Studies on the Antigenicity of the Browning Product between $\beta$-Lactoglobulin and Lactose

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Abstract Analysis of the quantitative precipitin reaction of a browning product between $\beta$-lactoglobulin and lactose (Lac-$\beta$-Lg) or S-carboxymethylated $\beta$-lactoglobulin (SCM-$\beta$-Lg) with rabbit anti Lac-$\beta$-Lg serum indicates that there are at least five or three antigenic sites on Lac-$\beta$-Lg or SCM-$\beta$-Lg molecule, respectively. These facts suggest that at least three antigenic sites on Lac-$\beta$-Lg are formed by a heat denaturation of $\beta$-lactoglobulin when Lac-$\beta$-Lg is prepared, because SCM-$\beta$-Lg hardly react with antiserum to $\beta$-lactoglobulin. Hence, in order to study the preliminary localization of the antigenic sites formed by the heat denaturation, peptides 1-7, 8-24, 25-107, 108-145 and 146-162 were isolated from $\beta$-lactoglobulin and SCM-$\beta$-Lg cleaved with cyanogen bromide, and the antigenic reactivities of the peptides with anti Lac-$\beta$-Lg serum were examined. In quantitative precipitin reaction between SCM-$\beta$-Lg and anti Lac-$\beta$-Lg serum, the mixture of peptides consisting of 1-7, 8-24, 25-107, 108-145 and 146-162 inhibited about 93% of the formation of immune precipitate. Of these five peptides, peptides 25-107 and 108-145 inhibited about 60% and 24% of the quantitative precipitin reaction, respectively. Peptides 25-107 and 108-145 also inhibited the binding of SCM-$\beta$-Lg to the specific Ig G against Lac-$\beta$-Lg by enzyme-linked immunosorbent assay. These results indicate that four methionyl residues in $\beta$-lactoglobulin do not play an important role in the antigenic activity of Lac-$\beta$-Lg and that antigenic sites, which are formed as a result of heat denaturation of $\beta$-lactoglobulin, are mainly present in 25-107 and 108-145 regions.

It is necessary for milk and milk products as foodstuffs to undergo heat treatment for pasteurization, sterilization, concentration or drying when they are processed. In previous works\(^1\),\(^2\), we found that the antiserum to a browning product between $\beta$-lactoglobulin and lactose (Lac-$\beta$-Lg) or S-carboxymethylated $\beta$-lactoglobulin (SCM-$\beta$-Lg) formed a precipitin arc with UHT-processed milk. Hence, in order to study the preliminary localization of the antigenic sites formed by the heat denaturation, peptides 1-7, 8-24, 25-107, 108-145 and 146-162 were isolated from $\beta$-lactoglobulin and SCM-$\beta$-Lg cleaved with cyanogen bromide, and the antigenic reactivities of the peptides with anti Lac-$\beta$-Lg serum were examined. In quantitative precipitin reaction between SCM-$\beta$-Lg and anti Lac-$\beta$-Lg serum, the mixture of peptides consisting of 1-7, 8-24, 25-107, 108-145 and 146-162 inhibited about 93% of the formation of immune precipitate. Of these five peptides, peptides 25-107 and 108-145 inhibited about 60% and 24% of the quantitative precipitin reaction, respectively. Peptides 25-107 and 108-145 also inhibited the binding of SCM-$\beta$-Lg to the specific Ig G against Lac-$\beta$-Lg by enzyme-linked immunosorbent assay. These results indicate that four methionyl residues in $\beta$-lactoglobulin do not play an important role in the antigenic activity of Lac-$\beta$-Lg and that antigenic sites, which are formed as a result of heat denaturation of $\beta$-lactoglobulin, are mainly present in 25-107 and 108-145 regions.

The aim of the present work is to clarify the antigenicity of Lac-$\beta$-Lg in an attempt to study the antigenic structure of $\beta$-lactoglobulin in milk products.

Materials and Methods

1. Materials

The methods of preparation of $\beta$-lactoglobulin, Lac-$\beta$-Lg, SCM-$\beta$-Lg and anti-
serum to Lac-β-Lg have been described in previous papers. Immunoglobulin G (Ig G) to Lac-β-Lg was prepared from anti Lac-β-Lg rabbit serum by a combination of ammonium sulfate fractionation and DEAE cellulose column chromatography. Bovine serum albumin (BSA) and anti rabbit Ig G goat serum conjugated with peroxidase were purchased from Sigma Chemical Co. Ltd., St. Louis, and Cappel Laboratories, Pennsylvania, respectively.

The other chemicals were of the highest grade commercially available.

2. Cleavage of β-lactoglobulin and SCM-β-Lg with cyanogen bromide

Beta-lactoglobulin and SCM-β-Lg were cleaved by cyanogen bromide (CNBr) according to the method of Braunitzer and Chen. The protein (1 g) and CNBr (1 g) were dissolved in 100 ml of 70% formic acid. The reaction was allowed to proceed at 37°C for 24 h. The reaction mixture was diluted with 10 volumes of distilled water and lyophilized.

3. Immunological methods

Quantitative precipitin reaction was carried out essentially according to the method described in a previous paper. To 0.5 ml of anti Lac-β-Lg serum diluted 2-fold with 0.01 M phosphate buffer, pH 7.2, containing 0.15 M NaCl (PBS), the same volume of antigen was added at various concentrations. The mixtures were allowed to stand for 2 h at 37°C, then kept at 4°C for 48 h. The precipitates formed were collected by centrifugation at 3,000 rpm for 30 min at 4°C, then washed three times with cold PBS and dissolved in 36 N H2SO4. The amount of protein was determined by the standard Kjeldahl procedure (Kj. N x 6.25). For inhibition studies, to anti Lac-β-Lg serum, the same volume of peptide was added at various concentrations and immune precipitates formed were removed by the same procedure mentioned above. To the supernatant obtained, the same volume of SCM-β-Lg was added at various concentrations, and the amount of immune precipitates was estimated according to the way described above. The results were expressed in percent inhibition.

A two-step inhibition test with enzyme-linked immunosorbent assay (ELISA inhibition test) was performed according to the method described previously. A flat bottomed microtiter plate (Sankojunyaku Co. Ltd., Tokyo) was coated overnight at 4°C with 100 μl of SCM-β-Lg solution (100 nM) in 0.1 M carbonate buffer, pH 9.5, and washed with three changes of PBS-Tween. In addition, Ig G solution appropriately diluted with PBS-Tween containing 2% polyvinyl-pyrolidone (PVP) and 0.2% BSA was mixed with increasing amounts of the competing antigen in test tubes and incubated at 37°C for 2 h and 4°C for 12 h. A 100 μl aliquot of each mixture was added to each well and incubated for 3 h at 25°C. The plate was washed with PBS-Tween, 100 μl of peroxidase-conjugated goat anti rabbit Ig G appropriately diluted with PBS-Tween containing 2% PVP and 0.2% BSA was added and incubated for 2 h at 25°C. After washing with five changes of PBS-Tween, 100 μl of substrate (0.4% 0-phenylene diamine and 0.003% H2O2 dissolved in 0.1 M citrate-phosphate buffer, pH 5.0) was added and the plate was incubated for 15 min at 25°C. The
reaction was stopped by adding 25 μl of 4N H₂SO₄ to each well. The reaction product formed in each well was measured spectrophotometrically at 490 nm.

4. Gel filtration

Gel filtration was performed on a Sephadex G-75 (Pharmacia Fine Chemicals, Sweden), Bio-Gel P-6 or Bio-Gel P-4 (100–200 mesh, Bio-Rad Laboratories, California) equilibrated with formic acid-acetic acid-water (25:87:888, v/v) at a flow rate of 25 ml/h. The amount of peptides in the eluate was monitored by measuring absorbance at 280 nm or 570 nm after color development with ninhydrin reagent⁹).

5. Thin layer chromatography

Thin layer chromatography was carried out with the following solvent: butanol-acetic acid-water (4:1:2, v/v). The peptide spot was revealed at 110°C with 0.3% ninhydrin.

6. Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed according to the method of Weber and Osborn¹⁰). After electrophoresis, peptides were stained with Amido Black 10 B.

7. Amino acid analyses

Amino acid composition and N-terminal amino acid residue were determined by the procedures described in a previous paper¹¹).

Results

1. Quantitative precipitin reaction of Lac–β–Lg and SCM–β–Lg with anti Lac–β–Lg serum

Figure 1 shows the curves of the quantitative precipitin reaction of Lac–β–Lg and SCM–β–Lg with anti Lac–β–Lg serum. Sixty μg of Lac–β–Lg gave 1144 μg of immune precipitate, while 20 μg of SCM–β–Lg gave 271 μg of the precipitate. Each molar ratio of antibody to antigen in immune precipitate in the antibody excess region was determined by using molecular weights of 150,000 for antibody and 18,400 for Lac–β–Lg or SCM–β–Lg. Extrapolation of each curve to zero concentration of Lac–β–Lg or SCM–β–Lg (Curve L or S) yields a value of 4.2 or 2.8, respectively.

Fig. 1. Quantitative precipitation of Lac–β–Lg (A) and SCM–β–Lg (B) with anti Lac–β–Lg serum.
2. Isolation and identification of CNBr−peptides

Figure 2A shows a typical elution pattern of β-lactoglobulin treated with CNBr on a Sephadex G-75 column. The products were divided into four fractions. The fractions were designated N-I, N-II, N-III and N-IV in order of elution. Of these fractions N-II and N-IV were collected and lyophilized.

N-IV was further fractionated into four fractions by gel filtration on a Bio-Gel P-4 column (N-IVA, N-IVB, N-IVC and N-IVD) as shown in Fig. 2B. Of these four fractions N-IVB and N-IVC were collected and purified by rechromatography until a single spot was observed on thin layer chromatography (Fig. 3).

On the other hand, N-II was S-carboxymethylated and fractionated on a Sephadex G-75 column (N-IIA, N-IIB, N-IIIC, N-IID and N-IIIE) as shown in Fig. 2C. Of these five fractions N-IIB and N-IID were collected and purified by rechromatography until a single band or a single spot was obtained on SDS-PAGE or thin layer chromatography, respectively (Fig. 3).

Figure 4A shows a Sephadex G-75 column chromatogram of SCM−β−Lg cleaved with CNBr. The products were divided into five fractions (S-I, S-II, S-III, S-IV and S-V). S-IV was collected and further fractionated into five fractions (S-IVA, S-IVB, S-IVC, S-IVD and S-IVE). Of these five fractions S-IVB was collected and purified by rechromatography until a single spot was observed on thin layer chromatography (Fig. 3).

Table shows the amino acid compositions and N-terminal amino acids of the
Antigenicity of Lac-β-Lg

Fig. 4. Elution patterns of SCM-β-Lg treated with cyanogen bromide and S-IV on Sephadex G-75 (A) and Bio-Gel P-6 columns (B), respectively. Column: (A) 2.7 x 85 cm, (B) 2.7 x 65 cm. Solvent: Formic acid-Acetic acid-Water (25:87:88, v/v), Flow rate: 25 ml/h.

Table Amino acid compositions and N-terminuses of the purified peptides (residues of amino acid per mole peptide)

<table>
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<tr>
<th>Amino acids</th>
<th>N-IIIB 25-107*</th>
<th>N-IIID 146-162*</th>
<th>N-IVB 8-24*</th>
<th>N-IVC 1-7*</th>
<th>S-IVB 108-145*</th>
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* According to BRAUNITZER et al. 12

peptides purified. It is confirmed from these results that the amino acid compositions and N-terminal amino acids of N-IIIB, N-IIID, N-IVB, N-IVC and S-IVB correspond to those expected for the sequences 25-107, 146-162, 8-24, 1-7 and 108-145 of β-lactoglobulin, respectively.

3. Antigenic reactivities of the CNBr-peptides with anti Lac-β-Lg serum

The immunoprecipitin experiment was performed between SCM-β-Lg and anti
Fig. 5. Quantitative precipitation of SCM-β-Lg with anti Lac-β-Lg serum which had been absorbed with the mixture of CNBr-peptides (A) and inhibition of the precipitin reaction by the mixture (B). Concentration of each peptide in the mixture: ○: 0 nM, △: 1 nM, ▲: 2 nM, □: 4 nM.

Lac-β-Lg serum which had been absorbed with the mixture of peptides 1-7, 8-24, 25-107, 108-145 and 146-162 (Fig. 5A). The inhibitory activity of the mixture was calculated from the amount of immune precipitates between SCM-β-Lg and some population of antibodies which remained after the peptides bound to antibodies against Lac-β-Lg (Fig. 5B). As shown in Fig. 5B, the percent inhibition of the mixture

Fig. 6. Inhibition of the precipitin reaction of SCM-β-Lg with anti Lac-β-Lg serum by the CNBr-peptides. ○: 1-7, △: 8-24, ●: 25-107, ▲: 108-145, □: 146-162.

Fig. 7. Reactivity of SCM-β-Lg with specific Ig G to Lac-β-Lg which had been absorbed with CNBr-peptides as measured by ELISA. ○: SCM-β-Lg, ●: 1-7, ▲: 8-24, △: 25-107, □: 108-145, ■: 146-162.
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reached about 93% at maximum.

On the other hand, the immunoprecipitin reaction was also carried out between SCM-β-Lg and anti Lac-β-Lg serum which had been absorbed either with peptide 1-7, 8-24, 25-107, 108-145 or 146-162, and the maximum inhibitory activity of each peptide was calculated from the amount of immune precipitates (Fig. 6). As can be seen in Fig. 6, peptides 25-107 and 108-145 inhibited about 60% and 24% of the formation of precipitate between SCM-β-Lg and anti Lac-β-Lg serum, respectively, whereas the peptides 1-7, 8-24 and 146-162 were inert.

To ascertain whether the inhibitory activity of each peptide obtained in the above quantitative precipitin inhibition assay is correct or not, we utilized ELISA inhibition test, in which the binding property of each peptide was examined (Fig. 7). The ELISA value was obviously decreased when specific Ig G to Lac-β-Lg was previously incubated either with peptide 25-107 or 108-145. In this case also, only peptides 25-107 and 108-145 showed inhibitory activities.

Discussion

In the quantitative precipitin reaction of Lac-β-Lg and SCM-β-Lg with anti Lac-β-Lg serum, extrapolations of Curves L and S to zero concentration of antigens (Fig. 1) gave values of 4.2 and 2.8 for the molar ratios of antibody to Lac-β-Lg and SCM-β-Lg, respectively. According to CHAVEZ and SCHERAGA, these values represent minimum values for the number of antibodies bound per antigen molecules, because some antigenic sites can overlap or lie close to each other. Hence, there can be at least five and three antigenic sites on Lac-β-Lg and SCM-β-Lg molecules, respectively. These facts suggest that at least three antigenic sites in Lac-β-Lg are formed as a result of heat denaturation of β-lactoglobulin when Lac-β-Lg is prepared, because little immune precipitate is formed between anti β-lactoglobulin serum and SCM-β-Lg.

The examination of antigenic activity with a large fragments of the antigens is an effective procedure for the initial step of the localization of antigenic sites. Therefore, we prepared the peptides 1-7, 8-24, 25-107, 108-145 and 146-162 from the CNBr-cleaved β-lactoglobulin and SCM-β-Lg, and the antigenic reactivities of the peptides with anti Lac-β-Lg serum were determined. The mixture of whole CNBr-peptides inhibited about 93% of the quantitative precipitin reaction of SCM-β-Lg with anti Lac-β-Lg serum (Fig. 5). This fact confirms that four methionyl residues in β-lactoglobulin do not play an important role in the antigenic activity of Lac-β-Lg and that the complete antigenic sites are present in the CNBr-peptides.

Only two peptides isolated, i.e., the peptides 25-107 and 108-145, showed antigenic activities in both quantitative precipitin inhibition assay (Fig. 6) and ELISA inhibition test (Fig. 7). These results indicate that the antigenic sites of Lac-β-Lg, which are formed as a result of heat denaturation of β-lactoglobulin, are mainly present in 25-107 and 108-145 regions.

Experiments leading to a more precise localization of the antigenic sites in Lac-
β-Lg are now in progress.

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References