more rapidly (Figure 1) when α-LA took on the molten globule state, a partially denatured form with nativelike secondary structure but disordered tertiary structure (*9*). It has been found that the accessibility of a protein molecule to enzymes increases in the molten globule state. For example, digestion of the channel peptide of colicin E1 by papain and bromelain is much faster in the molten globule state than in the native state (*21*). Matsumura et al. (*12*) reported the enhanced susceptibility of α-LA in the molten globule state to transglutaminase reaction. Our results are consistent with these findings. Although **Gln[39]**, **Gln[43]**, **Gln[54]**, and **Gln[65]** are not involved in the cluster of aromatic groups stabilizing the native structure of α-LA(13), the substantial alteration of the tertiary structure may be necessary for enhancing the accessibility of these glutamine residues to the enzyme by lowering the steric hindrance of adjacent side chains.

Unlike **Gln[39]**, **Gln[43]**, **Gln[54]**, and **Gln[65]**, Gln-[2] and Gln[117] remained untouched by the enzyme during transformation from the native state to the molten globule state of α-LA. The primary sequence around target sites may affect the reactivity of the glutamine residues toward protein-glutaminase. However, we could not find a clear rule concerning the sequence around targeted glutamine residues from the results of Table 1. Further studies are needed using various substrates to know the requirements of primary structures around glutamine residues targeted by protein-glutaminase.

The difference in reactivity among glutamine residues may be partially due to the bipartite structure of α-LA in the molten globule state (*22*). α-LA consists of an α-helical domain (including residues 1−37 and 86−123) and a *β*-sheet domain (residues 38−85). It has been shown that the two domains behave differently from one another when α-LA takes on the molten globule state. That is, the α-helical domain forms a helical structure with a nativelike tertiary structure, whereas the *β*-sheet domain is largely unfolded (*22*). All glutamine residues deamidated by protein-glutaminase, **Gln[39]**, **Gln[43]**, **Gln[54]**, and **Gln[65]**, were included in the *β*-sheet domain of α-LA, while nonreactive Gln[2] and Gln[117] were positioned in the α-helical domain. The nativelike folding structure of the α-domain may obstruct the access of protein-glutaminase to the target glutamin residues.

It is well-known that *Bacillus circulans* peptidoglutminase catalyzes the hydrolysis of glutamine residues in peptides (*23*). The isolated enzyme consisted of two distinct isozymes: peptidoglutaminase I and II (*24*). The former deamidated a C-terminal glutaminyl residue, and the latter deamidated internal glutaminyl residues as well as a C-terminal residue. The protein-glutaminase differs from the peptidoglutaminases in some points. Most importantly, the peptidoglutaminases are inactive against higher molecular mass peptides and proteins. For example, casein is not deamidated by peptidoglutaminase (*6*, *23*). In contrast, protein-glutaminase can deamidate proteins, including casein, with higher catalytic efficiency (*Kcat/Km*) than on short peptides (*8*). Furthermore, peptidoglutaminases are dimers with subunit sizes of molecular weight 42000 and 51000, and pI values of 4.1 and 4.0 (*25*), whereas protein-glutaminase is a single protein with a molecular weight of 19860 and a pI value of 10.0 (*8*). Peptidoglutaminases are located intracellularly (25), but the protein-glutaminase is a secreted protein (*7*). On the basis of these findings, it is thought that peptidoglutaminases and protein-glutaminase are quite different with respect to their physicochemical properties and substrate preferences.

Transglutaminase can also catalyze the deamidation reaction, if acyl acceptors are absent in the reaction mixture. Unfortunately, there have been no reports on deamidation of α-LA by transglutaminase, but amine-incorporation to and cross-linking of α-LA by microbial transglutminase from *Streptoverticillium* (MTGase) were investigated (*12*). This report showed that only Gln[54]

of six glutamine residues could be modified by MTGase. As shown in Table 1, however, four glutamine residues, including Gln[54], were deamidated by protein-glutaminase. Although it is impossible to compare the reactions of both the enzymes directly, we can say that protein-glutaminase has broader site-specificity than MTGase for target glutamine residues. Transglutaminases (especially MTGase) are now used widely as food agents to improve the textural properties of protein-based foods. However, the control of cross-linking is very difficult, so that the excess polymerization sometimes causes the generation of undesirable textural and physical properties. The high reactivity of protein-glutaminase over transglutaminases toward glutamine residues in food proteins suggests the possibility that protein-glutaminase can be used as a control agent for transglutaminase activity in real food systems.

We have already shown that casein and wheat gluten are highly susceptible to protein-glutaminase reaction (*8*). For gluten, in which one out of three amino acids is glutamine, the specific activity of protein-glutaminase was 10 times larger than that for α-LA, although the reaction was started in a suspension of gluten. Measuring deamidation of glutamine residues in a unique repeat sequence in gluten molecules (*26*) may be useful in revealing the mode of action of protein-glutaminase. Furthermore, the improvement of functional properties of gluten, such as solubility, foaming ability, etc., by deamidation can be expected. The application of protein-glutaminase to wheat gluten and other cereal proteins is now in progress.

CONCLUSION

Our results showed that a novel protein-glutaminase from *Chryseobacterium proteolyticum* could catalyze the deamidation of α-LA even in the native state, although the transformation to the molten globule state of α-LA enhanced the susceptibility of glutamine residues to the enzymatic reaction. Chemical treatments, such as the reduction of intact disulfide bonds of substrate proteins, were not necessary before the enzymatic reaction, which is advantageous to the food application. The deamidation of α-LA caused the loss of tertiary structure to a large extent, but did not influence secondary structure. Such a molten globule-like form is suggested to be closely related to the functional properties of food proteins such as gelling, emulsifying, and foaming. Therefore, the evaluation of functional properties of food proteins deamidated by protein-glutaminase should be the most important topic in the next stage. Protein-glutaminase with broad site-specificity may be applicable to the modification of various food proteins as well as modulation of the cross-linking reaction of transglutaminase in food systems.

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