Characterization of Ascorbylated Proteins by Immunochemical Methods

Birgit Huber and Monika Pischetsrieder*

Institut für Pharmazie, Abteilung Lebensmittelchemie, der Universität München, Sophienstrasse 10, D-80333 München, Germany

L-Ascorbic acid (AA) binds covalently to proteins in a Maillard-type reaction (protein ascorbylation). An immunochemical screening method was developed to determine the products formed during protein ascorbylation. Thus, sheep serum albumin (SSA) was incubated with L-dehydroascorbic acid (DHA) to get highly modified ascorbylated SSA (DHA−SSA), and a polyclonal anti-DHA−protein antiserum was prepared using DHA−SSA as antigen. Noncompetitive and competitive ELISAs were performed to characterize the reactive epitopes. It was found that (1) the antibody binds to ascorbylated lysine but not to arginine or histidine residues; (2) the formation of reactive products is highly dependent on the presence of oxygen; (3) proteins that were glycated by sugars, such as glucose or ribose, show significant binding to the anti-DHA−protein antibody, indicating that the reactive epitope can also be generated by carbohydrates other than AA; (4) CML-protein [CML = N^{ϵ}-(carboxymethyl)lysine] and CML can inhibit the antibody binding completely. These results show that the anti-DHA−protein antiserum consists mainly of anti-CML antibodies. Using specific anti-CML and anti-oxalic acid monoamide (OMA) antibodies (Pischetsrieder, M.; Larisch, B.; Seidel, W. *J. Agric. Food Chem.* **1997**, *45*, 2070−2075), it was found that both antisera bind significantly to DHA−proteins. Therefore, it can be concluded that CML is an important product formed during the reation of AA and proteins and that it is the main immunological epitope of ascorbylated protein. However, other predominant modifications on ascorbylated proteins besides CML and OMA cannot be excluded.

Keywords: *L-Ascorbic acid; protein ascorbylation; immunochemical method; Maillard reaction; N^{ϵ}-(carboxymethyl)lysine*

INTRODUCTION

L-Ascorbic acid (AA) is relatively labile and is degraded during food processing or storage and in vivo to give a large number of products. More recently it was found that AA can also bind covalently to proteins (protein ascorbylation). From model studies it was deduced that proteins are ascorbylated in a Maillard-type reaction (Ortwerth et al., 1988). When AA is incubated with proteins, browning, formation of fluorescence (Bensch et al., 1985), and protein cross-linking (Ortwerth and Olesen, 1980) can be observed. The ability of AA to bind to proteins is dependent on the presence of oxygen (Ortwerth et al., 1988). AA is probably first oxidized to give L-dehydroascorbic acid (DHA) and other reactive degradation products, which react then easily with lysine or arginine side chains of proteins, so it was found that under aerobic conditions AA reacts >60 times faster with proteins than glucose or other sugars (Ortwerth et al., 1998b), indicating that even small amounts of oxidized AA can generate intensive protein modifications.

Several studies suggest that ascorbate-induced nonenzymatic browning occurs during food processing and storage (Hartkopf and Erbersdobler, 1995), where it contributes to discoloration (Labuza et al., 1997) and off-flavor formation (Sakurai et al., 1996). Furthermore, it can be assumed that ascorbylation leads to changes of the physical and physiological properties of food proteins, such as protein cross-linking (Larisch et al., 1996).

In vivo oxidative stress can cause protein ascorbylation, and it was linked to enhanced protein glycation in diabetic patients, where an increase in the DHA over AA ratio is observed (Hunt, 1996). Furthermore, it was suggested that in human lenses AA is oxidized by UVA light, which can lead to protein binding and cataract formation (Ortwerth et al., 1998a).

Because protein ascorbylation seems to be an important process in food technology and in vivo, in several studies possible mechanisms have been investigated. In model reactions AA was heated with alkylamines or free amino acids and several products have been identified (Dunn et al., 1990; Larisch et al., 1996; Pischetsrieder et al., 1995; Pischetsrieder, 1996). However, because most of these compounds are not stable under the conditions of acidic or alkaline hydrolysis, only little is known about the reaction of proteins with AA and about adducts that are formed in vivo or in foods. In a previous study we have used immunochemical methods to detect a specific ascorbylation product (oxalic acid monoamide, OMA) in protein-bound form. In this work we developed a different immunochemical approach to investigate protein ascorbylation. Therefore, protein was incubated with DHA and a polyclonal and polyspecific antibody against the ascorbylated protein was produced. The antiserum was then used in a competitive ELISA to characterize protein ascorbylation and to

* Author to whom correspondence should be adressed (e-mail pischets@pharmchem.uni-muenchen.de).

10.1021/jf9803132 CCC: $15.00
Published on Web 09/03/1998

determine cross-reactivity with synthesized reaction products of amino acids and AA. In this way structural identification of protein−ascorbic acid adducts can be achieved.

MATERIALS AND METHODS

Apparatus. Reaction mixtures were incubated in a shaking water bath at 120 rpm (Julabo, Seelbach/Schwarzwald, Germany). ELISA plates were shaken by a microtiter plate shaker (Janke & Kunkel GmbH & Co., Staufen, Germany), washed by an ELISA washer (SLT, Crailsheim, Germany), and read on a spectra classic ELISA reader (SLT).

Reagents. Maxisorb 96-well microtiter plates were purchased from Nunc (Roskilde, Denmark), and DHA was from Sigma. Incubations were carried out in sterile 15 and 50 mL centrifuge tubes (Corning, NY) using 0.08 M phosphate-buffered saline (PBS) as solvent.

In addition to the polyclonal anti-DHA−protein antiserum, which we raised, two other antisera were used: a polyclonal anti-OMA antiserum, which we had previously prepared (Pischetsrieder et al., 1997), and a polyclonal anti-CML antiserum, which was a generous gift of Dr. Ann Marie Schmidt (College of Physicians and Surgeons, Columbia University, New York).

For preparation of ascorbylated proteins (DHA−proteins), 100 mg of β-lactoglobulin (from bovine milk, Sigma), human serum albumin (HSA, fraction V, Sigma), or bovine pancreatic ribonuclease A (RNase, Serva, Heidelberg, Germany), respectively, and 87 mg of DHA (0.5 mmol) were dissolved in 10 mL of PBS. The reaction mixtures were filtered through 0.2 μm filters into 50 mL tubes. After incubation for 14 days at 37 °C, the reaction mixtures were dialyzed three times against pure water and lyophilized.

The corresponding unmodified control proteins were obtained by incubating 100 mg of protein in 10 mL of PBS and treating the reaction mixture as described above.

β-Lactoglobulin was also ascorbylated by incubating 88 mg of L-ascorbic acid (AA) (0.5 mmol) with 100 mg of β-lactoglobulin as described above (AA−β-lactoglobulin) with the exception that the solution was adjusted to pH 7.4 with diluted NaOH prior to incubation. Two different preparations using AA were carried out: Incubation under aerobic conditions was done as described above. Low-oxygen conditions were achieved by bubbling nitrogen through the mixture prior to incubation, by adding 1 mM DTPA to the mixture and by incubating the mixture in a 15 mL tube.

For preparation of ascorbylated amino acids, 0.5 mmol of N^{α}-BOC-L-lysine (BOC = *tert*-butyloxycarbonyl), N^{α}-BOC-L-arginine, or N^{α}-acetyl-L-histidine, respectively, and 0.5 mmol of DHA were dissolved in 10 mL of PBS and incubated as described previously for DHA−protein. Equivalents of the reaction mixtures were used as inhibitiors in the competitive ELISA.

The corresponding unmodified control amino acids were obtained by incubating 0.5 mmol of amino acid in 10 mL of PBS as described above. In the same way a DHA-control reaction mixture was prepared, by using 0.5 mmol of DHA instead of amino acid.

To obtain glycated β-lactoglobulin, 0.5 mmol of D-glucose, D-glucose-6-phosphate, D-fructose, D-ribose, maltose, or lactose, respectively, and 100 mg β-lactoglobulin were dissolved in 10 mL of PBS. The reaction mixtures were filtered through 0.2 μm filters into 50 mL tubes. After incubation for 14 days at 37 °C, the reaction mixtures were dialyzed three times against pure water and lyophilized.

AGE−β-lactoglobulin (AGE = advanced glycation end product) was prepared according to a literature method (Makita et al., 1992). Briefly, 50 mg/mL β-lactoglobulin was incubated in PBS containing 0.5 M glucose for 60 days at 37 °C, dialyzed three times against pure water, and lyophilized.

Carboxymethylated β-lactoglobulin (CML-β-lactoglobulin) (Reddy et al., 1995), acetylated β-lactoglobulin (Basu et al., 1976), and oxalic acid monoamide-protein (OMA-protein) (Pischetsrieder et al., 1997) were prepared as described in the literature. Modification rates of lysine residues were determined according to the TNBS assay (Fields, 1972) as 32%, 75%, and 62.5%, respectively. CML modifications were additionally quantified by HPLC according to the method of Hartkopf et al. (1994).

For the preparation of CML, 280 mg of N^{α}-carbobenzoxy-lysine (Fluka) and 186 mg of iodoacetic acid were dissolved in 10 mL of 0.2 M phospate buffer. After the pH was adjusted with 2 N NaOH to pH 10, the solution was stirred for 48 h at room temperature. Two milliliters of 25% NH_3 was added, and the solution was stirred overnight at room temperature. N^{α}-Carbobenzoxy-N^{ϵ}-(carboxymethyl)lysine was isolated by preparative HPLC. Purification was achieved on a Supelcosil 250-21,2 column (Supelco) packed with LC-18-DB, 5 μm particle size. Ten percent methanol (HPLC grade) in 5 mM ammonium formate buffer, pH 7.0, was used as eluant with a flow rate of 10 mL/min. The product was detected at 217 nm, and the fractions between 21 and 26 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, N^{ϵ}-(carboxymethyl)lysine was obtained as a white powder. The spectral data were identical to those reported in the literature (Climie and Evans, 1982).

Pentosidine was prepared according to the modified method of Henle et al. (1997).

Preparation of Polyclonal Anti-DHA−Protein Antibody. DHA−sheep serum albumin (DHA−SSA) was prepared as already described for other DHA−proteins with the exception that the reaction mixture was not dialyzed and lyophilized. An amount of 0.320 mL of the reaction mixture was diluted with 1.680 mL of PBS and filtered through a 0.2 μm filter. Each time, 1.0 mL of this solution in 1 mL of 50% Freund's complete adjuvant (Sigma) was injected subdermally at 10 sites on the back of two female rabbits. The rabbits received booster injections in the same way using Freund's incomplete adjuvant (Sigma) after 4, 8, and 12 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 DHA−protein or unmodified protein in 50 mM carbonate buffer, pH 9.7, in a concentration range between 0.001 and 1000 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 a 3% solution of defatted milk powder in PBS for 2 h at room temperature with shaking. Two hundred microliters of antiserum [dilution 1:10000 in PBS containing 0.2% bovine serum albumin (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 1:5000 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 (Figure 2), microtiter plates were coated with solutions containing an amount of DHA−β-lactoglobulin as indicated and a complementary share of β-lactoglobulin to obtain a total protein concentration of 100 μg/mL (e.g., 0.01 μg/mL DHA−β-lactoglobulin + 99.99 μg/mL β-lactoglobulin, etc.). The assay was continued as described above. Antiserum was applied in dilutions of 1:1000 to 1:100000.

For competitive ELISA, microtiter plates were coated with 1 μg/mL (Figures 3 and 6) or 10 μg/mL DHA−β-lactoglobulin (Figures 4 and 5), respectively, and treated as described above. Antiserum was added in a dilution of 1:10000 with various amounts of inhibitor added as indicated. The value for 0.001 μg/mL DHA−β-lactoglobulin as inhibitor was defined as 100%

absorbance corresponding to 0% inhibition. In relation to this value the inhibiton of the examined substances was determined.

Using anti-CML antibody or anti-OMA antibody in competitive ELISAs, microtiter plates were coated with 0.1 μg/mL CML−β-lactoglobulin or OMA−β-lactoglobulin and the antiserum was diluted 1:75000 (anti-CML antibody) or 1:10000 (anti-OMA antibody). Except for these differences, the assays were performed as described above.

RESULTS AND DISCUSSION

During the incubation of L-ascorbic acid (AA) with proteins, covalent binding of AA or its degradation products occurs in a process that is called protein ascorbylation.

So far, several ascorbylation products have been identified from model reactions of AA and free lysine and arginine derivatives (Albrecht et al., 1992; Dunn et al., 1990; Larisch et al., 1996; Pischetsrieder et al., 1995; Pischetsrieder, 1996). However, up to now there is little known about the products that are formed during protein ascorbylation.

In this study an immunochemical screening method was developed to investigate the reaction between proteins and AA by raising a polyclonal, polyspecific antiserum against ascorbylated protein. In a competitive ELISA various isolated ascorbylation products of free amino acids can then be tested. Signal suppression indicates that the tested product is also present on ascorbylated protein.

Thus, sheep serum albumin (SSA) was incubated with DHA to get highly modified ascorbylated SSA (DHA−SSA), and a polyclonal anti-DHA−protein antiserum was prepared using DHA−SSA as antigen. In a noncompetitive ELISA the reactivity of the antiserum was tested against various DHA−proteins, such as DHA−β-lactoglobulin (Figure 1A), DHA−HSA (Figure 1B), or DHA−RNase (data not shown). The proteins so ascorbylated produced a dose-dependent signal, whereas the corresponding unmodified proteins did not bind. This result indicates that the antiserum recognizes specifically protein modifications which are formed during protein ascorbylation.

In the previous experiments (Figure 1) unspecific background absorbance was observed when the wells were coated with low protein concentrations. In the subsequent experiment (Figure 2) DHA−β-lactoglobulin was used as antigen. Unmodified β-lactoglobulin was added to the antigen solutions to obtain a constant protein concentration of 100 μg/mL (see Materials and Methods). Thus, background absorbance could almost completely be suppressed. In Figure 2 the dependency of the signals on the antiserum dilution is shown. Significant signal could be produced with antiserum diluted up to 1:100000. Preimmunization serum did not give a signal (data not shown).

Because the antibody shows specific reaction with DHA−protein, but not with unmodified carrier, a competitive ELISA was developed using DHA−β-lactoglobulin as antigen.

In the competitive ELISA system unspecific variations in maximum absorbance were observed when no inhibitor was added. As these variations could not be avoided, only inhibition of >20% was considered significant.

From amino acid analyses before and after protein ascorbylation it is known that AA can react with lysine, arginine, or histidine side chains (Ortwerth and Olesen, 1980). To find out which amino acid side chains of the proteins react with DHA to give epitopes binding to the anti-DHA−protein antibody, several DHA−amino acids were prepared and tested in a competitive ELISA for reactivity with the antiserum. DHA−N^{α}-BOC-L-lysine inhibited the binding almost completely. However, suppression was weaker compared to the protein-bound epitope (Figure 3). This observation can often be made when a monovalent antigen, for example, a modified amino acid, competes with a polyvalent one, for example, a modified protein (Desphande, 1996; Harlow

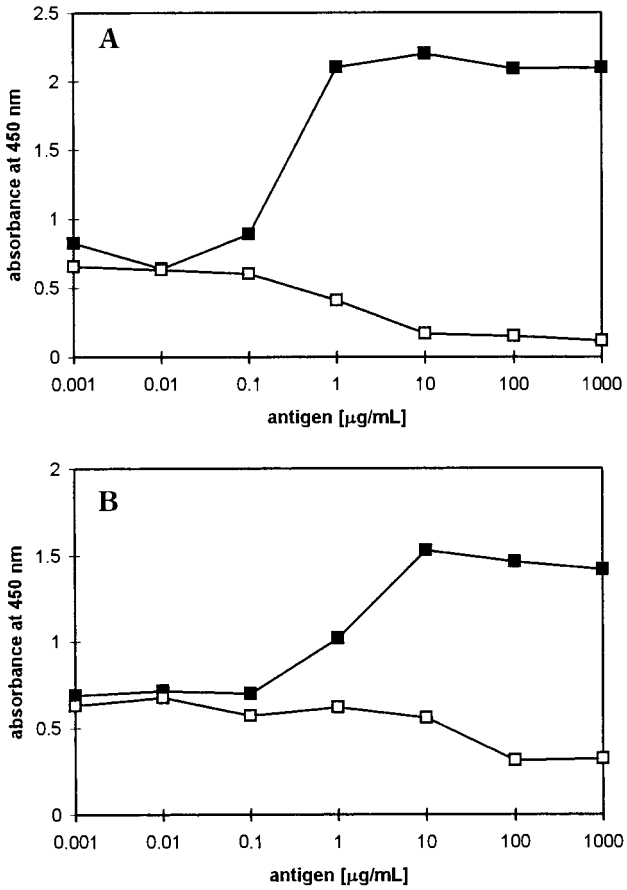

Figure 1. Immunoreactivity of anti-DHA−protein antiserum to different ascorbylated proteins and the corresponding unmodified proteins: (A) DHA−β-lactoglobulin (■), β-lactoglobulin (□); (B) DHA−HSA (■), HSA (□).

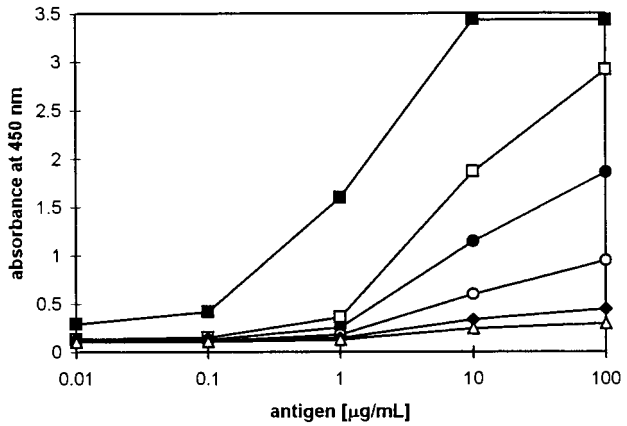

Figure 2. Antiserum dilution curves for anti-DHA−protein antiserum in a noncompetitive ELISA using DHA−β-lactoglobulin as antigen: antiserum dilution 1:1000 (■), 1:5000 (□), 1:10000 (●), 1:25000 (○), 1:50000 (◆), 1:100000 (△).

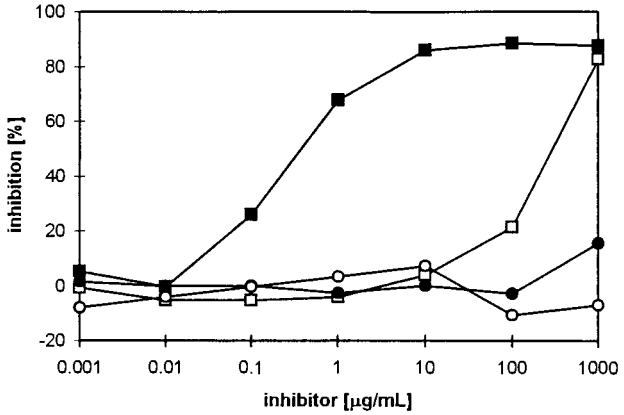

Figure 3. Inhibition of anti-DHA−protein antiserum binding to DHA−β-lactoglobulin by DHA−β-lactoglobulin (■) DHA−N^{α}-BOC-L-lysine (□), DHA−N^{α}-BOC-L-arginine (●), and DHA−N^{α}-acetyl-L-histidine (○).

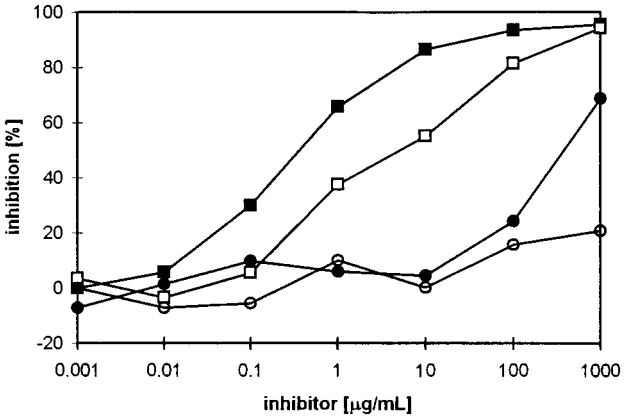

Figure 4. Inhibition of anti-DHA−protein antiserum binding to DHA−β-lactoglobulin by DHA−β-lactoglobulin (■), AA−β-lactoglobulin (aerobic) (□), AA−β-lactoglobulin (low-oxygen) (●), and β-lactoglobulin (○).

and Lane, 1988). It was found that a >100-fold excess of the monovalent antigen is required for complete inhibition (Reddy et al., 1995; Pischetsrieder et al., 1997). DHA−N^{α}-BOC-L-arginine, DHA−N^{α}-acetyl-L-histidine (Figure 3), the unmodified amino acids (data not shown), and the DHA-control (data not shown), which was incubated in the absence of amino acids, did not show significant cross-reactivity. Thus, it was concluded that the antibody binds specifically to lysine modifications which are formed during ascorbylation.

The role of oxygen was examined by competitive ELISAs using DHA−β-lactoglobulin, AA−β-lactoglobulin (aerobic), or AA−β-lactoglobulin (low-oxygen) as inhibitors (Figure 4). AA−β-lactoglobulin (aerobic) or AA−β-lactoglobulin (low-oxygen) were obtained by incubating AA and β-lactoglobulin under aerobic or low-oxygen conditions. For the latter, nitrogen was bubbled through the mixture prior to incubation, 1 mM DTPA was added, and airspace was avoided in the reaction vial. As expected, protein that had been incubated with DHA showed strong binding (Figure 4). AA-β-lactoglobulin (aerobic) produced a weaker signal, but antibody binding could still be completely inhibited. Protein incubated with AA under low-oxygen conditions reacted only to a lesser extent. Thus, it can be concluded that the presence of oxygen highly favors formation of the epitope.

Furthermore, it was examined if the epitopes, which are recognized by the antiserum, are AA-specific products or if they can also be generated by other carbohydrates. Therefore, β-lactoglobulin was incubated with various carbohydrates and treated as the DHA-modified protein. Several sugars, which are abundant in food (e.g., maltose, lactose, and fructose) or in vivo (e.g., glucose, glucose-6-phosphate), were used for incubation. Ribose was added as a representative of pentoses. Additionally, long-time incubated AGE−protein (AGE = advanced glycation end product) was synthesized. The glycated proteins were subjected to competition assay. In Figure 5, the dose-dependent curves for AGE-, glucose-, lactose-, fructose-, and ribose-treated β-lactoglobulin are shown. Total inhibition was obtained by addition of AGE−β-lactoglobulin, ribose−β-lactoglobulin, or fructose-β-lactoglobulin. Glucose−β-lactoglobulin and glucose-6-phosphate-β-lactoglobulin (data not shown) reacted to a lesser extent, whereas lactose-β-lactoglobulin and maltose-β-lactoglobulin (data not shown) showed low reactivity. These results allow the conclusion that there exist some common structures of ascorbylated and glycated proteins and that the reacting epitopes can also be generated by carbohydrates other than AA.

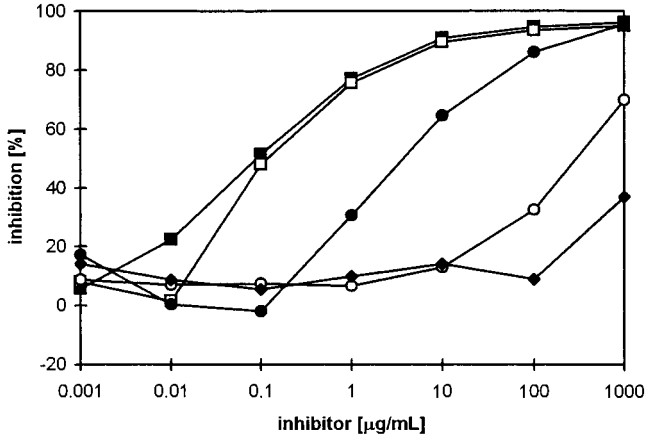

Figure 5. Inhibition of anti-DHA−protein antiserum binding to DHA−β-lactoglobulin by ribose−β-lactoglobulin (■), AGE−β-lactoglobulin (□), fructose−β-lactoglobulin (●), glucose−β-lactoglobulin (○), and lactose−β-lactoglobulin (◆).

From model reactions of AA and free lysine and arginine derivatives several ascorbylation products have been identified (Pischetsrieder et al., 1995; Pischetsrieder, 1996; Larisch et al., 1996). However, the majority of them are specific for AA and cannot be generated from other carbohydrates. As an exception N^{ϵ}-(carboxymethyl)lysine (CML), which is known to be an important AGE, and pentosidine have been identified as products that can be formed from various carbohydrates including glucose and AA (Albrecht et al., 1992; Dunn et al., 1990; Grandhee and Monnier, 1991). Because AGE−protein strongly inhibited the antibody binding, we carried out a competitive ELISA using DHA−β-lactoglobulin, CML−β-lactoglobulin, CML, pentosidine, and free lysine as inhibitors. Pentosidine and lysine did not show any cross-reactivity, whereas CML−β-lactoglobulin and CML showed strong binding (Figure 6): CML−β-lactoglobulin proved to be an equally potent inhibitor for the antibody binding as DHA−β-lactoglobulin. CML also inhibited the antibody binding completely; however, suppression was weaker compared to the protein-bound epitope (explanation see above). These results indicate that CML is the major antigen which is recognized by the anti-DHA−protein antibody. To confirm that the

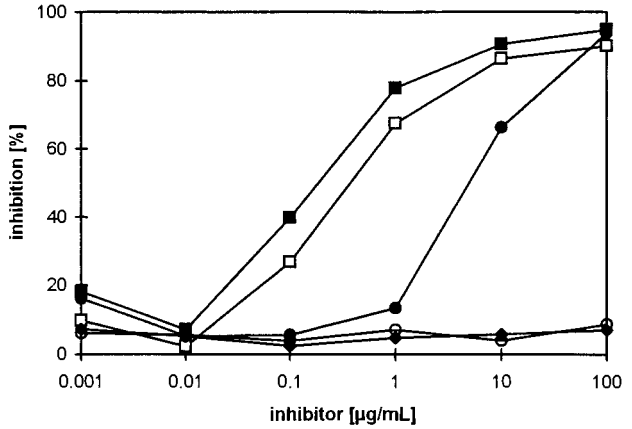

Figure 6. Inhibition of anti-DHA−protein antiserum binding to DHA−β-lactoglobulin by CML−β-lactoglobulin (■), DHA−β-lactoglobulin (□), CML (●), pentosidine (○), and lysine (◆).

anti-DHA−protein antibody does not unspecifically recognize C2-modifications of the lysine residues of proteins, acetylated β-lactoglobulin and OMA−β-lactoglobulin were tested for cross-reactivity with the antibody (data not shown). Because these modifications did not display cross-reactivity, nonspecific interactions can be widely excluded. These results show that CML is an important product formed during protein ascorbylation.

To confirm this conclusion, another assay was performed: Using an anti-CML antibody, a competitive ELISA was carried out with CML−β-lactoglobulin as antigen and DHA−β-lactoglobulin as inhibitor. DHA−β-lactoglobulin inhibited the antibody binding, however, to a lesser extent compared to CML-β-lactoglobulin (Figure 7A).

The results of this study lead us to the conclusion that CML is the main immunological epitope of ascorbylated proteins and that the anti-DHA−protein antiserum consists mainly of anti-CML antibodies. This can be concluded from the fact that CML−β-lactoglobulin is an inhibitor for the anti-DHA−protein antibody binding equally as potent as DHA−β-lactoglobulin. In a competitive ELISA 100% inhibition was achieved by adding CML−β-lactoglobulin or CML as inhibitor, corresponding to a complete binding of the antibody to CML−β-lactoglobulin or CML. This result seems to be surprising, because it is known from previous studies that the ascorbylation of proteins leads to the formation of at least one other product, the oxalic acid monoamide (OMA). Using a polyclonal anti-OMA antibody, considerable amounts of OMA on DHA−protein were detected (Figure 7B). This shows that the DHA−protein which was used for immunization contains significant amounts of OMA in addition to CML. However, antibodies against OMA were not found in the anti-DHA−protein antiserum (data not shown). This phenomenon has been previously observed in immunological studies. When a mixture of several equimolar antigens is used for immunization, the substances evoke very different antibody responses according to their immunogenic activity.

Therefore, it can be assumed that the existence of protein ascorbylation products other than OMA and CML cannot be excluded. Although both anti-DHA−protein antisera that we raised show analogous results, it could also be speculated that if a higer number of rabbits were immunized, antisera could be obtained

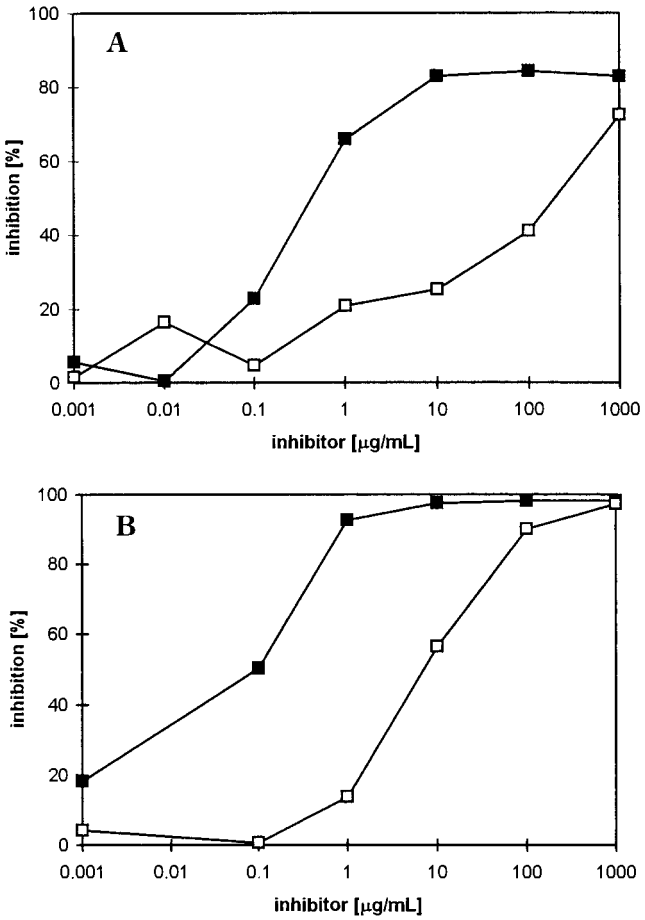

Figure 7. (A) Inhibition of anti-CML antibody binding to CML−β-lactoglobulin by CML−β-lactoglobulin (■) and DHA−β-lactoglobulin (□); (B) inhibition of anti-OMA antibody binding to OMA−BSA by OMA−BSA (■) and DHA−BSA (□).

which are also specific for ascorbylation products other than CML.

The immunochemical approach developed here can be used as a general method to identify protein modifications that are labile under the conditions of acidic or alkaline protein hydrolysis.

ABBREVIATIONS USED

AA, L-ascorbic acid; AGE, advanced glycation end product; BOC, *tert*-butyloxycarbonyl; BSA, bovine serum albumin; CML, N^{ϵ}-(carboxymethyl)lysine; DHA, L-dehydroascorbic acid; DTPA, diethylenetriaminepentaacetic acid; ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; HSA, human serum albumin; OMA, oxalic acid monoamide; PBS, phosphate-buffered saline, RNase, bovine pancreatic ribonuclease; SSA, sheep serum albumin; TNBS, 2,4,6-trinitrobenzenesulfonic acid; Z, carbobenzoxy.

ACKNOWLEDGMENT

We thank Dr. Erbersdobler from the Institut für Humanernährung und Lebensmittelkunde, University of Kiel, for HPLC analysis, Dr. Knopf from the Institut für Wasserchemie, TU Munich, for the introduction in ELISA, and Dr. Samtleben from the Institut für pharmazeutische Biologie, University of Munich, for the use of the ELISA reader. We are grateful to Dr. Schmidt from the College of Physicians and Surgeons, Columbia University, New York, for the anti-CML antiserum.

LITERATURE CITED

Albrecht, U.; Hartkopf, J.; Erbersdobler, H. F. Proof of the formation of N^{ϵ}-carboxymethyllysine by reaction between ascorbic acid and lysine. *Ernaehrungs-Umsch.* **1992**, *39 (4)*, 164.

Basu, S.; Goldstein, J.; Anderson, R.; Brown, M. Degradation of cationized low-density lipoproteins and regulation of cholesterol metabolism in homozygous familial hypercholesterolemia fibroblasts. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 3178−3182.

Bensch, K.; Fleming, J.; Lohmann, W. The role of ascorbic acid in senile cataract. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 7193−7196.

Climie, I. J. G.; Evans, D. A. ^{13}C-Nuclear magnetic resonance spectroscopy as a probe of enzyme environment-II. Effects of solvent and pH on ^{13}C-chemical shifts in derivatized amino acid models. *Tetrahedron* **1982**, *38* (5), 697−711.

Desphande, S. S. Antibody affinity and avidity. In *Enzyme Immunoassays from Concept to Product Development*; Chapman & Hall: New York, 1996.

Dunn, J. A.; Ahmed, M. U.; Murtiashaw, M. H.; Richardson, J. M.; Walla, M. D.; Thorpe, S. R.; Baynes, J. W. Reaction of ascorbate with lysine and protein under autoxidizing conditions: formation of N^{ϵ}-carboxymethyllysine by reaction between lysine and products of autoxidation of ascorbate. *Biochemistry* **1990**, *29*, 10964−10970.

Fields, R. The rapid determination of amino groups with TNBS. *Methods Enzymol.* **1972**, *15*, 464−468.

Grandhee, S. K.; Monnier, V. M. Mechanism of formation of the Maillard protein cross-link pentosidine. *J. Biol. Chem.* **1991**, *266*, 11649−11653.

Harlow, E.; Lane, D. Antibody−antigen interactions. In *Antibodies—A Laboratory Manual*; CSH Laboratory: Cold Spring Harbor, NY, 1988.

Hartkopf, J.; Erbersdobler, H. F. Model experiments with sausage meat on the formation of N^{ϵ}-carboxymethyllysine. *Z. Lebensm. Unters. Forsch.* **1995**, *201* (1), 27−29.

Hartkopf, J.; Pahlke, G.; Lüdemann, G.; Erbersdobler, H. F. Determination of N^{ϵ}-carboxymethyllysine by a reversed-phase high-performance liquid chromatography method. *J. Chromatogr. A* **1994**, *672*, 242−246.

Henle, Th.; Schwarzenbolz, U.; Klostermeyer, H. Detection and quantification of pentosidine in foods. *Z. Lebensm. Unters. Forsch. A* **1997**, *204*, 95−98.

Hunt, J. Ascorbic acid and diabetes mellitus. In *Subcellular Biochemistry, Vol. 25: Ascorbic Acid: Biochemistry and Biomedical Cell Biology*; Harris, J. R., Ed.; Plenum Press: New York, 1996.

Labuza, Th. P.; Kaanane, A.; Davies, C. Ascorbic acid as the principal reactant causing browning in an orange juice model system. Poster on the 6th International Symposium on the Maillard Reaction, London, 1997.

Larisch, B.; Pischetsrieder, M.; Severin, T. Reactions of dehydroascorbic acid with primary aliphatic amines including N^{ϵ}-acetyllysine. *J. Agric. Food Chem.* **1996**, *44*, 1630−1634.

Makita, Z.; Vlassara, H.; Cerami, A.; Bucala, R. Immunochemical detection of advanced glycosylation end products in vivo. *J. Biol. Chem.* **1992**, *267*, 5133−5138.

Ortwerth, B. J.; Olesen, P. Ascorbic acid-induced crosslinking of lens proteins: evidence supporting a Maillard reaction. *Biochim. Biophys. Acta* **1980**, *956*, 10−22.

Ortwerth, B. J.; Feather, M.; Olesen, P. The precipitation and cross-linking of lens crystallins by ascorbic acid. *Exp. Eye Res.* **1988**, *47*, 155−168.

Ortwerth, B. J.; Coots, A.; James, H. L.; Linetsky, M. UVA irradiation of human lens proteins produces residual oxidation of ascorbic acid even in the presence of high levels of glutathione. *Arch. Biochem. Biophys.* **1998a**, *351* (2), 189−196.

Ortwerth, B. J.; Lee, K.-W.; Coots, A.; Mossine, V. The relative oxidation, glycation and crosslinking activity of glucose and ascorbic acid in vitro. In *The Maillard Reaction in Foods and Medicine*; O'Brien, J., Ames, J. M., Nursten, H. E., Crabbe, J., Eds.; The Royal Society of Chemistry: London, 1998b.

Pischetsrieder, M. Reaction of L-ascorbic acid with L-arginine derivatives. *J. Agric. Food Chem.* **1996**, *44*, 2081−2085.

Pischetsrieder, M.; Larisch, B.; Müller, U.; Severin, T. Reaction of ascorbic acid with aliphatic amines. *J. Agric. Food Chem.* **1995**, *43*, 3004−3006.

Pischetsrieder, M.; Larisch, B.; Seidel, W. Immunochemical detection of oxalic acid monoamides that are formed during the oxidative reaction of L-ascorbic acid and proteins. *J. Agric. Food Chem.* **1997**, *45*, 2070−2075.

Reddy, S.; Bichler, J.; Wells-Knecht, K.; Thorpe, S. R.; Baynes, J. W. N^{ϵ}-(Carboxymethyl)lysine is a dominant advanced glycation end product (AGE) antigen in tissue proteins. *Biochemistry* **1995**, *34*, 10872−10878.

Sakurai, H.; Ishii, K.; Nguyen, H.; Reblova, Z.; Valentova, H.; Pokorny, J. Condensation reactions of dehydroascorbic acid with aspartame. In *Chemical Reactions in Foods III—Proceedings*; Velisek, J., Davidek, J., Eds.; Czech. Chemical Society: Prague, 1996.

Received for review March 26, 1998. Revised manuscript received July 10, 1998. Accepted July 16, 1998.

JF9803132