Location of Antigenic Sites in a Browning Product Between β -Lactoglobulin and Lactose

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Abstract Peptide 1-65 was isolated from cyanylated β -lactoglobulin kept in alkaline solution, and the antigenic reactivity with antiserum towards browning product between β -lactoglobulin and lactose (Lac- β -Lg) was compared with that of peptide 25-107. In a quantitative precipitin reaction of S-carboxymethylated β -lactoglobulin with anti Lac- β -Lg antiserum, peptides 1-65 and 25-107, and a mixture of peptides 1-65 and 25-107 were inhibited by 51.9%, 55.2% and 55.0%, respectively. Further, the binding ability of peptide 1-65 with anti Lac- β -Lg antibody was very similar to that of peptide 25-107 or the mixture of peptide 1-65 and 25-107 in an enzyme-linked immunosorbent assay. In the light of both these results and the antigenic activities of peptides 1-7, 8-24, 25-40, 41-107, 25-61, 62-107, 108-124, 125-145, 108-145 and 146-162, it is concluded that antigenic sites formed in Lac- β -Lg locate in sequences 41-61 and 125-145, and near tryptophane residue at position 61 in the primary structure of β -lactoglobulin.

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Cow's milk proteins are well known to cause an allergic reaction in some infants. Of the milk proteins, β -lactoglobulin is considered to be one of the potential causes of the allergy because of the absence of this protein in human milk¹⁾. Hence, it is of practical interest to clarify the antigenic sites of bovine β -lactoglobulin.

Two types of antigenic sites are generally known in protein molecules. In brief, one is the continuous or sequential site which is composed of residues of peptide bonds, and the other is the discontinuous or assembled topographic site which is made of spatially adjacent surface residues that are generally distant in sequence portions²⁾. OTANI and TOKITA³⁻⁵⁾ studied the antigenic reactivities of β -lactoglobulin, cleaved with some chemical reagents and proteolytic enzymes towards antiserum with respect to native β -lactoglobulin, and reported that the antigenic sites of the native protein depended upon steric conformation of its molecule, i.e., discontinuous sites.

On the other hand, OTANI *et al.* found that a browning product formed by the reaction of β -lactoglobulin with lactose (referred to as Lac- β -Lg) or β -lactoglobulin in UHT-processed milk had new antigenic sites beside the antigenic sites of native β -lactoglobulin^{6,7)}. They also examined the antigenicity of Lac- β -Lg with the intention of studying the antigenic structure of β -lactoglobulin in milk products, and found that

some of the new antigenic sites in Lac- β -Lg related to the sugar moiety linked to β -lactoglobulin through amino-carbonyl reaction⁸⁾ and that at least three continuous sites were formed as a result of irreversible alterations of the β -lactoglobulin molecule *via* the heat treatment used for the preparation of Lac- β -Lg^{9,10)}. Further, OTANI *et al.* reported the localizations of some antigenic reactive regions by examining the antigenic activities of 11 peptides from β -lactoglobulin cleaved with some chemical reagents and trypsin, and suggested that an antigenic site related to a residue (s) in region 25-107, which was cleaved and/or modified with 2-(2-nitrophenylsulphenyl)-3-methylbromindoleine (BNPS-skatole)¹⁰⁻¹²⁾.

Thus, in the present experiment, we isolated peptide 1-65 from cyanylated β -lactoglobulin kept in an alkaline solution and compared the antigenic activity of peptide 1-65 with that of peptide 25-107, thereby learning whether a residue cleaved with BNPS-skatole (tryptophane residue at position 61) relates to an antigenic site of Lac- β -Lg or not. Furthermore, based on our present findings, we investigated the location of antigenic sites formed in Lac- β -Lg.

Materials and Methods

1. Materials

β -Lactoglobulin, Lac- β -Lg, S-carboxymethylated β -lactoglobulin (SCM- β -Lg), peptides 1-7, 8-24, 25-107, 108-145, 146-162, 25-40, 41-107, 25-61, 62-107, 108-124 and 125-145, antiserum to Lac- β -Lg, and immunoglobulin G fraction (IgG) specific to Lac- β -Lg were prepared *via* the procedures described in previous papers^{6,10-12)}. Anti rabbit IgG antibodies (from goat) conjugated with peroxidase and 2-nitro-5-thiocyanobenzoic acid were purchased from Cappel Laboratories, Pennsylvania and Eastman Kodak Company, New York, respectively.

2. Cleavage of β -lactoglobulin at cysteine residues after cyanylation

The cyanylation and cleavage of β -lactoglobulin at cysteine residue sites were accomplished according to the procedure of JACOBSON *et al.*¹³⁾ with the following modification. β -Lactoglobulin (720 mg) was dissolved in 100 ml of 0.2 M Tris-acetate buffer, pH 8.0, containing 6 M guanidinium chloride, 1 mM ethylenediamine tetraacetic acid and 10 mM dithiothreitol, and the solution was incubated at 37°C for 5 hr. A 10-fold molar excess of 2-nitro-5-thiocyanobenzoic acid over the total thiol group was added, and the pH was readjusted to 8.0 with 1 N NaOH. After 1 hr at 37°C, the reaction mixture was cooled at 4°C and acidified by the addition of acetic acid. The cyanylated protein was separated from reagents by dialysis against 2 more 500 ml portions of 50% acetic acid for 2 days each and by gel filtration with a Sephadex G-25 column equilibrated with 50% acetic acid, and recovered by freeze-drying.

For cleavage, the cyanylated protein was dissolved at 20 mg/ml in 0.1 M sodium borate buffer, pH 9.0, containing 6 M guanidinium chloride. The pH was adjusted to 9.0 with 1 N NaOH, and the solution was incubated at 37°C for 24 hr. Then, the reaction mixture was divided into two parts: a very small part was immediately applied to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate

(SDS), and the other larger part was made 50% (v/v) with respect to acetic acid by the addition of 99% acetic acid and then used to separate peptide 1-65.

3. Immunological methods

A quantitative precipitin inhibition study and a two-step inhibition test involving an enzyme-linked immunosorbent assay (ELISA inhibition test) were done as described previously¹⁰.

4. Gel filtration

Gel filtration was performed on Sephadex G-50 (Fine, Pharmacia Fine Chemicals, Uppsala) or Bio-Gel P-10 (100-200 mesh, Bio-Rad Laboratories, California) equilibrated with 50% acetic acid and formic acid-acetic acid-water (25 : 87 : 888), respectively.

5. Ion exchange chromatography

DEAE-cellulose was equilibrated with a 0.02 M phosphate buffer, pH 7.0, containing 4.5 M urea and 0.1% 2-mercaptoethanol in a column (1.5 × 45 cm). Peptides dissolved in the phosphate buffer were applied to the column. Elution was performed with the phosphate buffer containing 0-0.1 M NaCl.

6. Electrophoresis

Polyacrylamide (12.5% acrylamide) gel electrophoresis in the presence of SDS was performed using the method of LAEMMLI¹⁴. The gel was stained with 1% Amido Black 10 B in 20% acetic acid and destained with 7% acetic acid.

7. Amino acid analyses

The amino acid composition was determined with a Hitachi Model KLA-5 amino acid analyzer after hydrolysis with 6 N HCl and a drop of 10% phenol at 110°C for 24 hr in an evacuated, sealed tube. The N-terminal residue was determined using the method of GRAY¹⁵. The dansyl derivative was identified by thin layer chromatography on a polyamide sheet¹⁶.

Results

1. Separation of peptide 1-65

An aliquot of the cyanylated β -lactoglobulin kept in 0.1 M sodium borate buffer, pH 9.0, containing 6 M guanidinium chloride at 37°C for 24 hr was applied to SDS-polyacrylamide gel electrophoresis. The resulting gel pattern is shown in Fig. 1.

Fig. 1. SDS-polyacrylamide gel electrophoresis of the cyanylated β -lactoglobulin after standing at pH 9.0 for 24 hr.

A : intact β -lactoglobulin,

B : cyanylated β -lactoglobulin.

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Several bands are seen in the cleaved sample, with a molecular weight of less than intact β -lactoglobulin. β -Lactoglobulin contains five cysteine residues at positions 66, 106, 119, 121 and 160¹⁷⁾. Hence, six peptides, i.e., 1-65, 66-105, 106-118, 119-120, 121-159 and 160-162 are expected to be produced when β -lactoglobulin is cleaved completely by the cyanylation and alkaline treatment. Of these six peptides, peptide 1-65 is the largest, having about 7,300 daltons of molecular weight, and only one peptide having leucine as its N-terminal residue was further fractionated from the cleaved protein.

Figure 2a shows a typical elution profile of the cleaved β -lactoglobulin on a

Fig. 2. Sephadex G-50 column chromatography of the cleaved β -lactoglobulin (a) and Bio-Gel P-10 column chromatography of the fraction A obtained by gel filtration with Sephadex G-50 (b).

a; The products (100 mg of peptide) were eluted with 50% acetic acid at a flow rate of 20 ml/hr. Column size: 2.7 $\times$ 96 cm. b; The fraction A (63 mg of peptide) was eluted with formic acid-acetic acid-water (25:87:888) at a flow rate of 20 ml/hr. Column size: 2.7 $\times$ 96.5 cm.

Sephadex G-50 column. The fraction designated as A was collected, concentrated and applied to another gel filtration with a Bio-Gel P-10 column (Fig. 2b). The fraction designated as A-2 was collected and lyophilized.

The lyophilized fraction A-2 was dissolved in a 0.02 M phosphate buffer, pH 7.0, containing 4.5 M urea and 0.1% 2-mercaptoethanol, and was applied to a DEAE-cellulose column chromatography (Fig. 3). The fraction designated as A-2a was collected, concentrated by membrane filtration (ULVAC DIAFILTER G-05 T; cut-off molecular weight 5,000), and made 50% (v/v) with respect to acetic acid. Then, the solution was chromatographed with a Bio-Gel P-10 column. The main peptide fraction was recovered and lyophilized. The purified A-2a showed a single band on SDS-polyacrylamide gel electrophoresis (Fig. 4).

The amino acid composition and N-terminal residue of the separated peptide are shown in Table 1, where a comparison is made with those of peptide 1-65. These results confirmed that the amino acid composition and N-terminal amino acid of A-2a correspond to those expected for sequence 1-65 in the primary structure of β -lactoglobulin.

2. Antigenic reactivities of peptides towards antibodies against Lac- β -Lg

During a preliminary precipitation between SCM- β -Lg and anti Lac- β -Lg

Fig. 3. DEAE-cellulose column chromatography of the fraction A-2 obtained by Bio-Gel P-10 column chromatography. The fraction A-2 (100 mg) dissolved in 10 ml of 0.02 M phosphate buffer, pH 7.0, containing 4.5 M urea and 0.1% 2-mercaptoethanol was applied to a column (1.5×45 cm), and was eluted with the same buffer containing various concentrations of NaCl at a flow rate of 15 ml/hr.

Fig. 4. SDS-polyacrylamide gel electrophoresis of the separated A-2a.

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Table 1. Amino acid composition and N-terminus of the separated peptide (amino acid residues per mole peptide)

Amino acids	A-2a	1-65*
Ala	6.00	6
Gly	2.60	3
Pro	2.88	3
Glx	9.23	10
Asx	6.80	6
Thr	4.05	4
Ser	3.23	4
Val	4.42	4
Met	0.55	2
Ile	4.32	4
Leu	9.62	10
Tyr	1.20	2
Lys	4.00	4
Arg	0.64	1
Trp	n.d**	2
N-terminus	Leu	Leu

* Calculated from the sequence of β -lactoglobulin A.¹⁷⁾

** n.d.; not determined.

Table 2. Inhibitory activities of the peptides.

Peptides	Inhibitory activity (%)*
1-65	51.9
25-107	55.2
Mixture of 1-65 and 25-107	55.0
1-7	0.0
8-24	0.6
25-40	1.8
41-107	55.8
25-61	29.5
62-107	4.5
108-124	1.5
125-145	28.3
108-145	26.9
146-162	1.7

* Results are expressed in per cent inhibition by each peptide of the precipitin reaction of SCM- β -Lg with antiserum to Lac- β -Lg, *i.e.*, 100 X (total precipitate with SCM- β -Lg alone at equivalence - total precipitate at equivalence in the presence of 10-fold molar excess of peptide)/total precipitate with SCM- β -Lg alone at equivalence. Data were obtained from three independent determinations.

antiserum, we determined the amount of Lac- β -Lg required to obtain maximum precipitation at equivalence. Thus, on the basis of this observation, the immunoprecipitin experiment was performed between SCM- β -Lg and anti Lac- β -Lg antiserum which had been absorbed with a 10-fold molar excess of peptide 1-65 or peptides separated in previous works to SCM- β -Lg at equivalence. Table 2 summarizes the activity of each peptide in percent inhibition of the reaction of SCM- β -Lg with antiserum to Lac- β -Lg. The peptide 1-65, peptide 25-107 and a peptide mixture consisting of 1-65 and 25-107 were inhibited by 51.9%, 55.2% and 55.0%, respectively, while inhibitory activities of peptides 1-7, 8-24, 25-40, 41-107, 25-61, 62-107, 108-124, 125-145, 108-145 and 146-162 were almost the same as those reported in previous papers¹⁰⁻¹². These results suggest that the binding ability of peptide 1-65 is compatible with that of peptide 25-107. To confirm this supposition, the abilities of peptide 1-65, peptide 25-107 and peptide mixture of 1-65 and 25-107 to inhibit the binding of SCM- β -Lg with anti Lac- β -Lg antibody by ELISA inhibition test were checked. As shown in Fig. 5, the ELISA pattern obtained by addition of peptide 1-65 alone was very similar to that observed by addition of peptide 25-107 alone or a mixture of peptides 1-65 and 25-107. These binding experiments confirm that the binding affinities are almost the same in peptides 1-65 and 25-107.

Discussion

The results of the inhibitory activities of peptides 1-7, 8-24, 25-40, 41-107, 25-61, 62-107, 25-107, 108-124, 125-145, 108-145 and 146-162 (Table 2) indicated that the

Fig. 5. Reactivity of SCM- β -Lg with IgG specific to Lac- β -Lg previously incubated with peptides 1-65 and 25-107, as measured by ELISA.

●, peptide 1-65 alone; △, peptide 25-107 alone; □, mixture of peptides 1-65 and 25-107; ■, SCM- β -Lg.

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residues 1-7, 8-24, 25-40, 108-124 and 146-162 were out of the antigenic reactive regions, and that the residues 41-107 and 125-145 carried antigenic reactive site(s), as reported previously^{10,12)}. The sum of inhibitory activities of peptides 25-61 and 62-107 was 34%. This activity was significantly lower than that of the parent peptide 25-107 (55.2%). It was thus confirmed that some antigenic sites in region 25-107 were inactivated by cleavage of a peptide bond (Trp⁶¹-Glu⁶²) and/or modification with BNPS-skatole used for the preparation of these two peptides as described previously¹¹⁾. Hence, in order to exclude the possibility that the loss of antigenic activity of peptide 25-107 after the treatment with BNPS-skatole might be caused by modification without the cleavage of a peptide bond, a peptide (peptide 1-65) longer than peptide 25-61 was prepared, and we compared its antigenic activity with peptide 25-107 by a quantitative precipitin inhibition test and ELISA inhibition test in the present work.

Peptide 1-65 and 25-107 were inhibited by 51.9% and 55.2% of quantitative precipitin reaction of SCM- β -Lg with antiserum to Lac- β -Lg (Table 2). The inhibition pattern of peptide 1-65 was very similar to that of peptide 25-107 on an enzyme-linked immunosorbent assay (Fig. 5). Further, the antigenic activity of either peptide 1-65 or peptide 25-107 alone was compatible with that of the mixture of peptides 1-65 and 25-107 (Table 2 and Fig. 5). From these results the fact that residues 1-7, 8-24 and 62-107 have little or no antigenic activity (Table 2), it is reasonable to consider that the antigenic reactive sites in region 1-65 are in common with those in region 25-107. Therefore, it is concluded that antigenic sites, which are formed as an irreversible heat denaturation of β -lactoglobulin with lactose, locate in sequences 41-61 and 125-145, and near tryptophane residue at position 61.

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β -ラクトグロブリンとラクトースの褐変反応物の抗原決定部位

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ラクトース存在下で加熱した β -ラクトグロブリン (Lac- β -Lg) には, β -ラクトグロブリン (β -Lg) 自体の熱変性により形成された一次構造依存型の抗原決定部位が少なくとも 3 個存在し, それらは β -Lg の一次構造上, 41-107 域と 125-145 域に位置することを示すと共に, 61 番目の Trp 残基付近にも抗原決定部位が存在する可能性を, 先に示した. そこで, 本論文では, β -Lg に 2-nitro-5-thiocyanobenzoic acid を作用させてシアニル化後, アルカリ処理を施し, Cys 残基の位置で特異的に切断し, その分解物より, N-末端から 65 番目までのペプチドを単離し, そのペプチド 1-65 と既報で単離した 25-107 の抗原活性を比較した. ペプチド 1-65, 25-107 および両ペプチドの混合物は, 抗 Lac- β -Lg 抗体と S-カルボキシメチル化 β -Lg の反応におけ

る沈降物の形成を, それぞれ, 51.9%, 55.2% および 55.0% 阻害した. また, 酵素免疫測定法においても, ペプチド 1-65 と 25-107 は抗 Lac- β -Lg 抗体と同程度の反応性を示し, かつ, 両ペプチドを混合しても反応性に顕著な増加は認められなかった. これらの結果, 並びに既報において単離した β -Lg の一次構造上, 1-7, 8-24, 25-40, 41-107, 25-61, 62-107, 108-124, 125-145, 108-145 および 146-162 域のペプチドの抗原活性より, Lac- β -Lg の調製時に β -Lg 自体の熱変性により形成された抗原決定部位は, 41-61 域, 125-145 域に位置するものに加えて, 61 番目の Trp 残基が関与したものも存在するものと考えられる.

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