Effect of Maillard Browning Reactions of the Kunitz Soybean Trypsin Inhibitor on Its Interaction with Monoclonal Antibodies

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Carbohydrates react with proteins to form nonenzymatically browned products, including Maillard reaction products. The effects of these transformations on the antigenicity of the Kunitz soybean trypsin inhibitor (KTI) were studied with use of two monoclonal antibodies. Solid mixtures of KTI and carbohydrates were heated in an oven at 120 °C, dialyzed, freeze-dried, and analyzed by enzyme-linked immunosorbent assay (ELISA). Glucose, lactose, and maltose decreased the antigenicity of KTI by 60–80%, compared to a control sample heated without carbohydrate. Starch was less effective than the three reducing sugars. This decrease was rapid, occurring within 10 min when glucose was heated with KTI, with retention of 60% of the chemically available lysine. Longer heating times increased browning and reduced the level of available lysine in KTI, without further reducing antigenicity. The results suggest that relatively mild conditions of heating food proteins with carbohydrates can reduce the antigenicity of the protein and possibly modify sites known to elicit allergenic responses.

Among the most significant chemical phenomena in food processing are nonenzymatic browning reactions, including the Maillard reaction (Phillips and Finley, 1989; Friedman, 1982; Mauron, 1981). The principal participants in the Maillard reaction, reducing carbohydrates and amino groups, are present in most foodstuffs. High temperature and low moisture content, conditions favoring browning, may occur during processing. Under certain conditions, nonreducing carbohydrates also induce browning. For example, adding sucrose and starch increased the loss of chemically available or reactive lysine from casein heated at 121 °C (Smith and Friedman, 1984).

Although the reactions of nonreducing carbohydrates with proteins are not well-defined, the Maillard reaction mechanism has been extensively studied. The first stable Maillard reaction intermediate is generally agreed to be the Amadori rearrangement compound (Mauron, 1981), a ketosyl or aldosylamine N-susbstituted protein. Subsequent reaction steps can include regeneration of the protein amino group (Feather, 1981), deamination by carbonyl compounds formed from the dehydrated carbohydrate moiety (Namiki et al., 1986), and formation of N^{ϵ}-(carboxymethyl)lysine (Liardon et al., 1987). These initial steps of the Maillard reaction can thus modify the charge of the protein. For example, transformation of a positively charged amino group or a lysine side chain (RNH_3^+) to a carboxymethyl group ($RNH_2^+CH_2COO^-$) is accompanied by a change of a positive charge to a zwitterion. Since mild heating with reducing carbohydrates can produce protein polymers (Kato et al., 1986), the molecular weight of the protein may change as well.

Nonenzymatic browning reactions can alter protein structure in a number of ways. We wanted to determine the effects of these reactions on the ability of protein to form antigen–antibody complexes, whose stability is strongly influenced by structural, electronic, and hydrogen-bonding effects [reviewed by Davies et al. (1988)]. In the present study, the effect of browning reactions on the antigenicity of Kunitz soybean trypsin inhibitor (KTI) was evaluated with two monoclonal antibodies elicited, isolated, and characterized by Brandon et al. (1987).

MATERIALS AND METHODS

KTI (Sigma Chemical Co.; St. Louis, MO; Lot 86F-8070) was used without further purification. The α-D-glucose (Sigma) β-lactose (Mallinckrodt, St. Louis, MO), and maltose (Fisher Scientific, Fairlawn, NJ) were of reagent quality. Starch was Aytex-P brand wheat starch (Ogilvie Mills, Minneapolis, MN). Other chemicals were reagent grade and were obtained from commercial sources.

KTI Samples. Samples to be treated were prepared by mixing KTI dissolved in water with weighed amounts of glucose, maltose, lactose, or starch. The starch was preheated a few minutes at 70 °C with a small amount of water prior to mixing. The exact amount of added KTI was determined by absorbance measurements (A_{280}(1 mg/mL) = 1.03; Kassell, 1970), giving the following weight ratios for mixed samples: KTI/glucose, 3.6:1; KTI/lactose, 3.0:1; KTI/maltose 2.8:1; KTI/starch, 3.0:1. The mixtures were lyophilized before heat treatment.

Heat Treatment. Solid samples were heated at 120 °C in a Fisher Isotemp thermostat forced-draft convection oven, Model 177. KTI, KTI/glucose, KTI/maltose, KTI/lactose, and KTI/starch were heated 50 min. In a second experiment, aliquots of a KTI/glucose sample were withdrawn after 10, 20, 30, 40, and 50 min of heating. These aliquots were then dialyzed against frequently changed glass-distilled water for 72 h at 4 °C, with use of a membrane with an exclusion limit of 3500 Da. The resulting samples were then lyophilized.

Browning Measurements. The ultraviolet–visible absorbance spectra of unheated KTI and the heat-treated KTI samples were recorded with a double-beam spectrophotometer (Cary 219, Varian Instruments, Palo Alto, CA). The degree of browning was measured as absorbance at 420 nm against that of an unheated control.

Amino Acid Analysis. Samples were acid-hydrolyzed with 6 N HCl in glass tubes and sealed under nitrogen at 120 °C for 20 h. The liberated amino acids were analyzed quantitatively with a Biotronik amino acid analyzer, Model LC 5001 (Biotronik Wissenschaftliche Geräte GmbH., West Germany), using the expanded Li buffer elution system recommended by the manufacturer. Furosine, which elutes after arginine (Erbersdobler,

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Table I. Relative Antigenicity of Native Kunitz Soybean Trypsin Inhibitor Heated Alone or with Various Carbohydrates at 120 °C for 50 min

sample	rel antigenicity, %
KTI, native (unheated)	100
KTI, heated	100
KTI + glucose, heated	10
KTI + maltose, heated	34
KTI + lactose, heated	27
KTI + starch, heated	55

1986), was assayed in a separate run by expanding the basic portion of the eluent profile. The amount of chemically available (reactive) lysine was determined as the yield of total lysine by amino acid analysis, corrected for unavailable lysine as calculated from the yield of furosine after acid hydrolysis and amino acid analysis (Erbersdobler, 1986).

Gel Filtration. The sample to be assayed was dissolved in 1 mL of phosphate-buffered saline (PBS, 5 mM sodium phosphate and 150 mM sodium chloride, pH 7.0). This solution was then chromatographed on a Sephadex G-100 column, 2.5 × 40 cm, with PBS containing 0.01% NaN_3 as eluant (23.5 mL/h). UV absorbance (280 nm) was continuously monitored, and fractions were collected. Column performance was established with standard proteins from a molecular weight calibration kit (Pharmacia Fine Chemicals, Piscataway, NJ).

Electrophoresis. SDS-polyacrylamide gel electrophoresis was performed by the Laemmli (1970) procedure, using a 12% acrylamide separating gel slab with a 5% acrylamide stacking gel. Protein was stained with Coomassie Blue R-250.

Immunoassays. We used previously prepared monoclonal anti-KTI antibodies 136 and 129 (Brandon et al., 1987, 1988). Competitive inhibition assays were performed with serial dilutions of the antigen (unheated or heat-treated KTI samples), using polystyrene plates (Costar, Cambridge, MA) coated with KTI. The detailed procedure is described in Brandon et al. (1987). The antigen concentrations in the assays were determined by measuring absorbance at 280 nm. Most of the heat-treated samples had increased absorbance in the ultraviolet and low visible region compared to native KTI.

To establish the protein content of the samples, amino acid analyses were performed on solutions with known absorbance. Conversion factors were calculated by comparing the yields of leucine, valine, glycine, glutamic acid, and aspartic acid from the heated samples with those from native KTI.

The degree of antigenicity was calculated by dividing the amount of native KTI producing a 50% inhibition by the amount of heated KTI producing the same inhibition. The results were expressed as percent of native KTI antigenicity.

RESULTS

Table I shows the reduction of KTI antigenicity measured with monoclonal antibody 136 after heating with glucose (Figure 1), lactose, maltose, or starch. Dry heating of pure KTI did not affect its antigenicity (Figure 1). Glucose had the strongest effect, and starch, the weakest. The order of effects reflected the concentration of reducing sugar in the samples, suggesting that the Maillard reaction was partly responsible for the decrease in antigenicity.

In a second experiment, samples with progressive degrees of Maillard-reacted KTI were generated by heating KTI/glucose mixtures from 10 to 50 min. This resulted in samples with a steady increase in browning and a decrease in available lysine (Figure 2). The results of the competitive inhibition assays with antibodies 136 and 129 on these samples are shown in Figure 3. Antigenicity decreased about 80% during the first 10–20 min of heating but remained the same thereafter.

SDS gel electrophoresis of the different KTI/glucose samples showed that the heat treatment produced a modified monomer and a dimer. The relative mobility of the monomer decreased only slightly between the 10 and 50

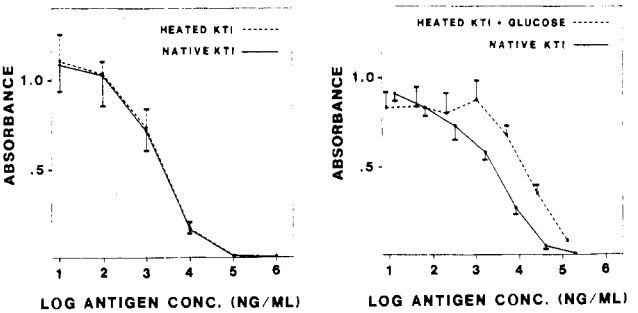

Figure 1. Competitive inhibition assays of native and modified Kunitz trypsin inhibitor with monoclonal antibody 136. The antibody was preincubated with the antigen for 2 h and then applied to the KTI-coated assay plates. Antibody bound to the plate was detected with peroxidase-conjugated rabbit anti-mouse IgG. Key: left, unheated KTI and KTI heated dry at 120 °C for 50 min; right, KTI and KTI + glucose heated dry at 120 °C for 50 min. Absorbance at 415 nm (mean ± standard deviation) of three to four wells on the plate is given.

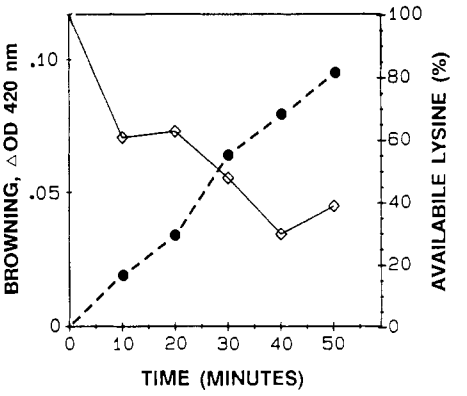

Figure 2. Effect on browning of heating KTI/glucose samples at 120 °C, given as optical density (OD) at 420 nm (- - -) and content of available lysine (–).

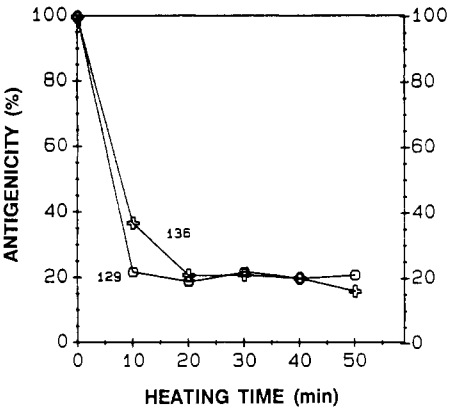

Figure 3. Effect on antigenicity of heating KTI/glucose samples at 120 °C, as elicited with monoclonal antibodies 136 and 129 in competitive binding assays.

min of heating (Figure 4). Formation of dimer apparently increased with heating time and was accompanied by production of higher molecular weight species.

The sample of KTI/glucose that had been heated for 50 min was also fractionated by gel chromatography. The results showed that a considerable amount of the protein was in the form of dimers or larger aggregates (Figure 5). From comparison with the elution profile of heated KTI alone, we concluded that the aggregation was produced by the interaction of glucose and protein during heating. Further, the apparent molecular weight of the monomer was 31 000, compared to 22 000 for KTI heated without glucose. This indicated a high degree of glucose

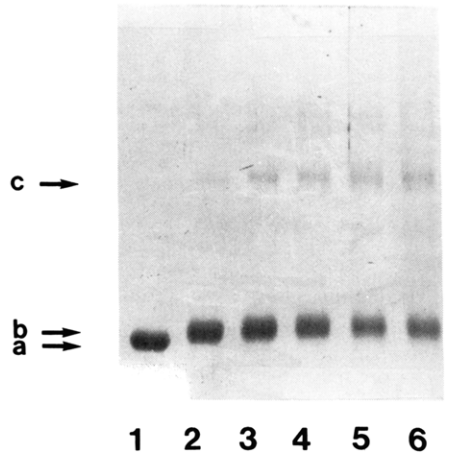

Figure 4. SDS-gel electrophoresis of native KTI (1) and KTI/glucose heated 10 (2), 20 (3), 30 (4), 40 (5), and 50 (6) min at 120 °C. The arrows indicate the separation of native KTI (a), modified KTI (b), and KTI dimer (c).

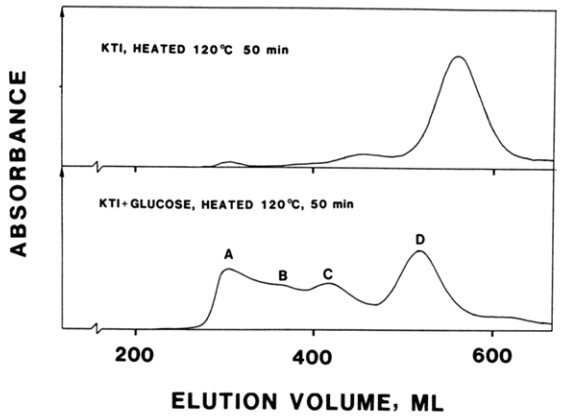

Figure 5. Gel filtration of heated KTI and heated KTI/glucose on Sephadex G-100. Native KTI has a molecular weight of 20 000 (Kassell, 1970). Heating KTI with glucose elicited the formation of high molecular weight species. Apparent molecular weights of fractions from the heated KTI/glucose sample: A, 200 000; B, 81 400; C, 60 400; D, 31 000.

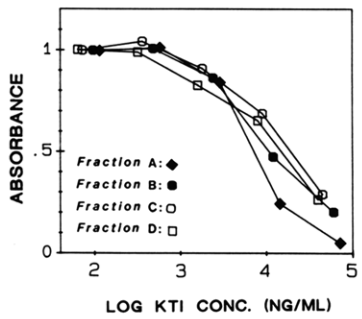

Figure 6. Competitive inhibition immunoassays with monoclonal antibody 136. Samples are the chromatographed fractions A–D of heated KTI/glucose.

substitution and/or protein conformational change. Competitive inhibition assays with antibody 136 were performed on the fractions of the chromatographed KTI/glucose (Figure 6). All these fractions had less antigenicity than native KTI. The monomeric fraction (D) was about 30% less antigenic than the polymeric fraction (A), as indicated by the shift in the immunoassay curve.

DISCUSSION

In the present investigation, a model system for nonenzymatic browning reactions was obtained by heating freeze-dried mixtures of the native KTI and carbohydrates. The changes in antigenicity of dialyzed samples were measured on monoclonal antibodies specific for two of the epitopes of native KTI. The results showed that the presence of carbohydrates in the heated samples reduced antigenicity of these epitopes. However, the decrease in antigenicity did not parallel the severity of the Maillard reaction. This was shown in two ways. First, in the KTI/glucose mixture, under conditions that favor the Maillard reaction, most of the reduction in antigenicity was produced within 10 min of heating. This heating time reduced the content of available lysine about 40% and resulted in only a minor degree of browning. Further heating did not affect the antigenicity but did decrease available lysine and increase browning. Second, even a nonreducing carbohydrate such as starch promoted the loss of antigenicity, though less dramatically. These results suggest that reactions other than the Maillard reaction also occur in this model system. Alternatively, the effect of starch might be due to the Maillard reaction, combined with steric hindrance resulting from complexation of the protein with this macromolecular carbohydrate.

SDS gel electrophoresis of these samples showed a small mobility change of the KTI molecule within 10 min of heating with glucose. Further heating caused only a slight additional change, which was not quantitated. The electrophoretic results are consistent with an initial rapid reaction with available glucose-reactive sites, giving a glucose-substituted KTI molecule. Further reactions, apparent from the increase in browning over time, probably involved the rearrangement of protein-bound glucose moieties, without further change in antigenicity.

Analysis of size-fractionated KTI/glucose showed that all fractions were less antigenic than native KTI. Surprisingly, the monomeric fraction (D, Figure 5) was least antigenic. This result suggests that even relatively mild conditions, which favor retention of monomeric protein during browning, may significantly change antigenicity.

The first step of the Maillard reaction is the condensation reaction between the amino group in the protein and the carbonyl group of the carbohydrate, forming a Schiff's base with the loss of one molecule of water. The blocking of amino groups by reducing carbohydrates, prior to the Amadori rearrangement and subsequent formation of brown products, may be a more general phenomenon than the browning of foods. The rapid loss of antigenicity of KTI upon heating with glucose indicates that this initial reaction may modify selected epitopes of the protein. Previous characterization of the antigenicity of the isoforms of KTI indicate that even a single amino acid substitution can eliminate an epitope (Brandon and Bates, 1988).

The reactivity of an ϵ-amino group in a protein depends on the surrounding structural and electronic environment, which may facilitate or limit accessibility (Baynes et al., 1986). The extent of heat-induced modification of lysine residues in food proteins varies widely among foodstuffs. This variation may be caused by differences in amino acid sequences, in the secondary and tertiary structures of proteins, and in the concentrations of reactants (Adrian, 1982; Smith and Friedman, 1984).

This study used two antibodies that bind to distinct, nonoverlapping epitopes. Similar changes in antigenicity occurred in both epitopes (Figure 3). In a related study, Ansari et al. (1975) found that acetylation of amino groups can alter the antigenicity of a protein. It is possible that both of the KTI epitopes include reactive basic amino acid residues that are rapidly modified during the Maillard reaction. Alternatively, the changes in each

epitope may reflect gross change in the conformation of KTI.

The assay for available lysine is based on correcting the lysine formed during acid hydrolysis by the amount liberated from the Amadori rearrangement product (Erbersdobler, 1986). Determination of the true amount of Schiff's base is difficult, since it exists in equilibrium with unsubstituted lysine and is hydrolyzed by acid to lysine. Measurements of available lysine reported in this paper would therefore include any Schiff's base formed. Studies by Finot et al. (1977) suggest that the Schiff's base formed in the early stages of the Maillard reaction may constitute 5–10% of the total lysine residues in roller-dried milk.

The nature and extent of browning reactions, as well as the magnitude of antigenicity changes, are probably highly dependent on the experimental conditions. In addition, the relative importance of the Maillard reaction and the reactions of nonreducing carbohydrates merit further study. Nevertheless, the present study leads us to hypothesize that the early stages of the Maillard reaction can significantly affect protein antigenicity. It should be noted that these reactions can also introduce new antigenic determinants into a food protein as demonstrated by Otani and Hosono (1987).

The Schiff's base formed in the first step of the Maillard reaction is biologically available (Finot et al., 1977). Therefore, it does not adversely affect protein nutrition. Many foods present a favorable medium for the Maillard reaction, including allergenic foods such as milk. An intriguing possibility suggested by our study is that the antigenic sites of food proteins responsible for adverse allergenic responses by eliciting production of IgE and possibly other isotypes which trigger allergic reactions could be selectively altered by modification with reducing carbohydrates under mild conditions. Thus, chemical and structural modification during food processing could be at least part of the basis for the observation that cow's milk is less antigenic in vivo after heat treatment (Kilshaw et al., 1982) and for apparent differences in the allergenicities between liquid and powdered soybean infant formulas (Burks et al., 1988). Further work is needed to determine whether conditions might be developed to exploit such beneficial effects of nonenzymatic browning and related food processing induced changes.

ACKNOWLEDGMENT

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Registry No. KTI, 9088-41-9; D-glucose, 50-99-7; maltose, 69-79-4; lactose, 63-42-3; starch, 9005-25-8; L-lysine, 56-87-1.

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