Note

Decreased Antioxidative Activity of Maize Zein in Response to Deamidation Rate

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Received June 16, 1993

Ten samples with different deamidation rates were prepared from commercially available maize zein by heat treatment with 0.05 M HCl in 70% ethanol, and were examined for their antioxidative activity in simply mixed protein–fatty acid (10:1) model systems at moderate humidity. A regression line with a correlation coefficient of \( r = -0.946 \) was obtained, when the linoleate/palmitate ratio on day 3 was plotted against the percentage of deamidation.

Many proteins and peptides are protective in varying degrees against the autoxidation of unsaturated lipids.\(^{1-3}\) We have previously demonstrated that wheat gliadin and maize zein were exceedingly antioxidative in powder model systems at high humidity\(^{4-6}\) and have offered a plausible explanation for the mechanism from a physical rather than chemical point of view.\(^{4,6}\) A very high content of glutamine, but not of glutamic acid, is characteristic of these proteins belonging to prolamine, endowing them with unique physical properties. It is, therefore, possible that the cleavage of amide bonds in prolamine would affect its antioxidative activity to a considerable extent. This preliminary report describes which correlation is valid between the antioxidative activity and the deamidation rate for partially deamidated zein samples.

Maize zein was purchased as a chemical reagent (Lot No. MOP1072) in April 1990 from Nacalai Tesque Ltd., Kyoto, and used without further purification. The protein was dissolved in 10 volumes of a 70%-ethanolic solution containing 0.05 M HCl, and divided into several portions in closed glass tubes, before heat treating at 90°C for the desired periods. Aliquots were used for an ammonia assay according to the method of Weathersburn,\(^7\) and the remainder was indolipholysed after adequately dialyzing against distilled water. Acid hydrolysis with 3 N HCl at 110°C for 3 h led to the complete deamidation of zein, the deamidation rate being expressed in percent as the ratio of free ammonia for each sample to that released under the specific conditions. An assay of the antioxidative activity was conducted on ten differently deamidated preparations of zein, each being dissolved or suspended in an equimolar mixture of chloroform and methanol containing linoleic-palmitic (2:1) acids at 10 wt% of protein. These samples were then put in a 40°C incubator without moisture control (but in a humidity range of 50 to 60%) after completely removing the organic solvent. Definite amounts of the respective samples were taken out after specific periods and assayed for both accumulated hydroperoxides and residual fatty acids. PV at each sampling time during the storage period at 40°C is, for convenience, expressed as the absorbance at 500 nm under the routine assay conditions.\(^8\) Unimpaired linoleic and palmitic acids, which were almost quantitatively extracted with chloroform–methanol (1:1), were methyl-esterified with a 15% BF\(_3\) solution in methanol and determined by GC as previously described.\(^9\) \(\alpha\)-Corn starch was used as a reference control not having antioxidative activity throughout this experiment. The tocopherol content of several deamidated zein preparations was obtained as the mean of triplicate determinations by comparing with the peak height of authentic tocopherol from HPLC.\(^{10}\)

Figure 1 shows the results of examining the antioxidative activity of zein preparations with various deamination rates in respect of (a) PV and (b) L/P ratio. Several representatives have been selected so as not to confuse interpretation of the graphs. Taken altogether from the left and right graphs, the more the deamidation proceeded, the more the antioxidative activity decreased. In Fig. 2, the deamidation rate is plotted with respect to (a) PV and (b) L/P ratio on day 3. The plots for both L/P ratio and PV give straight lines with correlation.

**Fig. 1.** Antioxidative Effects of Various Deamidated Maize Zein Preparations in a Powder Model System

Powder samples consisting of partially deamidated zein preparations and fatty acids at a 10:1 weight ratio were incubated at 40°C (50–60% for humidity) under aerobic conditions. The PV and L/P ratio for three series of samples withdrawn at appropriate intervals were measured by the ferric thiocyanate method and GC, respectively. The deamidation rate of each sample was as follows (in %): • 0; ○, 20; ●, 36; △, 61.1; □, 79.8; ■, α-corn starch instead of zein.

**Fig. 2.** Deamidation Rate against Antioxidative Activity Expressed as Peroxide Value (left) and Residual Linoleate Ratio (right).

Three series of ten deamidated zein preparations, including the ones given in Fig. 1, were used. The respective PV data and L/P ratios on day 3 were measured to compare the antioxidative activity among the samples. The two regression lines and their coefficients were estimated in the usual manner.\(^{11}\)

**Abbreviations:** L/P ratio, ratio of linoleate to palmitate; PV, peroxide value; HPLC, high-performance liquid chromatography; GC, gas chromatography; TCA, trichloroacetic acid; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; MW, molecular weight.
Antioxidative Activity of Deamidated Maize Zein

Table: Loss of Inherent Tocopherols in Zein by Deamidation

<table>
<thead>
<tr>
<th>Deamidation (%)</th>
<th>Tocopherol content (µg/g of protein)</th>
<th>α-Toc</th>
<th>β-Toc</th>
<th>γ-Toc</th>
<th>δ-Toc</th>
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<tr>
<td>0</td>
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<td>1.55</td>
<td>0.10</td>
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<td>20.6</td>
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<td>0.31</td>
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<td>38.0</td>
<td></td>
<td>—</td>
<td>—</td>
<td>0.09</td>
<td>—</td>
</tr>
<tr>
<td>61.1</td>
<td></td>
<td>—</td>
<td>—</td>
<td>&lt;0.01</td>
<td>—</td>
</tr>
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</table>

Tocopherols were determined with a Shimadzu LC-6A high-performance liquid chromatograph: column, Shimpack CLC-STL (4.6 x 250 mm); eluant, n-hexane-dioxane-ethanol (490:10:1 v/v); flow rate, 2 ml/min; detector, Shimadzu RF-535 spectrophotometer (Ex 298 nm, Em 325 nm). Tocopherols and 2,2,5,7,8-pentamethyl-6-hydroxycromen as the reference and internal standard were products of E. Merck and Wako Pure Chemical Industries, respectively. Samples taken at certain intervals were pretreated according to a modification of the method described by Abe et al.10 In brief, a definite amount of each sample (0.2 g) was suspended in 30 ml of ethanol containing 0.4 g of pyrogallol and 1.2 g of KOH, refluxed for 30 min while stirring, and extracted three times with 30 ml of ether containing the pentamethylhydroxycromen as an internal standard. The combined extracts were washed several times with distilled water and evaporated to dryness, after dehydrating over anhydrous sulfate. The residue was dissolved in 0.2 ml of n-hexane and infused into the HPLC apparatus; the retention times of α, β, γ, and δ-tocopherols were 5.33, 9.48, 10.43, and 16.54 min, respectively. Values were obtained as the means from three determinations.

Fig. 3. Fragmentation of Zein in the Process of Deamidation.

A 2% zein solution in 70% ethanol (0.05 HCl) was divided into ten-odd portions and heated at 90°C in sealed vessels. After cooling, each solution was taken out from the vessel and acidified with 9 volumes of 10% TCA, except for the samples used for ammonia determination. The clarified supernatant after centrifugation was subjected to UV-absorbance measurement; the absorbance at 280 nm of 0.1% zein in 70% ethanol being 0.422.

Wang et al.15,121 have referred to the important role of tocopherols, besides their physical properties, in the antioxidative activity of zein. We investigated to what extent tocopherols or other natural antioxidants present in zein would be impaired in the course of deamidation. The results of tocopherol measurement by HPLC are summarized in the Table. The whole tocopherol content in intact zein was approximately 10.48 µg/g of protein, in which γ-tocopherol predominated. When the deamidation rate was 20.6%, γ-tocopherol was reduced to less than one-twentieth of its initial content, and the other tocopherols were below their limit of detection; nevertheless, a sharp fall in the antioxidative activity did not occur. The zein preparation at the rate of 38.0%, at which level the tocopherol almost had been removed (about one-hundredth of their initial content), still retained moderate antioxidative activity. This may imply that traces of γ-tocopherol remaining in cracks of the polypeptide granules were intimately involved in the maximum effect. In this case, the decreased antioxidative activity would not be directly correlated with the loss of tocopherols as a whole. In this respect, traces of phenolic compounds are known to reside and serve as potent antioxidants in isolated oil-seed proteins after oil expression.13,140 It is likely that such is also the case with isolated cereal proteins. In practice, the extraction treatment with water-saturated n-butanol that is effective in the removal of phenolic compounds from isolated soybean protein caused a reduction in the antioxidative activity of zein. This could be interpreted as the inter- or intra-molecular attraction force having been disturbed by the use and incomplete removal of n-butanol.

The change in MW of zein by deamidation was evaluated by measuring the absorbance at 280 nm after centrifugal removal of the TCA precipitate. As shown in Fig. 3, the UV absorbance of the TCA-soluble fraction increased progressively with increasing reaction time, indicating that the constituent proteins would have been substantially fragmented during the process of deamidation. The fragmentation of zein at an advanced stage of deamidation, although MW was more than 10,000 dalton, was ascertained by gel filtration with Sephacyr S-200 and by SDS-PAGE (data not shown). While peptides are generally superior to protein in their antioxidative activity, probably because of augmented functional groups,15 zein lost its antioxidative activity during the process of deamidation. The latent antioxidative activity of zein is most apparent at high humidity, and a mode of action can be accounted for by embedded oil that can protect from external oxygen attack.121 As an analogy to this, it seems reasonable to consider that the deamidation-caused cleavage of amide bonds and subsequent conformational changes were mainly responsible for the decreased antioxidative activity. However, it is necessary to obtain further information on the type of substance buried in the protein matrix and in contact with oily microdroplets.

Acknowledgment. We are indebted to Professor G. Kajimoto and Dr. A. Shibahara of Kobe Gakuin University for their help when analyzing the tocopherols and fatty acids.

References