Immunochemical Detection of Oxalic Acid Monoamides That Are Formed during the Oxidative Reaction of L-Ascorbic Acid and Proteins

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Covalent binding of L-ascorbic acid (AA) to proteins (protein ascorbylation) occurs during food processing and storage and in vivo. It contributes, for example, to browning and various changes in the physical and physiological properties of proteins. Since oxalic acid monoalkylamides (OMAs) are formed in high yields from AA and primary amines under oxidative conditions, it was determined if OMAs also represent an ascorbylation product of proteins. Therefore a polyclonal anti-OMA antibody was raised, and a high-titer antiserum was obtained which is specific against OMA. In a competitive ELISA total binding inhibition was achieved by ascorbylated protein, indicating that ascorbylation of proteins leads to the formation of OMA. OMA is only formed under aerobic conditions. Proteins, which were glycosylated with other carbohydrates such as glucose, did not show cross-reactivity, indicating that the antiserum can be used to detect OMA as a specific marker for ascorbylation.

Keywords: *L-Ascorbic acid; ascorbylation; immunochemical detection; Maillard reaction; OMA protein; oxalic acid monoalkylamide*

INTRODUCTION

Ascorbic acid (AA) undergoes various degradation reactions during food processing and storage and in vivo. Of these reactions, protein ascorbylation, the covalent binding of AA to amino acid side chains of proteins, deserves particular attention.

This reaction leads not only to the loss of vitamin C but also to changes in physical and physiological properties of proteins. These changes include browning, formation of fluorescence (Bensch et al., 1985), protein cross-links (Ortwerth and Olesen, 1980), and precipitation (Ortwerth et al., 1988). Furthermore it was suspected that ascorbate-induced nonenzymatic browning has antinutritional effects (Ziderman et al., 1989) and causes discoloration (Rogacheva et al., 1995) and off-flavor formation during food processing (Sakurai et al., 1996). Under oxidative stress, protein ascorbylation also takes place in vivo, and it was associated with various complications (Bensch et al., 1985; Hunt, 1996).

So far little is known about the mechanisms of protein ascorbylation, and reaction products have not been identified. Amino acid analyses of ascorbylated proteins reveal that the reaction leads mainly to loss of lysine but also includes loss of arginine and histidine (Ortwerth and Olesen, 1980).

Thus in previous studies, alkylamines or lysine and arginine derivatives have been used as model compounds for amino acid side chains of proteins and were heated with AA under various conditions. From these reaction mixtures, several ascorbylation products were isolated and identified (Pischetsrieder et al., 1995; Larisch et al., 1996; Pischetsrieder, 1996). One of the main products is oxalic acid monoalkylamide (OMA), which is formed when AA is reacted under oxidative conditions with alkylamines. Therefore, it was suggested that OMA can also be formed between AA and the lysine side chains of proteins and that it can play a role in protein ascorbylation.

In this paper, we describe investigations which show that OMA is formed when proteins are reacted under oxidative conditions with AA and therefore represents a new ascorbylation product of proteins. Since OMA is labile under the conditions of acid or alkaline hydrolysis, polyclonal antibodies were raised which are specific against OMA, and a competitive ELISA was developed to detect OMA modifications of proteins.

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MATERIALS AND METHODS

Apparatus. ELISA plates were read on a spectra classic ELISA reader (SLT, Crailsheim, Germany). Preparative high-performance liquid chromatography (HPLC) was performed with a Merck L-6250 pump, a Merck L-4000 UV detector, and a Merck D-2500 chromatointegrator.

Reagents. Maxisorb 96-well microtiter plates were purchased from Nunc, and DHA was from Sigma. For preparation of OMA−bovine serum albumin (BSA, fraction V, Sigma), OMA−human serum albumin (HSA, fraction V, Sigma), OMA−bovine pancreatic ribonuclease A (RNase, Serva, Heidelberg, Germany), OMA−β-lactoglobulin (from bovine milk, Sigma), and OMA−ovalbumin (from chicken egg, grade V, Sigma), 50 mg of each protein was dissolved in 5 mL of phosphate-buffered saline (PBS). While the solution was vigorously stirred, a suspension of 12.5 mg of oxalic acid bis(*N*-hydroxysuccinimide) ester (O-NHS, Sigma) in 500 μL of dry *N*,*N*-dimethylformamide (DMF) was added dropwise. The solution was stirred for 2 h, dialyzed three times against pure water, filtered through a 0.2 μm filter, and lyophilized.

Carbohydrate-modified BSA was prepared by dissolving AA, DHA, D-glucose, D-glucose-6-phosphate, D-fructose, D-ribose, maltose, or lactose to a final concentration of 50 mM in a solution of 10 mg/mL of BSA in PBS. The AA solution was adjusted to pH 7.4 with diluted NaOH. The mixtures were incubated 14 days at 40 °C in the presence of oxygen, dialyzed three times against pure water, and lyophilized. For anaerobically ascorbylated BSA, a solution of AA and BSA was treated as described above, with the exception that nitrogen

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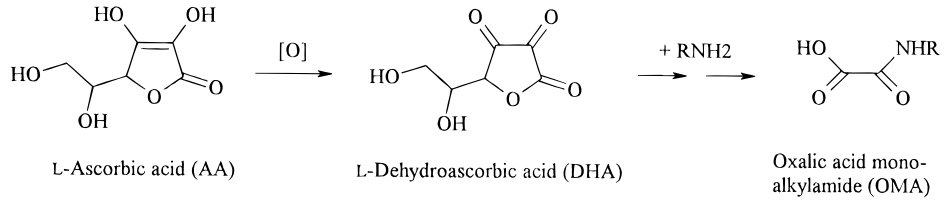

Figure 1. Formation of oxalic acid monoalkylamides (OMA) from AA. R = N^{α}-acetyllysine or protein, for example.

was bubbled through the mixture prior to incubation. AGE−BSA was prepared according to literature (Makita et al., 1992). Briefly 50 mg/mL of BSA was incubated in PBS containing 0.5 M glucose for 60 days at 37 °C, dialyzed three times against pure water, and lyophilized.

Carboxymethylated BSA (CML−BSA) (Reddy et al., 1995), acetylated BSA (Basu et al., 1976), and formaldehyde-treated BSA (FA−BSA) (Horiuchi et al., 1985) were prepared as described in literature. Modification rates of lysine residues were determined by the TNBS assay (Fields, 1972) as 32%, 75%, and 39%, respectively.

For the preparation of OMA−lysine, 390 mg of N^{α}-carbobenzoxylysine (Fluka, CH) was dissolved in 30 mL of PBS and stirred. 300 mg of O-NHS, which was suspended in 3.5 mL of acetonitrile, was added dropwise. The solution was stirred for 2 h more, concentrated to a final volume of 25 mL, and filtered. OMA−N^{α}-carbobenzoxylysine was isolated by preparative HPLC. Purification was achieved on a Hibar 250-25 column packed with Lichrospher RP 18, 10 μm particle size. 10% methanol (HPLC grade) in 5 mM ammonium formate buffer, pH 7.0, was used as eluant with a flow rate of 9 mL/min. The product was detected at 215 nm, and the fractions between 26 and 42 min were collected and lyophilized. The residue was dissolved in dry ethanol, palladium black was added, and the mixture was hydrogenated overnight. The precipitated product was filtered and dissolved in water. After lyophilization oxalic acid mono(N^{ϵ}-lysinyl)amide was obtained as a white powder (20% yield). ^{1}H NMR (D_2O) δ 1.2 (m, 2H, 4-CH_{2lys}), 1.4 (quin, 2H, 5-CH_{2lys}), 1.7 (m, 2H, 3-CH_{2lys}), 3.1 (t, 2H, 6-CH_{2lys}), 3.5 (t, 1H, 2-CH_{lys}); ^{13}C NMR (D_2O/DCl, standard: methanol at 49.0) δ 22.4 (C-4_{lys}), 28.4 (C-5_{lys}), 30.1 (C-3_{lys}), 40.1 (C-6_{lys}), 53.5 (C-2_{lys}), 160.0 (broad, COOH), 162.7 (broad, CON_{ϵ}lys $CON_{\alpha\text{-}lys}$), 172.7 (C-1_{lys}).

Preparation of Polyclonal Anti-OMA Antibody. OMA−KLH was prepared as follows: 20 mg of hemocyanin from keyhole limpet (KLH; Sigma) was dissolved in 2 mL of stabilizing buffer (31 mM sodium phosphate buffer containing 0.46 M NaCl and 91 mM sucrose). 900 μL of this solution was vigorously stirred, and 90 μL of a suspension of 20 mg/mL of O-NHS in dry acetonitrile was added in portions of 10 μL. Stirring was continued for 2 h more. The modification rate of 62.5% was determined by the TNBS method (Fields, 1972), and the solution was dialyzed three times against 2.5 L of stabilizing buffer. Each time, 1.0 mg of OMA−KLH in 1 mL of 50% Freund's complete adjuvant (Sigma) was injected subdermally at 10 sites on the backs of two female rabbits. The rabbits received booster injections in the same way using Freund's incomplete adjuvant (Sigma) after 4 and 8 weeks. Antiserum was prepared 10 days after final injection and stored frozen.

ELISA Procedures. All data represent the mean of triplicate determinations. The noncompetitive assay was performed as follows: each well of a microtiter plate was coated overnight at 4 °C with 200 μL of a solution of OMA protein or unmodified protein. The proteins were diluted in 50 mM carbonate buffer (pH 9.7) in a concentration range between 0.01 ng/mL and 10 μg/mL. After each step the plates were washed twice with PBS containing 0.05% Tween 20 unless otherwise noted. The coated wells were blocked with 300 μL of 3% defatted milk powder for 2 h at room temperature while shaking, then 200 μL of antiserum (dilution 1:50 000 in PBS containing 0.2% BSA and 0.05% Tween 20) was added and the plates were shaken for 1 h at room temperature. For masking, the wells were incubated for 45 min with 200 μL of anti-rabbit IgG alkaline phosphatase conjugate (from goat, Sigma; diluted 1:5 000 in 0.1% BSA in PBS) and washed three times with PBS/Tween. Antibody binding was detected using 150 μL of tetramethylbenzidine solution (Sigma). The reaction was stopped after 15 min by adding 50 μL of 2 N sulfuric acid.

To determine antiserum dilution curves, microtiter plates were coated with a 0.1 μg/mL OMA protein solution and treated as described above. Antiserum was applied in dilution of 1:500 to 1:1 000 000.

For competitive ELISA, microtiter plates were coated with 0.1 μg/mL of OMA−BSA and treated as described above. Antiserum was added in a dilution of 1:10 000 with various amounts of inhibitor added as indicated. The signals which were obtained by the addition of BSA were defined as 0% inhibition, and the relative inhibition of the substances was determined. To quantitate cross-reactivity of carbohydrate-modified BSA, ELISA was performed as described above with 0.02 mg/mL of inhibitor added. The standards which contained 0.02 mg/mL of OMA−BSA or BSA were tested each time on the same plate as the inhibitors, their values were defined as 100% and 0% inhibition, respectively, and relative inhibition of the substances was determined.

RESULTS

Characterization of OMA Antiserum. The purpose of this project was to show that ascorbylation products, which were isolated as derivatives from alkylamines, can also be formed from proteins. Oxalic acid monopropylamide is an important reaction product when propylamine is incubated with L-dehydroascorbic acid or with AA under oxidative conditions (Figure 1). However, it was not known if oxalic acid amides result also from ascorbylation of proteins. Thus oxalic acid modified KLH (OMA−KLH) was synthesized, and a polyclonal antiserum against the protein was developed. The antiserum reacted specifically with OMA−BSA, whereas native BSA did not give a signal (Figure 2A). Since the antiserum should be used later to determine AA modification of various proteins, it is important to show that the reaction is not dependent on the carrier protein, especially because it is known that antibodies against small epitopes recognize the antigen together with the amino acid environment. Therefore OMA-modified HSA, β-lactoglobulin, ovalbumin, and RNase were prepared and tested for reaction with the antiserum. As shown in Figures 2B and 2C for OMA−HSA and OMA−ovalbumin, recognition of OMA epitopes is only marginally dependent on the carrier. All five OMA proteins produce a dose dependent signal, which differs only in intensity. On the other hand, none of the underivatized proteins reacted.

Next, the relation between antiserum concentration and signal was tested. Using a relatively weak antigen, like OMA−β-lactoglobulin, the antiserum can be diluted up to 1:100 000 to give a significant reaction. For detection of OMA−BSA or OMA−HSA, the antiserum can even be diluted to 1:1 000 000 (Figure 3). The preimmunization serum did not give a signal (data not shown).

Because the antibody shows specific reaction with OMA protein, but not with unmodified carrier, a competitive ELISA was developed using OMA−BSA as antigen. The assay was first used to characterize

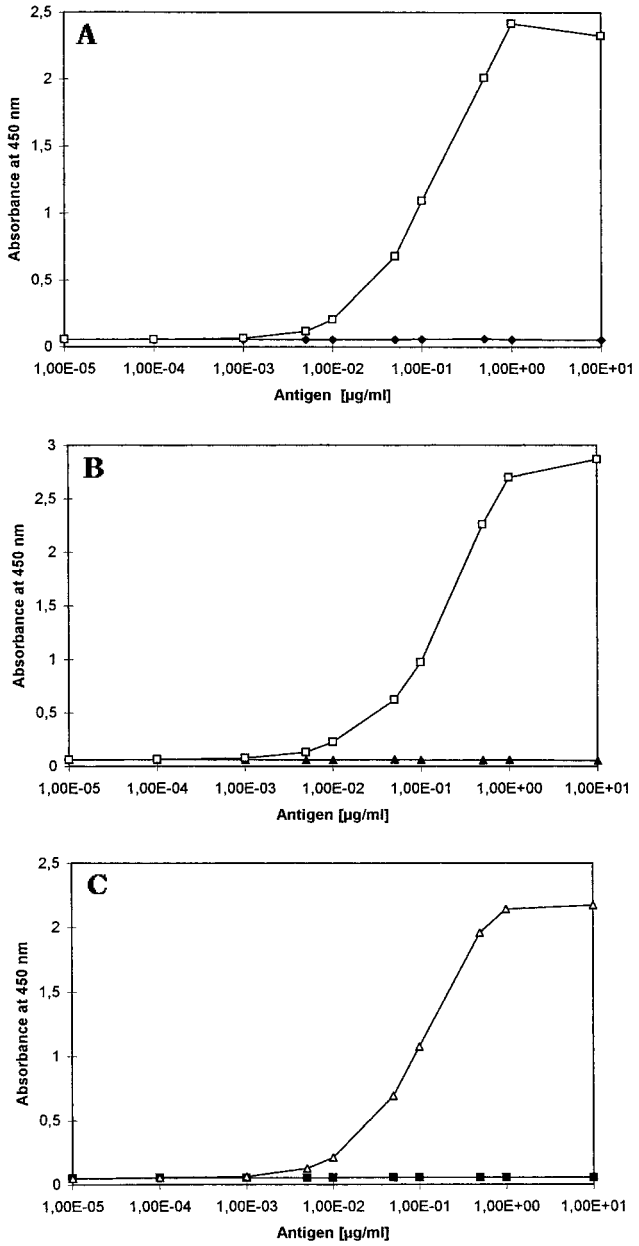

Figure 2. Immunochemical reaction of anti-OMA antibody with different OMA proteins: (A) OMA−BSA (□), BSA (◆); (B) OMA−ovalbumin (□), ovalbumin (▲); (C) OMA−HSA (△), HSA (■).

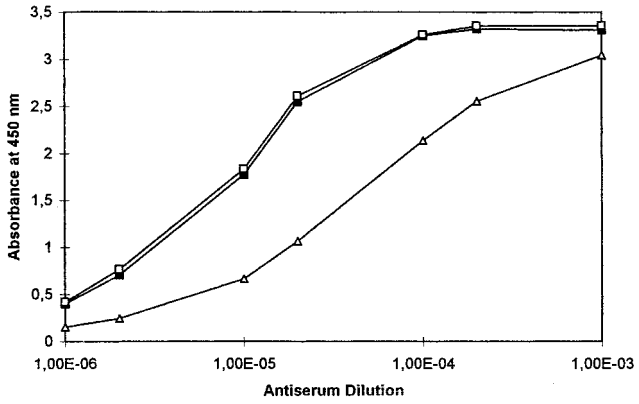

Figure 3. Antiserum dilution curves for anti-OMA antibody in a noncompetitive ELISA: for coating agents, OMA−BSA (■), OMA−HSA (□), and OMA−lactoglobulin (△) were used.

specificity and cross-reactivity of the antibody. Therefore synthesized OMA−lysine, which was purified and

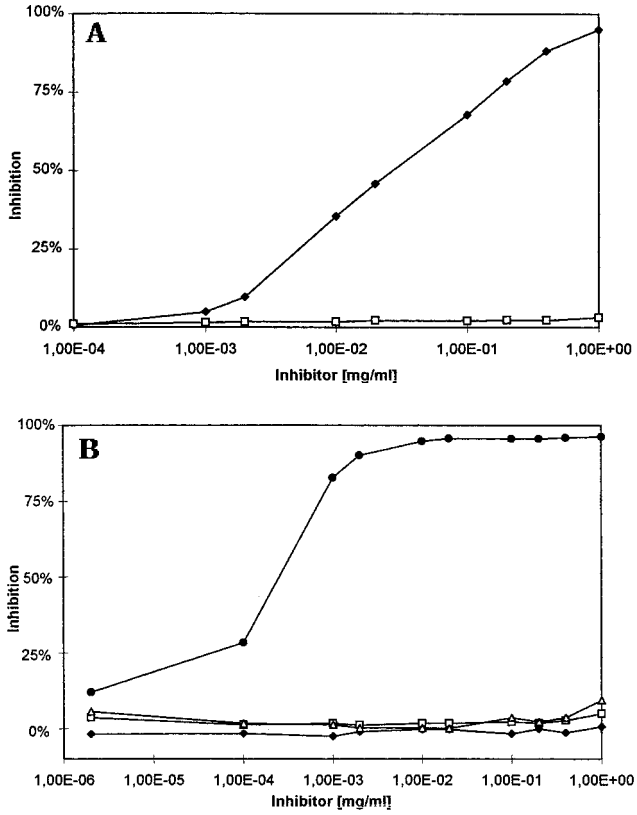

Figure 4. ELISA competition curves for anti-OMA antibody with different inhibitors added: (A) N^{ϵ}-OMA-lysine (◆), N^{α}-acetyllysine (□); (B) acetylated BSA (◆), formaldehyde-treated BSA (△), CML−BSA (□), OMA−BSA (●).

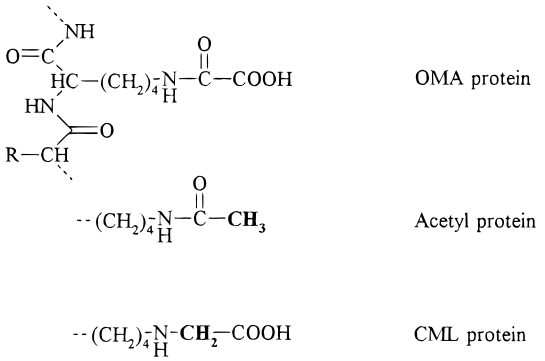

Figure 5. Similarities in the structures of OMA protein, acetylated protein, and CML protein.

characterized by spectroscopic data, was subjected to competition test. The signal could be completely suppressed by OMA−lysine, whereas N^{α}-acetyllysine did not show reaction (Figure 4A). However suppression was weaker compared to the protein bound epitope (Figure 4B). Furthermore, proteins with modifications which have similar structure as OMA were prepared and tested for cross-reactivity. Acetylated BSA, formaldehyde-treated BSA, and CML−BSA were used as competitors. Specifically, CML deserves attention because it is considered as a major advanced glycation endproduct (AGE) resulting from the reaction between glucose and protein and it has similar structure as OMA (Figure 5). As shown in Figure 4B, however, none of these products displays cross-reactivity. Thus it can be concluded that the antiserum is highly specific for OMA.

Identification of OMA as an Ascorbylation Product of Proteins. In order to obtain ascorbylated protein, BSA was incubated with AA under aerobic

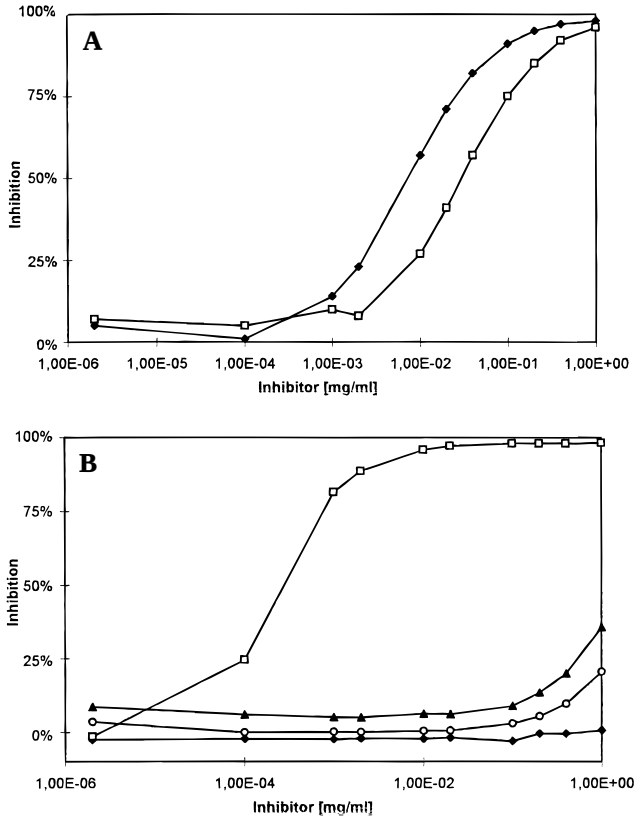

Figure 6. ELISA competition curves for anti-OMA antibody with ascorbylated and glycated BSA: (A) AA-modified BSA (□), DHA-modified BSA (◆); (B) glucose-modified BSA, (▲), glucose-6-phosphate-modified BSA (○), lactose-modified BSA (◆); control, OMA−BSA (□).

conditions or with DHA. During the course of the reaction, the mixtures turned dark brown, with DHA causing the more intense color. Protein polymerization, which was determined by SDS gel electrophoresis, was insignificant after 14 days of reaction under the described conditions. As shown in Figure 6A, the binding of OMA−BSA to antibodies was inhibited by AA- and DHA-modified protein in a dose dependent way, and total inhibition was achieved by adding 3 nmol of ascorbylated protein. These data strongly suggest that OMA represents an ascorbylation product of proteins. OMA modifications were formed in higher yields when protein was incubated with DHA in contrast to incubation with AA under oxidative conditions. When oxygen was excluded from the reaction mixture, OMA was not formed from AA.

Finally, the antibody was tested in an ELISA to see if it can be used to detect OMA as a marker for protein ascorbylation in foodstuffs or in vivo and to distinguish those modifications from otherwise glycosylated proteins. Therefore BSA was incubated with other carbohydrates and treated as the AA-modified protein. Additionally, long-time incubated AGE protein was synthesized. Besides glucose, the widest spread glycosylation agent, several other sugars which are abundant in food (e.g., maltose, lactose, and fructose) or in vivo (e.g., glucose-6-phosphate) were used for incubation. Ribose was added as a representative of pentoses. The glycosylated BSA was then subjected to competition assay. In Figure 6B, the dose dependent curves for glucose-, glucose-6-phosphate-, and lactose-treated BSA are shown. These carbohydrates do not generate modifications which display significant cross-reactivity. This result indicates that the antibody is specific for ascorbylation products whereas otherwise glycosylated proteins are not recognized. In Table 1, the binding inhibition caused by glycosylated substrates is summarized. Only significant competition of 49% was found for ribose, whereas even long-time incubated AGE−BSA did not compete. Summarizing the results, it can be stated that if the possibility of ribosylation is excluded, a signal which is produced in the competitive ELISA can be assigned to protein ascorbylation.

Table 1. Competitive ELISA with Different Glycosylated Proteins Added as Inhibitors

inhibitor	% inhibition
BSA	0
OMA−BSA	100
DHA−BSA	72
AA−BSA (anaerobic)	0
Glu−BSA	7
AGE−BSA	5
Glu-6-P−BSA	2
Fru−BSA	0
maltose−BSA	0
lactose−BSA	0
Rib−BSA	49

DISCUSSION

From model reactions it can be deduced that oxalic acid monoalkylamides (OMA) can play a role as ascorbylation products of proteins. OMA is one of the main products (Larisch et al., 1996) when propylamine is reacted with AA in the presence of oxygen under various conditions. It can be assumed that lysine side chains react in a similar way with AA as propylamine, resulting in the formation of OMA. This assumption, however, is difficult to prove because amides such as OMA are labile under the conditions of alkaline and acid hydrolysis. A useful approach to determine protein modifications which are not stable under hydrolytic conditions are immunological methods, which allow protein analysis without hydrolysis. In several studies, for example, proteins were reacted with glucose, and antibodies were produced against the glycosylated protein (Horiuchi et al., 1991; Makita et al., 1992; Nakayama et al., 1991; Matsuda et al., 1992; Reddy et al., 1995). Although this method is very useful to detect in vivo and in vitro glycated proteins, the structures of the glycosylation products remain unknown because the immunologically effective epitope has not been characterized.

Thus for this study, OMA protein was synthesized and polyclonal antibodies with high titer were produced which are suitable to detect OMA modification of proteins. In a competitive ELISA, total inhibition can be achieved by the addition of purified oxalic acid mono(N^{ϵ}-lysinyl)amide. This result shows, that the antiserum is highly specific to OMA. The relatively high amount of OMA−lysine which has to be added for inhibition results from the low affinity of antibodies to monovalent antigens (OMA−lysine), when they compete with a polyvalent antigen (OMA−BSA) (Desphande, 1996; Harlow and Lane, 1988).

The specificity of the antiserum was further determined by competitive studies with structurally similar epitopes. Acetylated and carboxymethylated proteins were used in competition studies, since both represent OMA-like C_2 modifications (Figure 5). Similar to OMA, the acetyl epitope is bound by an amide bond to lysine whereas the free carboxyl group is replaced by a methyl group. In the case of CML, the epitope is linked

by an alkyl−amine bond, but the free carboxyl group is present in the molecule. Neither CML nor the acetylated protein show cross-reactivity with anti-OMA antibody, indicating that both the amide bond and the free carboxyl group are necessary for antigen recognition.

For further specification, cross-reactivity with formaldehyde-treated protein (FA protein) was determined. In the case of FA protein, the structures of the modification sites are not fully characterized, but various epitopes must be assumed. However, it is known that FA proteins show specific antibody or receptor binding (Horiuchi et al., 1985). Again, the facts that FA protein does not show cross-reactivity with the anti-OMA antibody and that FA gives rise to a variety of products provide further evidence for the specificity of the antiserum.

Finally, protein which has been incubated for 2 weeks with AA was used for competitive ELISA. It was found that AA-modified proteins react specifically with the antiserum, which strongly suggest that OMA modifications are formed during the reaction of proteins with AA. Thus it can be concluded that OMA represents a protein-bound ascorbylation product.

OMA modifications of proteins are generated by AA under oxidative conditions and to an even higher extent by DHA, which is the primary oxidation product of AA. When protein was incubated with AA under anaerobic conditions, OMA was not detected. Therefore it can be further concluded that oxygen is necessary for the formation of OMA, and that AA must first be converted to DHA. This result is noteworthy because it has been stated in several studies that oxidative stress highly favors protein ascorbylation (Ortwerth and Olesen, 1988; Prabhakaram and Ortwerth, 1991).

Since the antiserum proved to be suitable to recognize OMA modifications, it was tested to see if it can be used to detect OMA as a marker for protein ascorbylation. For this purpose, it must be assured that the immunological reaction is independent from the carrier and that the epitope is not generated by other carbohydrates.

Firstly, dose dependent activity was found for all OMA-modified carrier proteins which were tested. This indicates that the binding is independent on the protein moiety. Additionally, cross-reactivity with glycosylated proteins was tested. Glycosylated proteins, which were prepared like AA−BSA, or long-time incubated AGE−BSA (advanced glycosylation endproducts) did not show significant reactivity. Therefore it can also be concluded that the antiserum can be used to distinguish protein ascorbylation from glycation. For further confirmation, various other carbohydrates were tested which can occur in vivo or in foods and which can induce nonenzymatic browning. The disaccharides maltose and lactose, which are of importance in many food stuffs, did not generate reactive epitopes. A similar result was obtained for fructose, which can be found in nutrition and under certain conditions in vivo, and for glucose-6-phosphate, which represents a powerful glycation agent in vivo. Although ribose is present only in minor concentrations in few foodstuffs, and the cross-reactivity for the pentose was found to be less than 50%, the method is probably not suitable to determine ascorbylation in foods which have a high ribose content compared to relatively low AA concentrations.

Investigations to show if the antiserum can be used in an ELISA or Western blotting assay to determine protein ascorbylation in food or in vivo are currently under progress.

ABBREVIATIONS USED

AA, L-ascorbic acid; AGE, advanced glycosylation endproduct; BSA, bovine serum albumin; CML, carboxymethyllysine; DHA, L-dehydroascorbic acid; ELISA, enzyme-linked immunosorbent assay; FA protein, formaldehyde-treated protein; HPLC, high-performance liquid chromatography; HSA, human serum albumin; KLH, keyhole limpet hemocyanin; OMA, oxalic acid monoalkylamide; O-NHS, oxalic acid bis(*N*-hydroxysuccinimide) ester; PBS, phosphate-buffered saline; RNase, bovine pancreatic ribonuclease; TNBS, 2,4,6-trinitrobenzenesulfonic acid.

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