Antigenically Reactive Regions of Bovine Milk Proteins

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Introduction

Cow's milk has been found to cause allergic reactions frequently at the clinical level. Cow's milk allergy can develop not only in formula-fed babies but also in breast-fed babies. Gerrard and Shenassa[1] recently reported that the allergy which develops in breast-fed babies is triggered by a trace amount of antigen and is probably IgE-mediated, whereas that which develops in formula-fed babies is triggered by large amounts of antigen and is probably mediated either by IgG or IgM or by immune complexes.

Alternative formulas have been developed for babies with cow's milk allergy. One of them is based on soybean protein isolates, but this approach is not entirely satisfactory since soybean proteins can themselves cause immunological hypersensitivity. The other formula is based on enzyme-digested bovine caseins, rich in free amino acids liberated from the protein. This approach has been satisfactory to some extent in lowering the sensitizing capacity of caseins. However, many biologically active peptides such as opioid, calcium-absorption stimulating, immunostimulating and angiotensin I-converting enzyme inhibitory peptides have been isolated from hydorolysates of caseins. Furthermore, it is increasingly apparent that the small intestinal mucosa takes up small peptides as well as free amino acids, and that the peptide absorption is more effective than free amino acid absorption because of a lower osmotic pressure and a greater kinetic advantage.

Judging from these facts, it is valuable to clarify the antigenically reactive regions of bovine milk proteins in order to prepare an alternative formula without altering the nutritive value of milk proteins for babies with cow's milk allergy and in order to protect the allergy.

Thus, the author has studied the antigenic properties of bovine milk proteins, and describes some information of the antigenically reactive regions of major bovine milk proteins in the present paper.

Numbers of antigenic determinants of bovine milk proteins

Male rabbits, weighing about 2 kg, were immunized by intradermal injections with

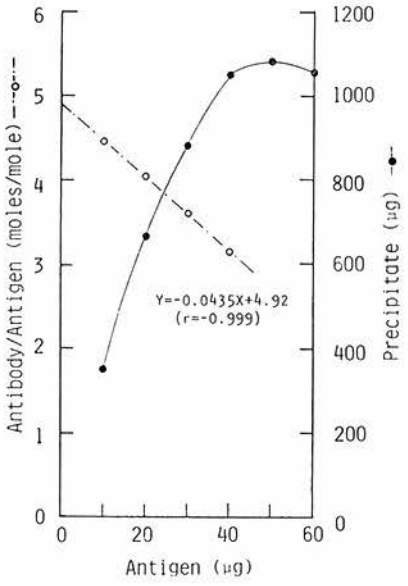

Fig. 1. Quantitative precipitation of κ-casein with its antiserum

Table 1. Numbers of antigenic determinants of bovine milk proteins

Protein	Minimum number of antigenic determinant
αs_1-Casein	6
β-Casein	6
κ-Casein	5
β-Lactoglobulin	4
Lac-LG[a]	5
	3[b]
SCM-LG[c]	4

a) : An amino-carbonyl product between β-lactoglobulin and lactose.
b) : This value was obtained by quantitative precipitation of anti-Lac-LG antiserum with S-carboxymethylated β-lactoglobulin.
c) : S-carboxymethylated β-lactoglobulin.

5 mg of bovine κ-casein in 0.85% NaCl, emulsified with Freund's complete adjuvant at 6-day intervals for 2 months. Fig. 1 shows the curve for the quantitative precipitation of the anti κ-casein rabbit antiserum with the antigen. Fifty μg of the antigen gave 1,072 μg of immune precipitate. The molecular ratio of antibody to antigen in immune precipitates in the antibody excess region was measured using molecular weights of 150,000 for antibody and 19,000 for the casein. The extrapolation of this curve to zero concentration of the antigen yields a value of 4.92. This value represents a minimum value for the number of antibodies bound per κ-casein molecule, because some antigenic sites can overlap or lie close to each other. Hence, there may be at least five antigenically reactive regions containing antigenic determinant on κ-casein molecule.

Table 1 shows minimum numbers of antigenic determinants of major milk proteins determined by the quantitative precipitation[15,19,20,23,29].

Antigenically reactive regions of bovine αs_1-casein, β-casein and β-lactoglobulin: from the results obtained by using rabbit antiserum

There are two types of antigenic determinants in protein molecule. One is the continuous determinant, which is composed of residues continuously linked by peptide bonds, and the other is the discontinuous determinant, which is made of spatially adjacent surface residues that are generally distant in sequence positions[3]. These antigenic determinants have been located by several approaches, for example by the effects of chemical modification of specific residues on the antigenic activity of the antigen, by isolation of peptide fragments with antigenic activities from their parent molecules by enzymatic or chemical degradation, and by direct synthesis of immunochemically active peptides[1,2]. The author prepared a large variety of overlapping peptide fragments from αs_1-casein, β-casein and β-lactoglobulin, and derivatives of β-lactoglobulin specially modified with chemical reagents, and the antigenic reactivity of each fragment or derivative with anti-intact protein rabbit antiserum was examined in an attempt to locate the antigenic determinants of the bovine proteins. The results obtained were summarized as follows.

1) αs_1-Casein

Bovine milk contains 26 different proteins which have antigenicities. Of the milk proteins, αs_1-casein is a major one (MW: 23,500), containing 8 or 9 phosphate groups, that is absent in human milk. Hence, αs_1-casein is considered to be one of the potential allergens to cause milk allergy.

As shown in Table 1, αs_1-casein contains at least 6 antigenic determinants. These antigenic determinants are mainly composed of residues continuously linked by peptide bonds, because chemically or enzymatically cleaved αs_1-casein retained the ability to bind the antibody specific to intact αs_1-casein. Therefore, the author prepared 14 kinds of peptide fragments from αs_1-casein cleaved with chymosin, pepsin, cyanogen bromide and/or 2 - (2 - nitrophenylsulphenyl) - 3-methyl-3-bromoindoleine, and the inhibition of intact αs_1-casein with its IgG antibody by the fragments, utilizing quantitative precipitation and enzyme-linked immunosorbent assay tech-

Table 2. Antigenic activity of peptide fragments derived from αs_1-casein

Peptide	Antigenic activity (%)[a]
1–199 (αs_1-casein)	100
24–199 (αs_1-I casein)	90
24–150 (αs_1-III casein)	56
1–23	0
1–54	19
55–60	4
61–123	31
124–135	10
136–196	20
197–199	0
136–151	2
152–193	12
165–199	11
180–199	2
194–199	1

a) : The results are expressed as percent inhibition by each peptide fragment of the precipitin reaction of αs_1-casein with its antiserum.

niques was measured[20,21,25,26]. Table 2 summarizes the antigenic activity of each peptide fragment expressed as percent inhibition of a quantitative precipitation. The anti-αs_1-casein antibody reacted noticeably with fragments 24–199, 24–150, 1–54, 61–123, 124–135, 136–196, 152–193 and 165–199, whereas fragments 1–23, 55–60, 197–199, 136–151, 180–199 and 194–199 showed little or no measurable affinity for the antibody. These reactivities indicate that at least one antigenic determinant of αs_1-casein is associated with each of regions 24–54, 61–123, 124–135 and 165–180. Further, fragment 61–123 had ability to form a sharp precipitin arc on immunodiffusion analysis using anti-αs_1-casein antiserum[25]. Thus, the region 61–123 is associated with more than 2 antigenic determinants.

On the other hand, in an enzyme-linked immunosorbent assay the reaction of native αs_1-casein with its IgG antibody was inhibited more strongly by native αs_1-casein than by dephosphorylated αs_1-casein[28]. Peptide fragment 1–25, having a phosphoserine residue-concentrated region from bovine β-casein, which has a very similar sequence consisting of 8 amino acid residues of region 63–70 in αs_1-casein, i.e., Glu-Ser(P)-X-Ser(P)-Ser(P)-Ser(P)-Glu-Glu, noticeably inhibited the reaction of native αs_1-casein and its antibody[28]. Furthermore, O-phospho-L-serine residue inhibited the reaction of peptide fragment 61–123 with antibody specific to native αs_1-casein, although L-serine and sodium phosphate showed no measurable inhibition[28]. These results suggest that the phosphoserine residue associates with part of an antigenic determinant of bovine αs_1-casein.

2) β-Casein

β-Casein, which composes of about 35% of bovine whole casein, is a major component of the caseins in mature human milk. At the immunochemical level, however, bovine and human β-caseins differ significantly; the formation of immune precipitate by bovine β-casein with its rabbit antiserum was inhibited only to an extent of 27% at maximum by the addition of the human casein[13]. This fact suggests that bovine β-casein may be one of the potential allergens. Hence, it is of practical interest to clarify the antigenic properties of bovine β-casein.

As shown in Table 1, β-casein contains at least 6 antigenic determinants. These antigenic determinants retained after some proteolytic or chemical degradations. Hence, the antigenic determinants are mainly composed of residues continuously linked by peptide bonds, and the author prepared 18 kinds of peptide fragments from β-casein cleaved with proteolytic enzymes and/or chemical reagents[15–18,27]. Table 3 summarizes the antigenic activity of each peptide fragment expressed as percent inhibition of a quantitative precipitation of β-casein with its antibody. It is found from these reactivities that at least one antigenic determinant of β-casein is associated with each of regions 1–25, 26–60, 61–93, 94–102, 103–109, 127–136 and 157–185.

On the other hand, the author isolated the IgG antibody specific to human β-casein from anti-bovine β-casein antibody by means of a human β-casein-immobilized Sepharose 4B

Table 3. Antigenic activity of peptide fragments derived from β-casein

Peptide	Antigenic activity (%)[a]
1–209 (β-casein)	100
1–139 (β-III)	83
106–209 (γ_2-casein)	27
1–93	36–52
94–102	10
103–109	10
110–144	16
145–156	1
157–185	14
186–209	2
1–60	27–35
61–93	0–10
1–25	16–22
26–93	12–24
49–93	0–12
114–144	14
110–121	0
122–131	2
132–144	1

a) : The results are expressed as percent inhibition by each peptide fragment of the precipitin reaction of β-casein with its antiserum.

column. By studying the reactivity of the IgG antibody specific to human β-casein with the 18 kinds of peptide fragments listed in Table 3, the author concluded that the antigenic determinant located in region 126–136 of bovine β-casein was one of the common antigenic determinants between bovine and human β-caseins.

3) β-Lactoglobulin

β-Lactoglobulin, a globular protein having 2 intramolecular disulfide linkages and molecular weight of 18,400, is considered to be a potential cause of an allergy to milk, because this protein is absent in human milk. Analysis of the quantitative precipitation of bovine β-lactoglobulin with its antibody indicated that there were at least 4 antigenic determinants on β-lactoglobulin molecule (Table 1). It was found that the antigenic reactivity of β-lactoglobulin with its antiserum was greatly decreased by reduced methylation of disulfide linkages in the protein and was lost completely by chemical or proteolytic degradation with cyanogen bromide, pepsin, trypsin or chymotrypsin[6,7,9,31]. These results suggest that the antigenic determinant of the protein is discontinuous one, which is made of spatially adjacent surface residues, and that it is difficult to isolate a large variety of overlapping peptide fragments with antigenic activities in the elucidation of the antigenic determinant of β-lactoglobulin. Hence, the author prepared 12 kinds of derivatives of β-lactoglobulin specifically modified with chemical reagents without cleavage of the primary structure. By studying the antigenic reactivities of the modified proteins with IgG antibody specific to native β-lactoglobulin and the changes of secondary structure, the author concluded that 1.1 of three arginine residues, two tryptophan residues and one sulfhydryl group were out of the antigenic determinant, but there was a possibility that the amino group, histidine residue and carboxyl group might play an important role in the antigenicity of bovine β-lactoglobulin[23].

4) Heated β-lactoglobulin

It is necessary for milk and milk products as food stuffs to undergo heat treatment for pasteurization, sterilization, concentration or drying when they are processed. Hence, new antigenic determinants, different from those of native β-lactoglobulin, may be formed by the heat treatment. The author examined the antigenic reactivity of S-carboxymethylated β-lactoglobulin (SCM-LG) with antiserum against β-lactoglobulin from UHT-processing milk or against an amino-carbonyl product between β-lactoglobulin and lactose (Lac-LG) prepared from 1% β-lactoglobulin heated in the presence of 10% lactose at pH 6.6, and found that both the antisera formed a precipitin arc with SCM-LG on double immunodiffusion and immunoelectrophoresis[8,10,14]. These findings represent that β-lactoglobulin in UHT-processed milk and Lac-LG have antigenic determinants which are not affected by the conformational changes caused by S-carboxymethylation of intramolecular disulfide linkages of β-lactoglobulin.

Analysis of the quantitative precipitation of SCM-LG with rabbit anti-Lac-LG antiserum

Table 4. Antigenic activity of peptide fragments derived from β-lactoglobulin

Peptide	Antigenic activity (%)[a]	
	Anti-Lac-LG	Anti-SCM-LG
1-162 (S-carboxymethylated β-lactoglobulin)	100	100
1-7	2	0
8-24	0	1
25-107	55-60	45
108-145	24-32	23
146-162	2	39
1-65	52	20
25-40	2	0
41-107	56	42
25-61	30	20
62-107	5	24
108-124	2	1
125-145	28	20

a) : The results are expressed as percent inhibition by each peptide fragment of the precipitin reaction of S-carboxymethylated β-lactoglobulin with anti-Lac-LG antiserum (Anti-Lac-LG) or anti-S-carboxymethylated β-lactoglobulin antiserum (Anti-SCM-LG).

indicated that there were at least 3 antigenic determinants on Lac-LG molecule[19]. This fact suggests that at least 3 antigenic determinants on Lac-LG are formed by heat denaturation of β-lactoglobulin when Lac-LG is prepared, because SCM-LG hardly reacted with antiserum to native β-lactoglobulin[14]. Hence, the author prepared 12 kinds of peptide fragments from β-lactoglobulin cleaved with chemical reagents and/or trypsin, and the antigenic reactivity of the fragments with anti-Lac-LG antiserum was examined (Table 4)[19,22,24,30]. It was found that the antigenic determinants, which were formed as an irreversible heat denaturation of β-lactoglobulin with lactose, located in sequences 41–61 and 125–145, and near tryptophane residue at position 61. In addition to this finding, it was demonstrated that there was an antigenic determinant which was associated with the sugar moiety linked to β-lactoglobulin through the amino-carbonyl reaction, because the amino-carbonyl product between lactose and bovine serum albumin or ovalbumin reacted with the antiserum to Lac-LG on passive cutaneous anaphylaxis test[11].

On the other hand, the author felt that a comparative study of the antigenically reactive regions in Lac-LG and SCM-LG might throw some light on the antigenic property of β-lactoglobulin in milk products, and studied the antigenicity of SCM-LG. It was estimated that SCM-LG had at least 4 antigenic determinants, which were associated with regions 41–61, 62–107, 125–145 and 146–162 (Tables 1 and 4)[29]. These results indicate that the regions 62–107 and 146–162 are associated with antigenic determinants in only SCM-LG, whereas the regions 41–61 and 125–145 carry antigenic determinants both in Lac-LG and SCM-LG.

β-Lactoglobulin has two disulfide linkages in its molecule, i.e., residues 66 and 160, and 106 and 119 or 121. SCM-LG is considered to be more unfolded form than Lac-LG, because all cysteine (cystine) residues in SCM-LG were S-carboxymethylated[29] and Lac-LG reacted strongly at the precipitation level with anti-native β-lactoglobulin antiserum whereas SCM-LG did not[10]. Thus, it is considered that the difference of antigenically reactive regions between SCM-LG and Lac-LG is due to the phase of unfolding, or the degree of denaturation, of β-lactoglobulin molecule. As mentioned already, it is necessary for milk and milk products as foodstuffs to undergo the heat treatment when they are processed, and thus, various phases of unfolding of β-lactoglobulin must be present in the milk products. Therefore, it is worth noting that the antigenic property of β-lactoglobulin in milk products may be different from that of native β-lactoglobulin in raw milk.

In addition to the antigenicity of the heat-denatured β-lactoglobulin, the author observed that β-lactoglobulin formed new antigens with molecular weights greater than 5×10^6 daltons during tryptic digestions[12].

Conclusion

Atassi et al.[2] elucidated the antigenic determinants of sperm whale myoglobin and egg white lysozyme. They found that the

antigenic determinants of the proteins usually consisted of six to seven amino acid residues containing lysine, arginine, aspartic acid and/or glutamic acid, and that the binding of antigenic determinants of the proteins with their antibodies took place primarily through ionic interactions. Hopp and Woods[5] indicated that the major region of hydrophilicity of proteins corresponded to the antigenic determinants for 12 proteins studied.

Table 5 and Fig. 2 show some amino acid sequences of antigenically active peptides from αs_1-casein, β-casein and β-lactoglobulin as mentioned above, and the hexapeptide analysis of the milk proteins and the positions of antigenically reactive peptides, respectively. It is found that many antigenically active peptides contain hydrophilic amino acids such as lysine, arginine, glutamic acid and/or aspartic acid. On hexapeptide analysis, all antigenically reactive peptides of Lac-LG are located in, or immediately adjacent to the point of high local average hydrophilicity. However, some of antigenically reactive peptides of αs_1-casein and β-casein are present in the low local average hydrophilicity. These facts indicate that the hydrophilic amino acids such as lysine, arginine, glutamic acid and/or aspartic acid are not always dominant in antigenic determinants of bovine αs_1-casein and β-casein. It is well known that αs_1-casein and β-casein have a rather unstable structure, which may approach a random coil behavior. Thus, it may be concluded that the ionic amino acids such as lysine, arginine, glutamic acid and/or aspartic acid are not always dominant amino acids in the antigenic determinants of unglobular proteins.

On the other hand, it is worth noting that the antigenic property of β-lactoglobulin in

Table 5. Amino acid sequences of antigenically active peptides

1. αs_1-Casein
   1) ^{24}Phe-Val-Ala-Pro-Phe-Pro-Gln-Val-Phe-Gly-Lys-Glu-Lys-Val-Asn-Glu-Leu-Ser-Lys-Asp-Ile-Gly-Ser-Glu-Ser-Thr-Glu-Asp-Gln-Ala-^{51}Met (p, p)
   2) ^{61}Glu-Ala-Glu-Ser-Ile-Ser-Ser-Ser-Glu-^{70}Glu (p, p, p, p)
   3) ^{124}Lys-Gln-Gly-Ile-His-Ala-Gln-Gln-Lys-Glu-Pro-^{135}Met
   4) ^{145}Phe-Tyr-Pro-Glu-Leu-Phe-Arg-Gln-Phe-^{155}Tyr
   5) ^{165}Tyr-Tyr-Val-Pro-Leu-Gly-Thr-Gln-Tyr-Thr-Asp-Ala-Pro-Ser-Phe-Ser-Asp-^{182}Ile
2. β-Casein
   1) ^{1}Arg-Glu-Leu-Glu-Glu-Leu-Asn-Val-Pro-Gly-Glu-Ile-Val-Glu-Ser-Leu-Ser-Ser-Ser-Glu-Glu-Ser-Ile-Thr-^{25}Arg (p, p, p, p)
   2) ^{26}Ile-Asn-Lys-Lys-Ile-Glu-Lys-Phe-Gln-Ser-Glu-Glu-Gln-Gln-Gln-Thr-Glu-Asp-Glu-Leu-Gln-Asp-Lys-Ile-His-Pro-Phe-Ala-Gln-Thr-Gln-Ser-Leu-Val-^{60}Tyr (p)
   3) ^{94}Gly-Val-Ser-Lys-Val-Lys-Glu-Ala-^{102}Met
   4) ^{103}Ala-Pro-Lys-His-Lys-Glu-^{109}Met
   5) ^{125}Leu-Thr-Asp-Val-Glu-Asn-Leu-His-Leu-^{136}Pro
   6) ^{157}Phe-Pro-Pro-Gln-Ser-Val-Leu-Ser-Leu-Ser-Gln-Ser-Lys-Val-Leu-Pro-Val-Pro-Glu-Lys-Ala-Val-Pro-Tyr-Pro-Gln-Arg-Asp-^{185}Met
3. β-Lactoglobulin (Lac-LG)
   1) ^{41}Val-Tyr-Val-Glu-Glu-Leu-Lys-Pro-Thr-Pro-Glu-Gly-Asp-Leu-Glu-Ile-Leu-Leu-Gln-^{60}Lys
   2) ^{55}Glu-Ile-Leu-Leu-Gln-Lys-Trp-Glu-Asn-Asp-^{65}Glu
   3) ^{125}Thr-Pro-Glu-Val-Asp-Asp-Glu-Ala-Leu-Glu-Lys-Phe-Asp-Lys-Ala-Leu-Lys-Ala-Leu-Pro-^{145}Met

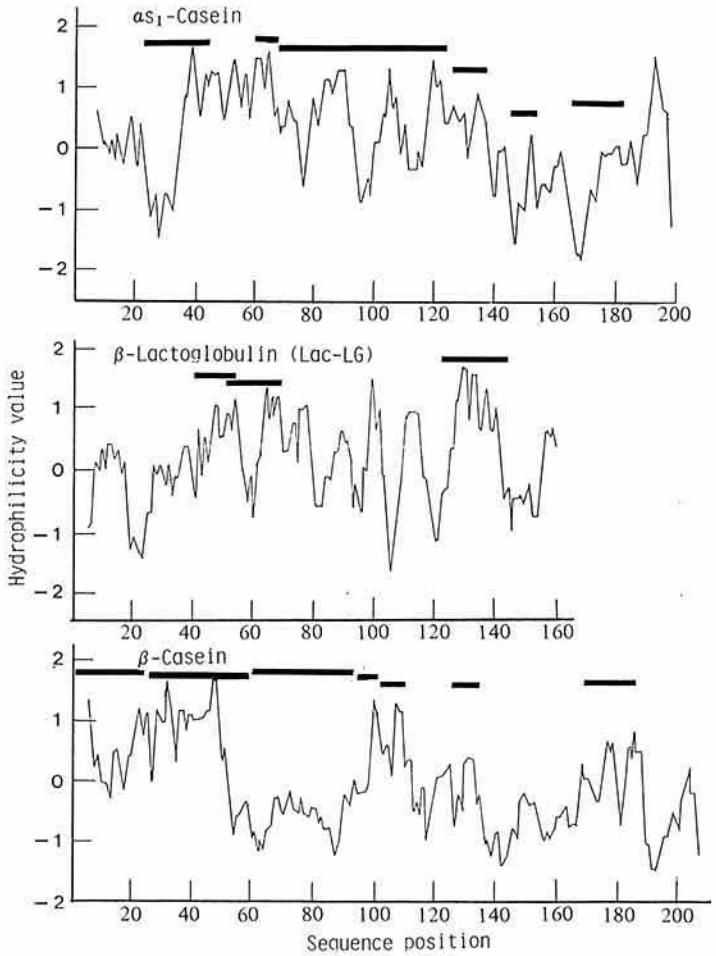

Fig. 2. Hexapeptide profiles and positions of antigenically active peptides (—) of bovine milk proteins

milk products may be different from that of β-lactoglobulin in raw milk.

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