Glycation of Proteinous Inhibitors: Loss in Trypsin Inhibitory Activity by the Blocking of Arginine and Lysine Residues at Their Reactive Sites

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Lysine-type trypsin inhibitors [Japanese quail ovomucoid (QOM) and Bowman−Birk inhibitor (BBI)], and arginine-type trypsin inhibitors [chicken ovomucoid (COM) and Kunitz soybean trypsin inhibitor (KSTI)] were modified with glucose by amino−carbonyl reaction at 50 °C and 65% relative humidity for 15 days. Free amino groups of inhibitors were blocked rapidly and decreased to <20% within 5 days. On the other hand, their free guanidino groups began to decrease after a time lag for a few days, and 6−40% of the free guanidino groups remained after the 15-day incubation. Both lysine- and arginine-type trypsin inhibitors were inactivated by the reaction. A linear relationship between the loss in inhibitory activities for the lysine-type inhibitors but not for the arginine-type inhibitors and the decrease of their free amino groups was observed. The soybean trypsin inhibitors were almost completely inactivated by the 15-day incubation with glucose without severe structural or chemical damage. Denaturation of COM and KSTI incubated with glucose was scarcely observed with immunoassay, though protein polymerization of COM−, QOM−, and BBI−glucose complexes was detected by SDS−PAGE.

Keywords: *Chicken ovomucoid; Japanese quail ovomucoid; Kunitz soybean trypsin inhibitor; Bowman−Birk inhibitor; amino−carbonyl reaction; Maillard reaction; protein modification; inactivation; trypsin inhibitory activity*

INTRODUCTION

Proteinous trypsin inhibitors are widely present in animal and plant foods. On the basis of the residues at their inhibitory reactive sites, these inhibitors can be classified into lysine- and arginine-type inhibitors.

Ovomucoid is one of the major protease inhibitors in avian eggs and accounts for about 10% of egg white proteins. Ovomucoids from chicken and quail eggs are known as trypsin inhibitors and inhibit only trypsin and no other protease. Their reactive sites are Arg^{89} in the second domain of chicken ovomucoid (COM) (Kato, I., et al., 1987), and Lys^{89} and Lys^{149} of Japanese quail ovomucoid (QOM) (Kato, I., et al., 1976). The inhibitory activity of COM was lost by severe heat treatments in neutral or alkaline conditions, such as at 100 °C for 60 min at pH 7 and 9 (Stevens and Feeney, 1963; Matsuda et al., 1982). The reductive cleavage of the ovomucoid disulfide bonds caused a complete loss of its trypsin inhibitory activity (Sjöberg and Feeney, 1968; Matsuda et al., 1981), though the inhibitory activity was regained by reoxidation. More than 95% of its original activity was recovered after reoxidation under appropriate conditions (Matsuda et al., 1981).

QOM is also heat stable as it has been reported that QOM retained about 100% of its original activity over a pH range from 1 to 12 after a 24-h incubation at 37 °C, and 70% of its original activity was maintained after a 1-h incubation at 100 °C (Takahashi et al., 1994).

The soybean trypsin inhibitors account for approximately 6% of the protein in defatted soybean meal (Rackis and Anderson, 1964). There are two types of trypsin inhibitors; the reactive site of Kunitz soybean trypsin inhibitor (KSTI) for trypsin inhibition is Arg^{63} (Odani and Ikenaka, 1973), and that of Bowman−Birk inhibitor (BBI) is Lys^{16} (Odani and Ikenaka, 1972).

BBI, which inhibits both trypsin and chymotrypsin, is a small protein consisting of 71 amino acids with 7 disulfide bonds. This small inhibitor is highly heat stable; for example, no large conformation change was induced even by heating at 80 °C for 1 h at pH 6.5 (Wu and Sessa, 1994). Moist heat treatment of soybeans at 100 °C for 20 min inactivates about 90% of the trypsin inhibitors. After processing by most commercial methods, however, 5−20% of the original trypsin inhibitory activity remains (Rackis et al., 1986). The more protracted heating required for complete inactivation of the inhibitors would induce a certain damage of soybean proteins, resulting in reduction of the nutritive value.

Trypsin inhibitors in raw soybeans cause growth inhibition in all tested animals: pancreatic hypertrophy and hyperplasia in rats, mice, chickens, and other experimental animals (Rackis et al., 1963; Morgan et al., 1986). Inactivation of trypsin inhibitors have been investigated from biochemical and food technological viewpoints. 1,2-Cyclohexanedione was employed to chemically modify the arginyl residues of chicken ovomucoid and soybean trypsin inhibitors, and nearly all of their trypsin inhibitory activities were abolished (Liu et al., 1968). Reducing agents such as cysteine and thiols enhance heat inactivation of soybean trypsin inhibitors (Lei et al., 1981; Friedman et al., 1982). Ascorbic acid plus $CuSO_4$, with moderate heat, effectively inactivates soybean trypsin inhibitors in model systems but not in soy flour (Sessa et al., 1990).

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Soybean inhibitors can be inactivated by cleavage of the two disulfide bridges in KSTI (Steiner, 1965) and by cleavage of four of the seven disulfide bonds in BBI (Hogle and Liener, 1973).

Thus, inactivation of the trypsin inhibitors is not easily accomplished by the usual physical processing of food because of the structural stability partly given by their small molecular sizes and high proportion of intramolecular disulfide bonds.

The amino−carbonyl reaction spontaneously occurs in foods containing both amino and carbonyl compounds, particularly in dry foods. Food proteins reacted with reducing sugar at lysine residues in the early stage of the reaction obtain high physicochemical properties, such as solubility and heat stability (Kato, Y., et al., 1981). The reducing sugars are main food components and not additives and, consequently, the foods reacted with sugars would be accepted as safe foods.

In this paper, we examine the decreases in lysine and arginine residues and in trypsin inhibitory activity of egg and soybean trypsin inhibitors by the amino−carbonyl reaction with glucose under a dry and mild-heating conditions. The results show that not only lysine-type trypsin inhibitors but also arginine-type inhibitors were effectively inactivated by the amino−carbonyl reaction without severe structural and chemical damages to the proteins and that soybean-type inhibitors were inactivated more effectively by the reaction with glucose.

MATERIALS AND METHODS

Materials. Trypsin, chymotrypsin (type II), and *N*-tosyl-L-phenylalanyl chloromethyl ketone (TPCK)-treated trypsin from bovine pancreas were obtained from Sigma Chemical Co. The substrates for trypsin and chymotrypsin, *N*-benzoyl-DL-arginine *p*-nitroanilide hydrochloride (BAPA), purchased from Katayama Chemical (Osaka), and *N*-benzoyl-L-tyrosine *p*-nitroanilide (BTpNA), purchased from Wako Pure Chemical Industries Ltd., respectively, were used.

Fresh eggs of quail (Japanese quail) and chicken (White Leghorn) were obtained from the university farm of the School of Agricultural Sciences, Nagoya University. Quail and chicken ovomucoids were isolated from each egg white according to the method of Waheed and Salahuddin (1975). All fractions with trypsin inhibitory activity recovered from SP-Sephadex-C50 ion-exchange chromatography were pooled, dialyzed against distilled water, and freeze-dried.

Crude Bowman−Birk inhibitor was prepared from commercially available defatted soy flour (type II-s, Sigma) by alcohol extraction and acetone preparation as described by Frattali (1969). The precipitates were dissolved in water and dialyzed against distilled water. This protein solution was chromatographed on a CM-Cellurofine (Seikagakukogyo Co.) column. The column (2.6 × 36) was equilibrated with 5 mM sodium acetate, pH 4.0, and the absorbed proteins were eluted with a linear gradient from 0 to 0.5 M NaCl according to the method of Ikenaka and Odani (1977). Trypsin and chymotrypsin inhibitory activities were examined for each fraction as follows: 10 μL of trypsin (0.5 mg/mL) and 10 μL of each fraction were incubated for 1 min at 37 °C, and 100 μL of BAPA (1 mg/mL) was added to the mixed solution. The increase in absorbance at 405 nm was continuously monitored as described by Y. Kato et al. (1993). Ten microliters of each fraction mixed with 10 μL of chymotrypsin (0.5 mg/mL) was incubated for 1 min at 37 °C, and then 100 μL of BTpNA solution (1 mg/mL) was added to the enzyme−inhibitor mixture. Absorbance of each fraction was monitored at 405 nm according to a modified method of Bundy (1962). The fractions that inhibited both trypsin and chymotrypsin were pooled and further purified by rechromatography on the same column of CM-Cellurofine. After the purity was confirmed by tricine−SDS−PAGE (Schägger and von Jagow, 1987), the BBI preparation was dialyzed against distilled water and freeze-dried.

KSTI was also purified from defatted soy flour by gel filtration Sephadex G-75 chromatography. The trypsin and chymotrypsin inhibitory activities of each fraction were measured as described above, and the fractions with inhibitory activity to trypsin but not to chymotrypsin were pooled, dialyzed against distilled water, and freeze-dried.

Amino−Carbonyl Reaction. The powdered protein samples and glucose were dissolved in distilled water at concentrations of 2 and 1 mg/mL, respectively. The solution containing both protein and glucose was adjusted to pH 8.0 with a small amount of 1 mol/L NaOH. The samples of the mixed solution were pipetted into test tubes (1 mg of protein/tube), and freeze-dried. The dried samples were kept in a humidity chamber (Yamato Scientific Co., Ltd. Model IG-43H.M.) at 50 °C and 65% relative humidity for various periods (0−15 days) to accelerate the amino−carbonyl reaction (Kato, Y., et al., 1986). The glycated protein preparations were kept at −20 °C until use.

Measurement of Trypsin Inhibitory Activity. Ten micrograms of each trypsin inhibitor incubated with glucose was incubated in a cuvette with a slight excess of bovine trypsin at 37 °C for 1 min, and then 400 μL of BAPA solution (1 mg/mL) was added to the cuvette. Trypsin inhibitory activity was assayed by measuring the remaining trypsin activity, i.e., the initial rate of increase in optical absorbance at 420 nm with increase of *p*-nitroaniline, as described by Waheed and Salahuddin (1975). The trypsin inhibitory activity of the inhibitor incubated with glucose was represented by percentage against that of the native one.

Determination of Free Amino and Guanidino Groups. Free amino groups were determined by the fluorometric method using fluorescamin (Sigma) according to the method of Böhlen et al. (1973). Free amino group contents in samples were expressed as a percentage of fluorescence intensity per milligram of protein against those of unmodified inhibitors, respectively.

Free guanidino groups were determined by Sakaguchi reaction (Sakaguchi, 1925; Albanese, 1946). One hundred microliters of NaOH solution (10%), 100 μL of α-naphthol (0.01% in 95% ethanol), and 400 μL of distilled water were added to 100 μL of each sample solution (1 mg of protein/mL), and the mixture was kept at room temperature for 5 min. One hundred microliters of NaBrO solution (as 1% Br_2 in 5% NaOH) was added to the mixture, followed by immediate addition of 200 μL of 40% urea solution. Absorbance at 510 nm of the sample solution was measured spectrophotometrically. A standard curve obtained using arginine was linear at the concentration between 5 and 100 mg/mL.

Measurement of Antigenic Reactivity by ELISA. Rabbit antiserum to COM was prepared as described previously (Matsuda et al., 1982). Antiserum to KSTI was prepared by immunizing three ddY (Japan SLC) mice injected subcutaneously with 50 μg of KSTI per a mouse in 100 μL of PBS, emulsified with Freund's complete adjuvant. The mice were then given two booster injections of the same antigen 14 and 28 days after the first immunization. The antigenic reactivity of COM or KSTI modified with glucose with each specific antibody was examined by ELISA (Engvall and Perlman, 1971). Flat-bottom microtiter plates were coated with an appropriate concentration (1−100 mg/mL) of protein antigens, and the mice antisera that reacted with the plate-binding antigens were determined by using peroxidase-coupled anti-mouse or anti-rabbit IgGs (DAKO, A/S, Denmark), respectively, and *o*-phenylenediamine as the enzyme substrate.

Electrophoresis. Sodium dodecyl sulfate (SDS)−polyacrylamide gel electrophoresis (SDS−PAGE) (using 12.5% acrylamide) was performed according to the method of Laemmli (1970) with subsequent Coomassie Blue staining.

RESULTS AND DISCUSSION

Modification of Lysine and Arginine Residues by Amino−Carbonyl Reaction. Ovomucoids (COM

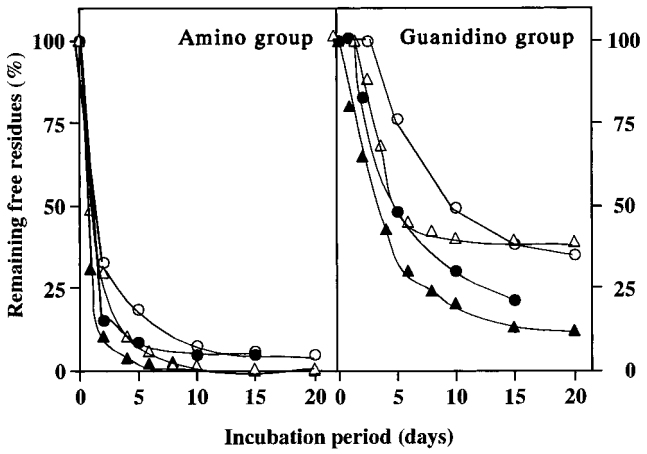

Figure 1. Decrease in free amino (left) and guanidino (right) groups of inhibitors incubated with glucose: COM (●), QOM (○), KSTI (▲), and BBI (△) were incubated with glucose at 50 °C and 65% realtive humidity for 1−15 days. Both groups of inhibitors incubated in the absence of glucose at the same condition remained completely free (not shown). Free amino group represents relative fluorescence intensity (percent) against each native protein, and free guanidino group represents relative absorbance intensity (percnet) against each native protein.

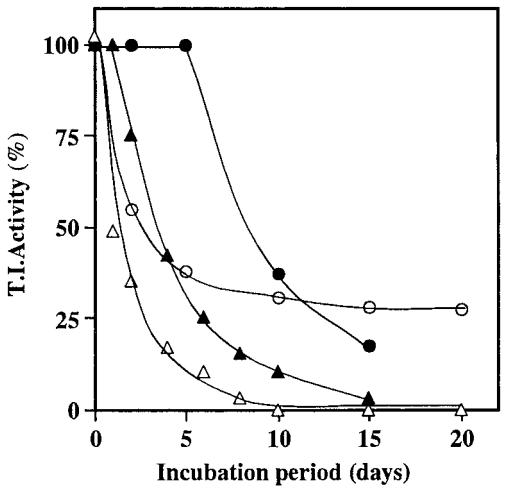

Figure 2. Suppression of trypsin inhibitory activity by inhibitors incubated with glucose: COM (●), QOM (○), KSTI (▲), and BBI (△) were incubated with glucose at 50 °C and 65% relative humidity for 1−15 days. The remaining inhibitory activity of the inhibitors incubated with glucose is represented by percentage against trypsin inhibitory activity of native inhibitors, respectively.

and QOM) and soybean trypsin inhibitors (KSTI and BBI) incubated with glucose at 50 °C and 65% relative humidity were still soluble in water even after the 15-day incubation. The residual free amino and guanidino-groups in the incubated samples of OMs and soybean trypsin inhibitors were determined by the fluorometric method and Sakaguchi reaction, respectively. Figure 1 shows the decrease in free amino or guanidino groups as a relative value (percent) to those of each native protein, respectively. Their free amino groups were lost quickly in the initial 2-day incubation with glucose, and the residual free amino groups of COM, QOM, BBI, and KSTI were about 5% or less and almost 0%, respectively, after the 15-day incubation with glucose.

On the other hand, the decrease in free guanidino groups appeared to start after a time lag for a few days except for the KSTI−glu mixture. There were large differences in the decrease profiles of free guanidino groups among the four inhibitors. The guanidino groups of COM and KSTI were blocked with glucose more rapidly than those of QOM and BBI, and residual free guanidino groups of COM, QOM, BBI, and KSTI decreased to about 20, 40, 40, and 10%, respectively, after 15 days of incubation with glucose. No less than 40% of guanidino groups of BBI and QOM remained free even after the 15-day incubation with glucose, whereas only about 20 and 10% of free guanidino groups were detected, respectively, in COM and KSTI incubated with glucose under the same conditions. Thus, the decrease in free guanidino groups by the reaction with glucose was largely different among these trypsin inhibitors.

Both amino and guanidino groups of the proteins were modified with glucose through the amino−carbonyl reaction, though the rate of decrease in guanidino groups was slower than that of the amino groups. These results agreed well with the results on the reaction of ovalbumin with glucose reported previously (Kato,Y., et al., 1989). The losses of lysine and arginine residues in amino−carbonyl reaction of ovalbumin with some disaccharides were measured by amino acid analysis after acid hydrolysis, and the free guanidino groups of ovalbumin were retained to a greater extent than free amino groups after 15 days of incubation (Kato, Y., et al., 1989). The compound reactive to guanidino group was suggested to be 3-deoxyglucosone generated from Amadori rearrangement product (Kato, H., et al., 1987).

Inactivation of Trypsin Inhibitors by Amino−Carbonyl Reaction. Remaining trypsin inhibitory activity was also measured for the protein samples incubated with glucose. Figure 2 shows the inhibitory activities expressed as a percentage of those of each native inhibitor. The inactivation profiles of the four inhibitors were considerably different from each other. The trypsin inhibitory activities of QOM and BBI, the lysine-type inhibitors, decreased gradually during the incubation periods, and the percentages of remaining inhibitory activity were about 0 and 30% after the 10-day incubation, respectively. Thus, the decreases in trypsin inhibitory activity were markedly different between the two inhibitors with lysine-type reactive sites, even though only a slight difference between the two was observed in decrease profiles of free lysine. Furthermore, the inactivation profiles of the COM− and KSTI−glu were also different; that is, the residual activity of KSTI was about 30% after the 5-day incubation, whereas almost full activity of COM was retained even after the 5-day incubation.

The free amino group contents were plotted against trypsin inhibitory activity of the two lysine-type trypsin inhibitors, BBI and QOM, incubated with glucose as described above (Figure 3). The plot shows that there is a good linear correlation between the two with correlation factors 0.997 and 0.996 for BBI and QOM, respectively. The loss of free amino groups in BBI resulted in the complete inactivation of its trypsin inhibition. On the other hand, about 30% of the trypsin inhibitory activity of QOM remained even after QOM lost its free amino group almost completely. One molecule of QOM has 13 lysine residues, and 2 of them (Lys 89 and Lys 149) are the reactive sites for trypsin inhibition (Table 1). The remaining free lysine residues of QOM incubated for 15 days are calculated to be one per molecule or less. These results suggest that the lysine residues of one or both reactive sites of QOM are less susceptible to the glucose modification than the other lysine residues.

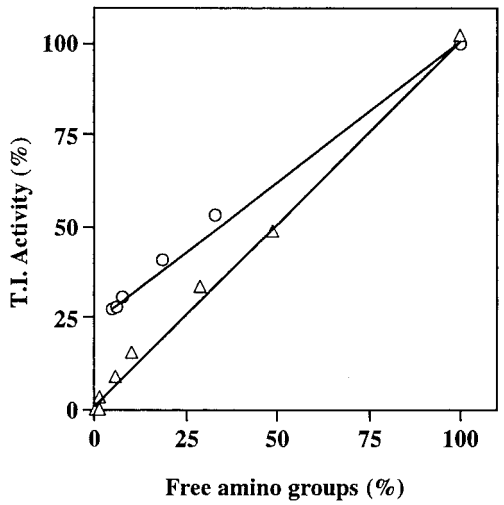

Figure 3. Relationships of trypsin inhibitory activity and remaining free amino groups of lysine-type inhibitors, QOM (○) and BBI (△), incubated with glucose.

Table 1. Structural Properties of Proteinous Trypsin Inhibitors

	MW	no. of amino acid residues	no. of lysine residues	no. of arginine residues	no. of S−S bonds	trypsin inhibitory site
KSTI	20100	181	10	9	2	Arg(63)
BBI	7975	71	5	2	7	Lys(16)
COM	28000	186	13	6	9	Arg(89)
QOM	28000	186	13	5	9	Lys(89), Lys(148)

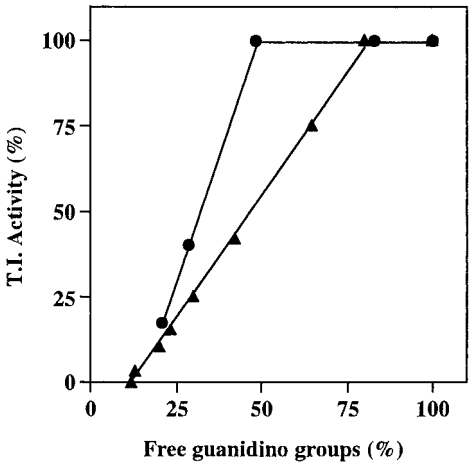

Figure 4. Relationships of trypsin inhibitory activity and remaining free guanidino groups of arginine-type inhibitors, COM (●) and KSTI (▲), incubated with glucose.

In the case of arginine-type trypsin inhibitors, the completely linear relationship between residual trypsin inhibitory activity and free guanidino groups was not seen, though correlation factors for COM and KSTI were calculated to be 0.84 and 0.98, respectively (Figure 4). KSTI has nine arginine residues per molecule, and about one free arginine residue remained after the 15-day incubation with glucose. However, no residual inhibitory activity was observed in KSTI incubated for 15 days. Such a difference between residual guanidino groups and trypsin inhibitory activity of KSTI might be due to preferential modification of the reactive site (Arg 63) or due to activity loss depending on conformational change of KSTI incubated by the amino−carbonyl reaction.

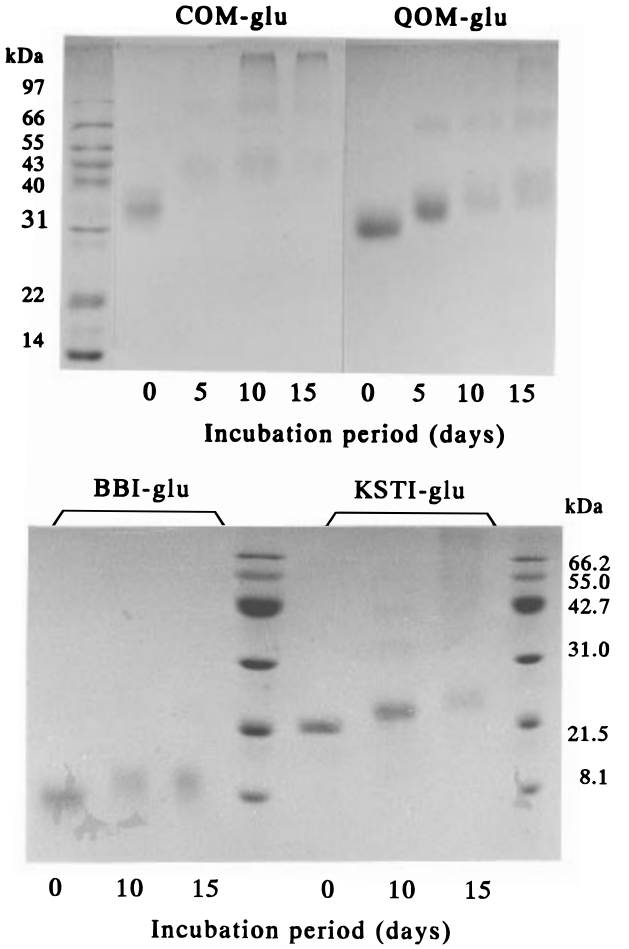

Figure 5. SDS−PAGE patterns of inhibitors incubated with glucose. Inhibitors were incubated with glucose for 15 days. The inhibitors incubated for 0 days are equivalent to native ones.

Comparison of the inactivation profiles between the egg inhibitors and the soybean inhibitors showed that soybean inhibitors lost their activities almost completely, but egg inhibitors did not. The reason for the stability of egg inhibitors is uncertain, but it might relate to the fact that egg inhibitors, COM and QOM, are glycoproteins with about 10−25% (w/w) sugar content.

Properties of Trypsin Inhibitors Reacted with Glucose. Protein glycation and subsequent polymerization during the incubation with glucose were examined by SDS gel electrophoresis, and the electrophoretogram is shown in Figure 5. The electrophoretic mobility of monomer proteins incubated with glucose decreased gradually, and the protein bands corresponding to dimer and polymers increased with increase in the incubation periods. The protein polymerization induced by the amino−carbonyl reaction with glucose has also been observed in the other protein systems such as ovalbumin (Kato, Y., et al., 1989) and lysozyme (Kato, H., 1987). Polymerization of protein was accelerated in the COM−glu system compared to the QOM− and BBI−glu systems.

Protein conformational changes induced by glycation were estimated by measuring antigenic activity of these inhibitors. Antigenic activity of COM and KSTI incubated with glucose was examined by ELISA using specific antisera and expressed as percentage of the ELISA values relative to those of native ones (Figure 6). The antigenic activities of both inhibitors remained

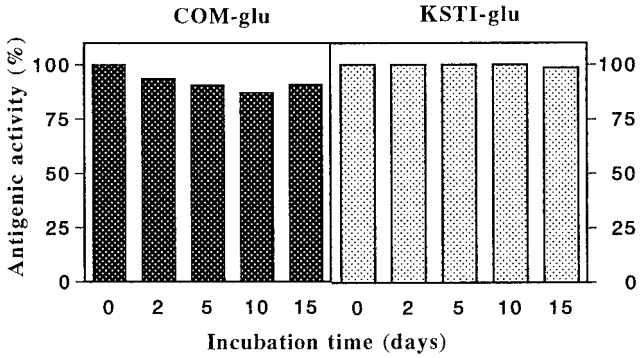

Figure 6. Changes in antigenic activities of inhibitors incubated with glucose. Antigenic activity was measured with the ELISA using rabbit (for COM) and mouse (for KSTI) antisera and peroxidase-coupled anti-mouse or anti-rabbit IgGs, respectively, and *o*-phenylenediamine as the enzyme substrate. COM (left) and KSTI (right) were incubated with glucose for 15 days. Antigenic activity represents a relative value (percent) to ELISA absorbance of each native inhibitor.

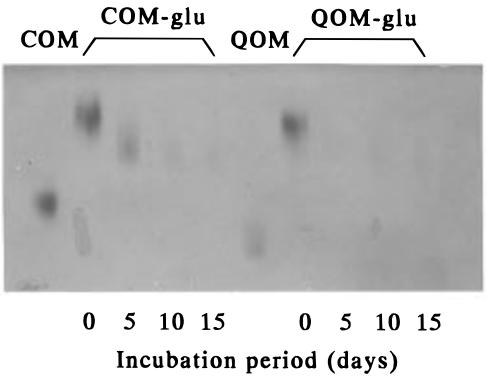

Figure 7. Polyacrylamide gel electrophoresis of an ovomucoid reacted with glucose and native one, both of which were incubated with TPCK-treated trypsin. The COM and QOM were incubated with excess trypsin (TPCK-treated trypsin: ovomucoid = 20:1; w/w) for 15 min at 37 °C.

90% or more of those of native ones. Their antigenic activities were maintained almost completely after 15 days of incubation with glucose, although protein polymerization was observed during the incubation (Figure 6). Since the antigenic activities of COM and KSTI are mostly conformation dependent (Matsuda et al., 1982, and unpublished data), the small effect of amino−carbonyl reaction on the antigenic activity suggests that the polypeptide conformation of these inhibitors remains almost intact.

The susceptibility to tryptic hydrolysis of ovomucoids incubated with glucose was analyzed by polyacrylamide gel electrophoresis. The ovomucoid reacted with glucose and native ovomucoid were incubated with excess of bovine trypsin (TPCK-treated trypsin:ovomucoid = 20: 1; w/w) and analyzed by a native gel electrophoresis (7.5% acrylamide gel) to detect stable enzyme−inhibition complexes (Figure 7). Distinct bands of the complexes of trypsin and native or 5-day-incubated COMs were detected, whereas those of COM incubated for 10 days or more were weak or faint. On the other hand, QOM incubated for 5 days showed very weak or almost no band of the enzyme−inhibitor complex. These results were in good agreement with the decrease in trypsin inhibitory activity determined for COM and QOM incubated with glucose (Figure 2).

In the present study, a model system for decrease in trypsin inhibitory activity was investigated by the amino−carbonyl reaction between four trypsin inhibitors and a reducing sugar, glucose. The results showed that incubation of inhibitors with glucose at 50 °C and 65% relative humidity reduced free amino and guanidino groups of the inhibitors. Decrease in trypsin inhibitory activity of lysine-type inhibitors, QOM and BBI, and arginine-type inhibitors, COM and KSTI, correlated to the decrease in free amino and guanidino groups, respectively, except that a short lag time before decrease in free guanidino groups was observed for the arginine-type inhibitors. The first step of amino−carbonyl reaction has been suggested to be conjugation of reducing sugar to protein ϵ-amino groups. In advanced steps of the reaction, free guanidino groups of proteins are reported to be modified with some reactive components in the reaction products (Cho et al., 1986). The inactivation of the arginine inhibitors could be due to the modification of reactive site of arginine with such active components produced in the latter stage of amino−carbonyl reaction.

Oste et al. (1990) also reported that the antigenicity of KSTI was remarkably reduced by heating with glucose at 120 °C for 50 min as measured by the monoclonal antibodies. Ansari et al. (1975) found that acetylation of amino groups can alter the antigenicity of protein. In the present study, mild heating at 50 °C and 65% realative humidity of KSTI and COM with glucose showed very little antigenicity changes as measured by ELISA using specific antibody, though protein polymerization was observed during the incubation. Therefore, the decrease in the trypsin inhibitory activity by incubating the inhibitors with glucose would not be due to conformational destruction of whole molecules but to specific modification of lysine or arginine residues of the inhibitors by the amino−carbonyl reaction with glucose and/or glucose-dependent carbonyl compounds.

Because of the deleterious effects of trypsin inhibitors in animal nutrition and the restrictive effect of trypsin inhibitors on protein digestion, some attempts to eliminate trypsin inhibitory activity have been reported. The major methods of suppression of trypsin inhibitory activity are reported to be heat treatment and reduction of S−S bond of the inhibitors. Hogle and Liener (1973), for example, examined reduction of the disulfide bonds of BBI with sodium boron hydride and reported that loss in its antitryptic activity was dependent on the average number of disulfide bonds. The more protracted heating required to destroy all inhibitor activity would damage the nutritive value of egg or soybean proteins. The amino−carbonyl reaction between inhibitors and reducing sugars might be to suppress the lysine- and arginine-type trypsin inhibitors in food and feed proteins, though some available lysine is lost.

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