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Enzymatic detection of L-isoaspartyl residues in food proteins and the protective properties of trehalose

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Protein L-isoaspartate (D-aspartate) O*-methyltransferase (PCMT) is a ubiquitous enzyme with substrate specificity toward proteins and peptides containing altered aspartyl residues. These are generated from the spontaneous deamidation of normal L-asparaginyl residues and/or isomerization of L-aspartyl residues. Such degradation reactions occur both* in vivo *and in purified protein molecules. Because of its peculiar substrate specificity, PCMT has been proposed as a tool for identifying and characterizing the L-isoaspartyl-containing byproducts of natural and recombinant proteins. Data presented here show the usefulness of PCMT for the identification of these altered residues in food proteins and the occurrence of these at detectable levels in several proteins from various animal sources. In addition, we noted that ovalbumin, either as a purified protein or in the whole chicken egg, becomes a better substrate for this enzyme during prolonged storage, thus indicating a progressive accumulation of the altered amino acid residues over time. The protective effects of trehalose against the occurrence of such specific protein damage in ovalbumin stored for several days were also assessed.* (J. Nutr. Biochem. 8:535–540, 1997) © *Elsevier Science Inc. 1997*

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Introduction

The enzyme protein L-isoaspartate (D-aspartate) *O*-methyltransferase (PCMT; EC 2.1.1.77) catalyzes, with high affinity, the transfer of a methyl group from S-adenosylmethionine to the free α-carboxyl moiety of altered L-isoaspartyl residues in protein and peptide substrates. These residues appear as the result of spontaneous and time-dependent deamidation of normal asparaginyl or isomerization of aspartyl residues.[1]

PCMT is ubiquitous in nature (it has actually been found in both prokaryotes and eukaryotes), and its function has been linked, in cellular systems, to the repair of the isopeptide bonds present at the level of altered L-isoaspartyl residues.[1]

It has also been demonstrated that such L-isoaspartyl residues are generated during the normal shelf life of proteins of pharmacological interest, and the enzyme PCMT has been successfully used for characterizing these degradative protein byproducts.[2–5]

The rationale of the present study lies in the use of PCMT for the in vitro identification and quantification of L-isoaspartyl residues in food proteins.

We also examined the protective effects of trehalose, a nonreducing disaccharide produced from enzymatic hydrolysis of starch.[6,7] Because trehalose addition to proteins is an effective means of preventing their deterioration, we investigated methyl acceptability of a model food protein, ovalbumin, lyophilized in the presence of trehalose, to test the ability of this sugar to prevent the intramolecular protein degradation reactions that are specifically monitored by methylation.

Data presented here show that food proteins are signifi-

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cantly affected by spontaneous reactions that give rise, to a different extent during their storage, to altered aspartyl sites specifically recognized by this enzymatic probe. Trehalose efficiently protects the tested proteins against the appearance of these altered sites.

The potential implications of our findings as a way of evaluating the time and conditions of food storage, as well as their possible influence on digestibility of food proteins are also discussed.

Methods and materials

Materials

Ovalbumin was purchased from both Serva Feinbiochemica GmbH & Co (Germany) and Sigma Corp. (St. Louis, MO, USA), here referred to as source A and source B, respectively. Other proteins used in the study were from Sigma Corp. S-adenosyl-L-[methyl-^{14}C] methionine (58 mCi/mmol) was purchased from Amersham International (UK). All reagents used were of analytical grade. Trehalose, as a crystalline powder, was from Hayashibara Co., Ltd., Japan.

Enzyme purification

The enzyme PCMT was purified from human erythrocytes, as described previously.[8,9] An enzyme unit (U) was defined as the amount of enzyme capable of transferring 1 pmol of $-CH_3$/min.

Ovalbumin preparation

Ovalbumin was prepared from fresh chicken egg whites according to the method of Kekwick and Cannan[10] and recrystallized three times from a Na_2SO_4 aqueous solution. Briefly, the Na_2SO_4 solution, prepared by dissolving 80 g of the anhydrous salt in 200 mL of warm water, was added to an equal volume of egg white. The mixture was stirred, and after 2 h the precipitate was removed by centrifugation (15,000 $\times$ g for 30 min). A 0.2 N H_2SO_4 solution was slowly added to the supernatant to reach a pH of about 4.6–4.8; anhydrous Na_2SO_4 was then added, until a permanent opalescence developed. The mixture was then decanted for 1 day. The crystalline material was removed by centrifugation (15,000 $\times$ g for 30 min) and redissolved in water equal in quantity to the original starting volume. Recrystallization was then obtained by addition of the Na_2SO_4 solution, and the process was repeated twice. The final product was then dialyzed and lyophilized as indicated.

In the experiments in which ovalbumin was purified from chicken eggs, which had been kept in the refrigerator for different time periods, and tested for its methyl accepting capability, we proceeded as follows. Chicken eggs, from the same animal, were kept at 4°C for different time periods. At the indicated time points ovalbumin was purified and assayed in vitro as a substrate of PCMT, according to the method described below.

PCMT enzyme assay

The radiochemical enzymatic assay method of vapor diffusion was used[11] which uses S-adenosyl-L-[methyl-^{14}C] methionine as the methyl donor. One hundred μg of substrate protein were incubated at 37°C for 3 h in the presence of 2.1 U PCMT (isoenzyme II),[12] in citrate buffer 0.2 M, pH 6.2, in a 40 μL final volume. After incubation, the reaction was quenched by adding an equal volume of a 0.2 M NaOH, 1% SDS solution. Protein methyl esters were determined after hydrolysis and liquid scintillation counting of labeled methanol released from methyl ester hydrolysis and trapped during the vapor diffusion step.[11] Proteins were determined by the method of Bradford.[13]

Sodiumdodecylsulphate/polyacrilamide gel electrophoresis (SDS/PAGE)

Sodiumdodecylsulphate polyacrilamide gel electrophoresis (SDS/PAGE) was performed according to the procedure of Laemmli.[14] After electrophoretic separation, proteins were evidenced by Coomassie blue staining.

Trehalose experiments

PCMT assays were preliminarily performed to rule out any interference of trehalose with enzyme activity and/or the methyl accepting capability of peptide substrates. In order to study the effect of trehalose on the methyl accepting capability of ovalbumin, aqueous solutions containing trehalose and ovalbumin at different w/w ratios were Iyophilized and stored at −20°C or +25°C for different times. These samples were redissolved in citrate buffer at the indicated time points and immediately assayed for PCMT. Parallel control samples, in which trehalose was added to ovalbumin only at the end of storage, just before performing the PCMT assay, were included to each set of methylation experiments.

Statistics

Results are expressed as the mean ± SD of three determinations.

Results

Enzymatic methylation of food proteins in vitro

Various purified proteins that are normally present in common foods of animal origin were selected and analyzed for the presence of L-isoaspartyl sites, as evidenced by methyl ester incorporation, after incubation with purified PCMT and the labeled methyl donor. Among selected proteins, which all showed to function as substrates for the enzyme, casein, in particular, demonstrated the highest methyl ester formation with respect to other proteins (*Table 1*).

Formation of methyl accepting sites in ovalbumin during its storage

To correlate possible variations of protein methyl acceptability as a function of storage time and temperature, we selected ovalbumin as model food protein, which we purified and tested as an in vitro substrate of PCMT purified from human erythrocytes. It should be noted that the procedure for purification of ovalbumin is scarcely manipulative, so any eventual rise of its methyl accepting capability would be solely dependent on storage conditions.

We had also preliminarily noted that the methyl accept-

Table 1 Methyl accepting capability of some proteins of animal origin

Protein	Origin	Protein methyl esters (nmol/mg)
actin	bovine muscle	0.430
α-casein	bovine milk	2.179
β-casein	bovine milk	1.947
β-lactoglobulin	bovine milk	0.299
β-lactoglobulin A	bovine milk	0.273
β-lactoglobulin B	bovine milk	0.223
myosin	bovine muscle	0.227
tropomyosin	chicken muscle	0.262
myoglobin	horse heart	0.148
albumin	bovine serum	0.152

ing capability of freshly purified ovalbumin increased eight fold upon deamidation induced according to Ref. 5. These results allowed us: 1) to establish the minimal methyl accepting capability of freshly prepared ovalbumin; and 2) to assess that basal methylation levels were susceptible to increase, indicating the appearance of altered aspartyl residues selectively recognized by PCMT.

In addition, we observed that ovalbumin obtained from different commercial sources is a better substrate for the enzyme, in vitro, with respect to the native ovalbumin freshly purified in our laboratory (*Figure 1*). This suggests that commercial preparations of this protein, of similar degree of purification according to the suppliers' specifications, may actually become deamidated during the course of their shelf life.

The latter interpretation is strengthened by the fact that a significant increase of the in vitro methyl accepting capability of freshly prepared ovalbumin was noted, after prolonged conservation in aqueous solution at pH 6.2, at

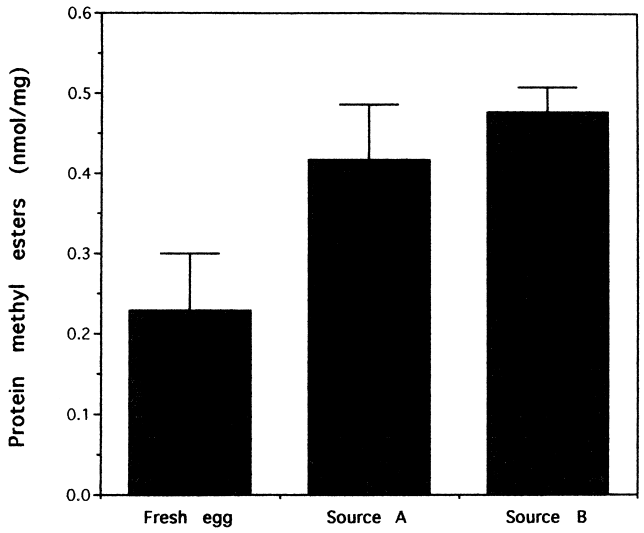

Figure 1 Enzymatic methyl esterification of ovalbumin purified from fresh eggs or obtained from commercial sources. Fresh egg refers to ovalbumin prepared in our laboratory from fresh eggs. Source A and source B: ovalbumin from different commercial sources, as defined under "Methods and materials." All different ovalbumin preparations were tested, in vitro, as substrates of PCMT.

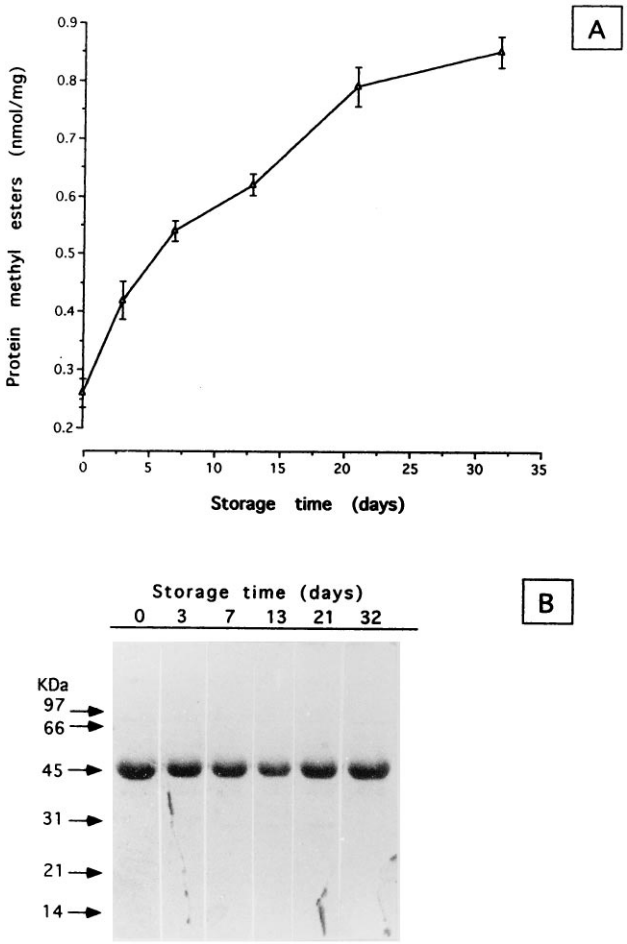

Figure 2 In vitro enzymatic methyl esterification (A) and SDS/PAGE analysis (B) of ovalbumin samples, as a function of storage time. Freshly prepared ovalbumin was stored in solution at −20°C (pH 6.2), for different time periods, and then assayed in vitro as a substrate of PCMT. Methyl accepting capability is expressed as a function of storage time. SDS/PAGE analysis of the same ovalbumin samples was also performed. Arrows refer to the position of molecular weight standards.

−20°C (*Figure 2A*). This result demonstrates that this protein is prone to spontaneously generate L-isoaspartyl-containing byproducts, in a time-dependent manner. This process is specifically monitored by the methyl esterification reaction.

It has been previously demonstrated that polypeptides deriving from partial proteolytic cleavage of a larger precursor can sometimes be better PCMT substrates compared to the original protein.[15] It is therefore possible that the increase in the methyl accepting capability of ovalbumin could be explained by the formation of shorter polypeptides produced by its partial hydrolysis because of proteases contaminating sample preparations. The SDS/PAGE analysis of ovalbumin samples, stored at −20°C for different time periods, showed: 1) that the molecular size of ovalbumin used for PCMT assay was the same at each time point; and 2) the absence of any hydrolysis byproducts of this protein, under our experimental conditions (*Figure 2B*).

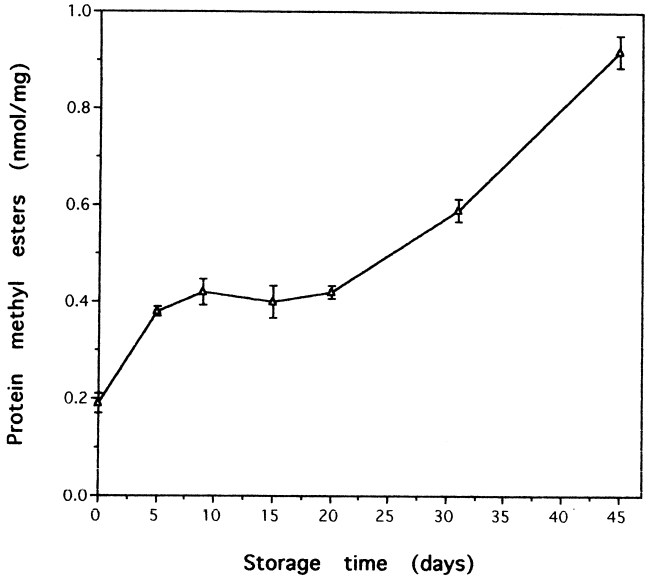

Figure 3 Methyl ester incorporation of ovalbumin extracted from chicken eggs kept at 4°C for several days. Eggs from the same animal source were stored at 4°C for different times. At the indicated time points ovalbumin was rapidly purified and assayed, in vitro, as a substrate of PCMT.

This further confirms the interpretation that the increase in methyl accepting capability of ovalbumin kept in solution at −20°C is due to the increase in methyl accepting sites that occurs during shelf life.

We checked whether the modifications we detected in isolated ovalbumin, stored for prolonged time periods, did also occur when the protein is kept within its natural environment (*i.e.* the whole egg). *Figure 3* shows that methyl accepting capability of freshly extracted ovalbumin increases significantly as a function of egg storage time.

Protective effect of trehalose against the formation of L-isoaspartyl residues

Trehalose is a nonreducing disaccharide which has been shown to exert a stabilizing effect during storage of dehydrated proteins.[6,7] To test the ability of trehalose to protect ovalbumin against the formation of L-isoaspartyl residues during prolonged storage, samples of freshly purified ovalbumin were Iyophilized in the presence of trehalose at different w/w ratios. Protein samples were stored dehydrated at −20°C or +25°C, for various time periods and then assayed for PCMT. The results show that trehalose is highly effective in preventing the time-dependent rise of methyl accepting capability of ovalbumin (*Figure 4* A, B). This suggests that the addition of this disaccharide to stored comestibles could be a means of increasing protein stability, with respect to deamidation and isomerization processes that give rise to L-isoaspartyl residues, thus improving shelf life.

Discussion

In the present study, we demonstrate that several proteins present in various foods of animal origin are substrates for

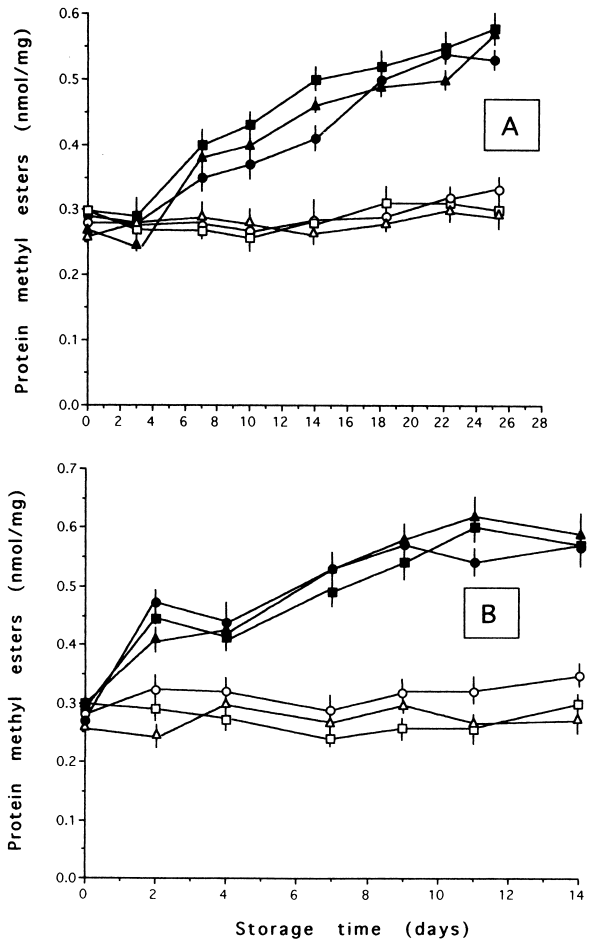

Figure 4 Effects of trehalose on methyl ester incorporation of ovalbumin extracted from chicken eggs. Freshly prepared ovalbumin samples were lyophilized in the presence of trehalose at different molar ratio. After different time of storage, samples were redissolved and assayed for PCMT. Panel A: samples stored at −20°C. Panel B: samples stored at +25°C. Full circles: ovalbumin stored lyophilized; no trehalose added. Full squares: ovalbumin stored lyophilized; trehalose (2:1; w/w) added to ovalbumin just before PCMT assay. Full triangles: ovalbumin stored lyophilized; trehalose (10:1; w/w) added to ovalbumin just before PCMT assay. Open squares: ovalbumin stored lyophilized with trehalose (5:1; w/w). Open circles: ovalbumin stored lyophilized with trehalose (10:1; w/w). Open triangles: ovalbumin stored lyophilized with trehalose (2:1; w/w).

PCMT. This enzyme selectively recognizes and methyl esterifies, in vitro, the free α-carboxyl groups, at the level of L-isoaspartyl residues in protein substrates. Regarding the specificity of the methylation sites in proteins, it has been previously reported that other altered residues, such as D-aspartate, are neither recognized at all by PCMT,[16] nor modified to a substantial extent, *in vitro*, by this enzyme, compared to L-isoaspartyl residues.[17] In the last study it was found that PCMT displayed, toward the D-aspartyl-containing peptides, K_m values in the millimolar range, whereas the K_m is in the micromolar range in the case of L-isoaspartyl-containing peptides. Hence, the evidence supports the notion that, under the conditions generally used in the in vitro assays, methyl esterification catalyzed by PCMT occurs essentially at L-isoaspartyl residues, while, in the same

situation, D-aspartyl sites are not methylated at an appreciable rate, because of the low affinity of this enzyme for the D-aspartyl-containing peptides.

The molecular mechanism for the formation of L-isoaspartyl residues, has been previously established, using several model peptides and proteins.[1] It has been shown that these abnormal L-isoaspartyl sites derive from deamidation of asparaginyl residues and/or isomerization of normal aspartyl residues. On this basis we can hypothesize a similar origin also for the methyl accepting sites that we detected in food proteins.

L-isoaspartyl residues are, indeed, by far the main product of the spontaneous deamidation of L-asparaginyl (or isomerization of L-aspartyl) residues, whose occurrence can be specifically monitored by the PCMT *in vitro* assay.

Ovalbumin offers several advantages as a model for these studies in food proteins, including the fact that chicken eggs are usually not subject to any chemical and/or physical treatments before being sold on the market, thus representing the ideal example of minimally manipulated food. Nevertheless we noted that ovalbumin methyl esterification increases in a time-dependent manner, either when it is purified and stored in solution or when it is stored as an intact whole egg, i.e. under conditions more pertaining to reality.

We also observed that shelf life always affects the isoaspartyl content of ovalbumin despite storage conditions, which can only slow down the rate of occurrence of such spontaneous degradation processes. The only exception is represented by the addition of trehalose to dehydrated ovalbumin preparations, kept at −20°C or at +25°C, which effectively prevents the accumulation of deamidated sites, at least within the time range of observation comparable to the other situations tested. Therefore, this disaccharide is an effective means to protect food proteins from the degradative processes such as the one described, either at low temperature or at room temperature, and the protective effect is stable over time.

In conclusion, the data indicate that: 1) proteins from common food sources contain L-isoaspartyl residues selectively recognized and modified by PCMT; and 2) this enzyme can be actually used as a specific tool to selectively monitor the occurrence of such alterations arising from spontaneous deamidation of food proteins. The enzymatic assay offers the advantage of an increased sensitivity with respect to chemical methods proposed for detecting L-isoaspartyl residues in proteins;[18] 3) freshly prepared, native ovalbumin significantly increases its methyl accepting capability upon storage under different conditions. This is true either if ovalbumin is used as such, or even when it is extracted from whole eggs stored for different times; and 4) trehalose has a protective effect on the accumulation of these altered residues.

It is possible to hypothesize that the presence of L-isoaspartyl residues in food proteins is capable of influencing protein digestibility, since the isopeptide bond is relatively resistant to proteolytic cleavage.[19,20] This is consistent with the previous observation of urinary excretion of a significant amount of isoaspartyl-containing peptides from dietary sources.[20] The intestinal absorption of partially undigested isoaspartyl peptides may be particularly relevant to situations in which a considerable amount of protein intake derives from long-term stored foods (for example powdered eggs). In addition, the presence of a quantifiable amount of indigestible proteins should be taken into account in subjects receiving low protein diets (for example chronic renal failure patients), especially when these include long-term stored foods. It is also possible to speculate that such isospartyl peptides derived from partial digestion of diet proteins, once absorbed by the intestine, may interfere with the physiologic PCMT-dependent repair of endogenous proteins.

The demonstration that these abnormal aspartyl residues increase over time indicates that the structural integrity of food proteins is affected during shelf life at molecular level under the ways of conservation usually used, and that trehalose could be envisaged as a means to circumvent the accumulation of these altered products.

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