Effects of Deamidation and Fragmentation on Antioxidative Activity of α-Zein

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Abstract: The effects of deamidation or fragmentation on the antioxidative activity of α-zein were investigated individually. Heat treatment at 70°C in 70% ethanol - 0.05 M HCl caused deamidation of α-zein without fragmentation. Antioxidative activity of α-zein was improved by this deamidation. α-Zein with deamidation treatment for 12 hours protected 80% of docosahexaenoic acid ethyl ester from autoxidation until 7 days. Model peptide of α-zein had a lower antioxidative activity than α-zein. Fragmentation by N-bromosuccinimide decreased the antioxidative activity of α-zein.


Key words: α-zein, antioxidative activity, deamidation, fragmentation

1 Introduction

Some groups have reported the antioxidative activity of prolams in a powder system (1-4). This novel function draws interest from a fundamental and practical standpoint.

Prolamins are classified into three groups based on their amino acid sequence, i.e., sulfur-rich prolamin, sulfur-poor prolamin, and a third group (5). In a previous study (6), we showed the highest antioxidative activity of C hordein in the sulfur-poor group. α-Zein, the representative of the third group, also showed an ability to retard lipid oxidation, although inferior to that of C hordein. C Hordein is different from α-zein in content and distribution in the primary sequences of glutamine. The molecular weight (53 kDa) of C hordein was higher than that of α-zein (20 kDa) (5).

The application of prolams is very limited in the food industry because of the low solubility in aqueous solutions. The high glutamine content should be considered responsible for such low solubility. Accordingly, deamidation (with or without fragmentation) of prolams has been attempted to improve functional properties such as solubility, emulsifying, and foaming properties (7-9).

However, Chiue et al. reported that antioxidative activity of prolams was reduced by deamidation with fragmentation (10-12). Therefore, the question arises as to whether the reduction in the antioxidative activity of prolams is deamidation or fragmentation. Thus, we used appropriate conditions to deamidate prolams without fragmentation in the present study in order to assess the effects of deamidation on antioxidative activity solely. We also prepared fragment peptides from prolams to understand the effect of fragmentation, in other words, the importance of molecular size, for antioxidative activity of prolams.

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2 Experimental

2.1 Materials

Commercial zein and stearic acid ethyl ester (SA-EE) were purchased from Nacalai Chemicals Ltd. (Kyoto, Japan). Eicosapentaenoic acid ethyl ester (EPA-EE) and docosahexaenoic acid ethyl ester (DHA-EE) were donated from Nippon Chemical Feed Co., Ltd. (Hakodate, Japan). N-bromosuccinimide (NBS) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). A model peptide of the repeat sequence of α-zein (MPRSaZ, Lot No. L11006T1, amino acid sequence: Gln-Leu-Leu-Pro-Ala-Asn-Gln-Leu-Ala-Val-Ala-Asn-Pro-Ala-Ala-Tyr-Leu-Gln) was synthesized by the Takara Shuzo Co., Ltd. Biomedical Group. Other analytical grade reagents were purchased from Nacalai Chemicals Ltd. (Kyoto, Japan) and Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.2 Preparation of Prolamins

Commercial zein was found by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis (13) to consist almost entirely of α-zein sub-units. Therefore, commercial zein was used in the experiments without further purification. This zein was treated with solvent-extraction to remove low molecular weight antioxidants before the subsequent experiments. The solvent-wash procedure was composed of four stages. First, α-zein (100 g) was vigorously stirred for 24 hours with two 2-L portions of n-hexane. After washing by n-hexane, the mixture was filtered and the precipitate (n-hexane-washed) was obtained. Second, the precipitate (n-hexane-washed) was vigorously stirred for 24 hours with two 2-L portions of chloroform. After washing by chloroform, the mixture was filtered and the precipitate (n-hexane and chloroform-washed) was obtained. Third, the precipitate (n-hexane and chloroform-washed) was vigorously stirred for 24 hours with two 2-L portions of ethyl acetate. After washing by ethyl acetate, the mixture was filtered and the precipitate (n-hexane, chloroform and ethyl acetate-washed) was obtained. Last, the precipitate (n-hexane, chloroform and ethyl acetate-washed) was vigorously stirred for 24 hours with two 2-L portions of acetone. After washing by acetone, the mixture was filtered and the precipitate (n-hexane, chloroform, ethyl acetate and acetone-washed) was obtained. This zein, washed in 4 stages, was dried and used for the following experiments.

2.3 Deamidation of α-Zein

α-Zein was deamidated chemically using hydrochloric acid in aqueous ethanol. α-Zein (1 g) was solubilized in 350 mL of ethanol, 125 mL of distilled water and 25 mL of 1 M hydrochloric acid. The mixture in a sealed glass vessel was heated at 70°C. This reaction was stopped with 25 mL of 1 M sodium hydroxide and then dialyzed against 0.1 M acetic acid and freeze-dried. Then the deamidated α-zein was treated by solvent extraction using ethyl acetate to remove low molecular weight antioxidants.

2.4 Fragmentation of α-Zein

α-Zein was chemically fragmented by NBS according to the following procedure (14,15). α-Zein (500 mg) was put into a 100-mL sealed glass vessel and then 50 mL of distilled water, 30 mL of acetic acid and 1.8 g of NBS were added. The glass vessel was then wrapped with aluminum foil to shield it from light. This mixture was shaken sufficiently to dissolve α-zein, and the reaction was carried out at room temperature. After two hours, 2 mL of 2-mercaptoethanol was added to stop the reaction. The reaction mixture was dialyzed against 0.1 M acetic acid using Spectra/Pro® CE membrane (MWCO: 500) (molecular weight cutoff, 500) and freeze-dried.

2.5 Electrophoresis

SDS-PAGE was performed on a ready-made gradient slab gel (PAGEL NPG-1020L with a concentration from 10 to 20%, ATTO Corporation, Tokyo, Japan) using Laemmli’s continuous system (16). Alkaline urea PAGE was performed on a slab gel using pH 8.7 and the 8 M urea buffer system according to Eipper et al. (17). Gels were stained with Coomassie Blue R-250 and trichloroacetic acid (12%) and destained with distilled water containing a small amount of Triton X-100 (18,19).

2.6 Removal of Peroxides from Unsaturated Fatty Acid Ethyl Esters

All ethyl esters of unsaturated fatty acid (UFA-EE) were freed from peroxides by passing them through Sep-Pak Vac 20cc (5 g) Florisil Cartridge, twice. The absence of peroxides in UFA-EEs was confirmed by measuring peroxide values. Peroxide-free UFA-EEs
were used immediately in the following experiments.

2.7 Preparation of Powder Systems
All prolamin were washed with ethyl acetate to remove antioxidants before preparation of the powder systems. A mixture of UFA-EE and stearic acid ethyl ester (SA-EE) dissolved in n-hexane was added to α-zein or related materials to a ratio of 1:9 by weight, respectively. SA-EE was added to allow estimation of the extraction efficiency of the UFA-EE’s. Sample powders were subdivided into small portions and stored in a humidity-controlled plastic box at 40°C. Water activity (A_w) was adjusted to 0.9 with 22% (w/w) sulfuric acid (1,3,20). Samples were removed at stated intervals, and the oxidation of UFA-EE was followed by gas chromatographic analysis and peroxide measurement.

2.8 Gas Liquid Chromatography
Protein-UFA-EE-SA-EE mixed powder (5 mg) was extracted three times with 1 mL of n-hexane. After centrifugation, the supernatants were combined and filtered through a Cosmonice Filter S, No. 440-85 (Nacalai Chemicals Ltd., Kyoto, Japan) to remove insoluble materials. The filtered solution was evaporated to dryness under a stream of nitrogen and dissolved in 100 µL of n-hexane. An aliquot (1 µL) was directly injected into the column inlet of a Shimadzu GC-9A PTF gas chromatograph (Shimadzu Co. Ltd., Kyoto, Japan) equipped with a hydrogen flame ionization detector. Analytical conditions were: a glass column (0.32×210 cm) packed with Silar 10C (10%) on Chromosorb W (AW-DMCS, 60-80 mesh); temperature program, 160-240°C at 4°C/min; injection and detection temperature, 250°C; carrier gas (N_2) at a flow rate of 60 mL/min; N_2 pressure, 6 kg/cm²; H_2 pressure, 0.6 kg/cm²; air pressure, 0.5 kg/cm².

2.9 Measurement of Peroxide Value (POV)
The POV was measured by the ferric thiocyanate method (1). A 5-mg portion of each sample powder was extracted with 1 mL of chloroform-methanol (2:1, v/v) and centrifuged at 1350×g for 10 min. The supernatant (0.25 mL) was diluted with 4.55 mL of the same solvent, and 0.1 mL of 30% ammonium thiocyanate and

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Fig. 1 (a) Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis of α-zein and Deamidated α-zeins. A detailed description is given in the text. Lane 1, α-zein; lane 2, 4h deamidated α-zein; lane 3, 8h deamidated α-zein; lane 4, 12h deamidated α-zein. (b) Alkaline Urea Polyacrylamide Gel Electrophoresis of α-zein and Deamidated α-zeins. Lane 1, α-zein; lane 2, 4h deamidated α-zein; lane 3, 8h deamidated α-zein; lane 4, 12h deamidated α-zein. (c) Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis of α-zein and Fragmented α-zein. Lane 1, α-zein; lane 2, 12h deamidated α-zein; lane 3, fragmented α-zein.
0.1 mL of mixture of equal volumes of 0.02 M ferrous sulfate and 0.02 M barium chloride were added. The absorbance at 500 nm was measured exactly 3 min after the start of reaction. The POV was expressed as absorbance at 500 nm without calibration.

3 Results

3.1 Deamidation or Fragmentation on \( \alpha \)-Zein

Figure 1-a shows the SDS-PAGE pattern of the \( \alpha \)-zein band and the bands after deamidation. The mobility of \( \alpha \)-zein bands was not changed after HCl-deamidation. That is, the molecular weight of \( \alpha \)-zein did not change by HCl-deamidation. With NBS-fragmentation, the \( \alpha \)-zein was degraded to under about 3500 dalton (Fig. 1-c).

Figure 1-b shows the alkaline urea PAGE pattern of \( \alpha \)-zein and HCl-deamidated \( \alpha \)-zeins. With the longer treatment of HCl in 70% EtOH, the band moved more. From the two results, the molecular weight was not changed and a plus charge was induced, and the \( \alpha \)-zein was deamidated by HCl-deamidation at 70°C without fragmentation.

3.2 Antioxidative Activity of Deamidated \( \alpha \)-Zeins

Figure 2 and 3 show the antioxidative activity of the original \( \alpha \)-zein and the deamidated \( \alpha \)-zeins in a powder system. Lipid oxidation was followed by measurement of the remaining unoxidized lipid using GLC (Fig. 2-a and 3-a) and the measurement of POV (Fig. 2-b and 3-b). Antioxidative activity of \( \alpha \)-zein was improved remarkably by deamidation (Fig. 2 and 3). In the case of EPA-EE, original \( \alpha \)-zein did not prevent EPA-EE from oxidation for only 1 day. 8h-Deamidated \( \alpha \)-zein protected 80% of EPA-EE from oxidation until day 4.5. The inhibition of EPA-EE oxidation was prolonged to day 8.5 with 4h- and 12h-deamidated \( \alpha \)-zein (Fig. 2-a). When EPA-EE was encapsulated with non-modified \( \alpha \)-zein, POV reached maximum at day 2 and decreased thereafter, indicating the production and degradation of lipid peroxide. However, deamidated \( \alpha \)-zeins inhibited the formation of peroxides (Fig. 2-b). The same tendency was found with a DHA-EE powder model system (Fig. 3). \( \alpha \)-Zein did not protect DHA-EE even at day 1 from oxidation, whereas deamidated \( \alpha \)-zeins showed higher antioxidative activity. In the case of 8h-deamidated \( \alpha \)-zein, 80% of DHA-EE remained at day 3. Both 4h- and 12h-deamidated \( \alpha \)-zeins kept about 80% of DHA-EE from oxidation until day 7 (Fig. 3-a). Deamidated \( \alpha \)-zeins delayed the formation of peroxides compared with non-modified \( \alpha \)-zein as shown in Fig. 3-b.

3.3 Antioxidative Activity of Fragmented

![Graph](image)

Fig. 2 (a) Time-dependent Changes in Amount of EPA-EE. Ratio of SA-EE: EPA-EE = 1:2.5 (w/w). Open triangles, \( \alpha \)-zein; open diamonds, 4h-deamidated \( \alpha \)-zein; open squares, 8h deamidated \( \alpha \)-zein; open circles, 12h deamidated \( \alpha \)-zein. Each value was expressed as the mean ± SD (n = 3). (b) Time-dependent Changes in Peroxide Value in Powder System. Symbols as for (a). Each value was expressed as the mean ± SD (n = 3).
**α-Zein and Model Peptide of Repeat Sequence of α-Zein**

Antioxidative activity of synthesized peptide based on the repeat sequence of α-zein was lower than α-zein (Fig. 4). The effect of fragmentation on the antioxidative activity of α-zein was demonstrated in Fig. 5. Peptide fragment from α-zein could not protect α-linolenic acid ethyl ester from oxidation, whereas original α-zein inhibited the oxidation of α-linolenic acid ethyl ester for 5 days (Fig. 5-a). POV increased only at 3 days when the peptide fragments were encapsulated (Fig. 5-b). The same tendency was observed in the case of AA-EE (data not shown).

![Graphs showing time-dependent changes in amount of DHA-EE and absorbance at 500 nm.](image)

**Fig. 3** (a) Time-dependent Changes in Amount of DHA-EE. Ratio of SA-EE:DHA-EE = 1:2.7 (w/w). Open triangles, α-zein; open diamonds, 4h-deamidated α-zein; open squares, 8h deamidated α-zein; open circles, 12h deamidated α-zein. Each value was expressed as the mean±SD (n = 3). (b) Time-dependent Changes in Peroxide Value in Powder System. Symbols as for (a). Each value was expressed as the mean±SD (n = 3).

![Graphs showing time-dependent changes in amount of α-AA-EE and absorbance at 500 nm.](image)

**Fig. 4** (a) Time-dependent Changes in Amount of α-AA-EE. Ratio of SA-EE:AA-EE = 1:2.2 (w/w). Open triangles, α-zein; closed triangles, model peptide of repeat sequence of α-zein. Each value was expressed as the mean±SD (n = 3). (b) Time-dependent Changes in Peroxide Value in Powder System. Symbols as for (a). Each value was expressed as the mean±SD (n = 3).
4 Discussion

In this paper, the changes in antioxidative activity of α-zein by deamidation and fragmentation were investigated.

An enzymatic reaction is the best way to modify proteins without non-specific and undesirable change. In the case of deamidation, transglutaminase (TGase) is generally used. TGase catalyzes an acyl transfer reaction between the γ-carboxyamide group of peptide-bound glutaminyl residue and a variety of primary amines (for example, ε-amino group of peptide-bound lysyl residue) (21). If no amines are present in the reaction system, TGase catalyzes the hydrolysis of γ-carboxyamide group of the glutaminyl residue, that is, TGase catalyzes deamidation (21). However, α-zein was chemically deamidated by heating with diluted HCl at 70°C in the present study because transglutaminase was inactive in the buffer or solvent systems which can dissolve the α-zein. The fragmentation by deamidation was checked using SDS-PAGE (Fig. 1-a), and was not found to occur during the deamidation reaction at 70°C for 12 hours. On the other hand, the progress of deamidation with reaction time was confirmed by alkaline urea PAGE. (Fig. 1-b). The more migration of bands indicated the progress of deamidation of α-zein to a larger extent with increasing reaction time.

Fragmentation of α-zein was also carried out by a chemical method for the same reason in the case of deamidation. Cyanobromide (CNBr) method was not adopted for chemical fragmentation of α-zein, because α-zein has only two cystein residues in the N-terminal region. Therefore, if α-zein had been fragmented by the CNBr method, the molecular size would have remained virtually unchanged. Therefore, in this study, α-zein was cleaved using NBS which cleaves protein at histidine, tyrosine and tryptophan.

The results showed that antioxidative activity of α-zein was improved by deamidation without fragmentation (Fig. 2 and 3). Even DHA-EE could be protected for 7 days from oxidation by deamidated α-zein in the powder system in the absence of other antioxidants. However, Chiue et al. reported that the deamidation of α-zein reduced the antioxidative activity in the powder system (10-12). The contradiction between our results and those in their previous studies would be due to the deamidation conditions. Chiue et al., heated α-zein at 95°C in 0.05 M HCl, causing both fragmentation and deamidation. We introduced the more mild condition (70°C in 0.05M HCl – 70% EtOH) which is referred to by Chiue et al. (10-12) and Bollecker et al., (8) and successfully prepared the deamidated α-zein molecules without fragmentation. Zein’s structure consists of an N-terminal part, central part (nine or ten repeating sequences each of which forms a single alpha-helix and these repeating sequences are joined by glutamine-rich loops), and a C-terminal part (5,22,23). Deamidation changed glutamine residues to glutamic acid residues in
polypeptides and resulted minus charge increase in alpha-zein. Such an increase in the minus charge may be caused by the change in some interaction between polypeptides and lipids, and thus affected the antioxidative activity of polypeptides.

The antioxidative activity of deamidated alpha-zein was better than that of C hordein because we earlier reported (6) that C hordein could not protect DHA-EE from oxidation. This made it possible to find positive effects of deamidation on antioxidative activity of alpha-zein.

A model peptide of repeat sequence of alpha-zein (MPRSαZ) which contains only one alpha-helix sequence showed lower antioxidative activity than alpha-zein (Fig. 4). It is of interest that, despite the fact that the number of AA-EE and double bonds per alpha-helix sequence in the MPRSαZ was less than the number of AA-EE and double bonds per alpha-helix sequence in the alpha-zein, the antioxidative activity of MPRSαZ was inferior to the antioxidative activity of alpha-zein (Table 1). Chemical fragmentation caused a reduction in the antioxidative activity of alpha-zein (Fig. 5). From these results, fragmentation lowers antioxidative activity of alpha-zein, that is, it is insufficient to exhibit the antioxidative activity with only alpha-helix sequence. To exert antioxidative activity in the case of alpha-zein type prolamin, polypeptide must have a structure containing the plural alpha-helix sequence.

From this study, fragmentation of lower molecule size reduces antioxidative activity of alpha-zein, and deamidation without fragmentation increases antioxidative activity of alpha-zein. Alpha-zein is not utilized much because of its low solubility and low nutritional value (alpha-zein does not contain tryptophan residue). We think that improvement of the antioxidative activity of alpha-zein by simple mild treatment is useful for the food industry.

References


Table 1  Comparison of Several Properties of alpha-zein and MPRSalphaZ.

<table>
<thead>
<tr>
<th>Property</th>
<th>alpha-zein</th>
<th>MPRSalphaZ</th>
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<tbody>
<tr>
<td>Molecule weight</td>
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</tr>
<tr>
<td>Number of alpha-helix sequences</td>
<td>9/10</td>
<td>1</td>
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<tr>
<td>Number of AA-EE (per protein or peptide)</td>
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<tr>
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<td>0.45</td>
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<td>1.8</td>
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<tr>
<td>Double bond of AA-EE (per alpha-helix)</td>
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<td>1.8</td>
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