Antigenicity of Modified β -Lactoglobulin Examined by Three Different Assays

Junichi KURISAKI, Shuji NAKAMURA, Shuichi KAMINOGAWA,
Kunio YAMAUCHI, Soichiro WATANABE,
Keiko HOTTA and Makoto HATTORI

*Department of Agricultural Chemistry, The University of Tokyo,
Bunkyo-ku, Tokyo 113, Japan*

Received November 6, 1984

The effects of chemical modifications on the antigenic properties of bovine β -lactoglobulin was studied by three different assay systems with IgG antibodies; immuno-double diffusion, quantitative immunoprecipitin assay, and radioimmunoassay. Carboxymethylation, succinylation, and guanidination seemed not to disturb the antigenicity of β -lactoglobulin in immuno-double diffusion, but the decrease in the affinity of these derivatives for anti- β -lactoglobulin antibody was observed in the quantitative immunoprecipitin assay and radioimmunoassay. Nitrophenylsulfenylation or reduction and carboxymethylation seemed to delete one of the antigenic sites of β -lactoglobulin judging from the data of immuno-double diffusion, but the results from the quantitative immunoprecipitin assay and radioimmunoassay showed that reduction and carboxymethylation affected the antigenicity of β -lactoglobulin more strongly than nitrophenylsulfenylation did.

Attassi and his colleagues¹⁾ have studied the antigenic structure of sperm whale myoglobin and chicken egg white lysozyme intensively. Their results for these proteins suggested that antigenic determinants are composed of four to eight amino acid residues and that two types of determinant exist, "continuous" and "discontinuous."²⁾ The antigenic determinants are located at or near the corners of the folded peptide chains. Accumulation of knowledge of antigenic structures is necessary for formulating the antigenicity of proteins. It will be also useful to know how to decrease the antigenicity of allergens.

Beta-lactoglobulin (β -Lg) is a main allergen in cow's milk allergy.^{3~5)} Several studies have been reported on the antigenic structure of β -Lg.^{6~8)} In our previous study,⁸⁾ the antigenic activity was measured with IgE antibodies. The results showed that the antigenic structures were susceptible to proteinase digestion and were maintained by disulfide bonds. It was also shown that one sulfhydryl group, two of the three arginine residues, two tryptophan

residues, and most of the amino groups were not involved in the antigenic site. We proceeded with the study using immuno-double diffusion, quantitative immunoprecipitin assay, and radioimmunoassay with IgG antibodies. In this paper, we describe the antigenic properties of β -Lg detected by IgG antibodies.

MATERIALS AND METHODS

Preparation of β -lactoglobulin. Fresh milk from a Holstein cow of the genotype AA for β -Lg was supplied from the National Institute of Animal Industry (Ibaraki, Japan). β -Lg A was prepared by the method of Aschaffenburg and Drewry,⁹⁾ and purified by gel filtration on Sephadex G-75 and by ion-exchange chromatography on DEAE Sephadex A-50.

Antisera. Rabbit anti- β -Lg antibody were obtained by immunizing rabbits. One to two hundred micrograms of purified β -Lg in phosphate buffer saline were emulsified with an equal volume of Freund's Incomplete Adjuvant and injected in the back skin of the animals. A booster injection was given 2 weeks later in the same way. Goat anti-rabbit antibody was purchased from Eiken Kagaku, Tokyo.

Chemical modification of β -lactoglobulin. The methods of chemical modification have been previously described in detail.⁸⁾ The sulfhydryl groups were carboxymethylated with (RCM- β -Lg) or without (CM- β -Lg) reduction. Amino groups were succinylated with solid succinic anhydride¹⁰⁾ (Su- β -Lg). Another modification of amino groups was guanidination with 1-guanyl-3,5-dimethyl pyrazole-1-carboxy nitrate (Gu- β -Lg).¹¹⁾ Tryptophan residues were modified with nitrophenylsulphenylchloride (NPS- β -Lg).¹²⁾ RCM-, CM- and NPS- β -Lg were hydrolyzed and applied to an amino acid analyzer to estimate the number of modified residues. The data are shown in Table I. The number of carboxymethylated residues was estimated by the determination of S-carboxymethyl-L-cysteine. The number of tryptophan residues modified was estimated from the decrease in the number of residues after modification. We have already confirmed that there are no unmodified β -Lg molecules left after modification of amino groups, by disc electrophoresis.⁸⁾ The effects of the modification on the conformation of β -Lg were checked by CD spectrum and the results were described in our previous paper.⁸⁾

Immuno-double diffusion. Immuno-double diffusion was done on 1% agarose gel plates in a 0.15 M NaCl solution using rabbit anti- β -Lg antibody.

Quantitative precipitin assay. The quantitative precipitin assay was done as described by Garvey.¹³⁾ Equal volumes of a rabbit anti- β -Lg antiserum (1:4 dilution) and antigen solutions which were seriously diluted in borate buffer saline (pH 8.4) were mixed and incubated at 37°C for 1 hr, then at 4°C for another 46~68 hr. Precipitates were centrifuged and washed with the buffer. After drying, 2% sodium dodecyl sulfate solution was added to solubilize the precipitate and the protein content in the precipitate was determined by Lowry's method.¹⁴⁾

Radioimmunoassay. β -Lg was labelled with ¹²⁵I using chloramine T. One hundred microliters of rabbit anti- β -Lg antibody, 200 μ l of serial dilutions of modified β -Lg, and 100 μ l of ¹²⁵I- β -Lg were incubated at 37°C for 20 hr. Normal rabbit serum and goat anti-rabbit antibody were

added to this reaction mixture and incubated for another 24 hr. Precipitates were separated by centrifugation and the radioactivity of the precipitates or supernatants were measured by an autowell type γ -counter.

RESULTS

Immuno-double diffusion

Precipitin lines formed by diffusion of rabbit anti- β -Lg antiserum and modified β -Lg are shown in Fig. 1. Native β -Lg formed one precipitin line with the antiserum. All of three modified antigens, CM-, Gu- and Su- β -Lg, also formed one precipitin line each that completely fused with the native β -lactoglobulin line. On the other hand, the reaction of NPS- and RCM- β -Lg with antiserum gave one broad precipitin line over which native β -Lg line spurred.

Quantitative immunoprecipitin assay

To estimate the number of antigen binding sites of β -Lg, we determined the valency of β -Lg by the quantitative immunoprecipitin method. The valency of the antigen is the ratio of antibody to antigen molecules at extreme antibody excess where every antigenic determinant binds a separate antibody molecule. The intercept of the line with the ordinate showed that the value of the valency of β -Lg was 4 (Fig. 2). This value was identical with the value at pH 6.0 where β -Lg exists as a dimer. This implies that the intramolecular binding site of β -Lg is not concerned in the antigen binding site.

Quantitative immunoprecipitin curves of modified β -Lg with antibody are shown in Fig. 3. The reaction between native β -Lg and antiserum gave 914 μ g of maximal precipitate at 31.5 μ g of antigen. Carboxymethylation, succinylation and guanidination decreased the amount of maximal precipitate to 88%, 93%, and 88%, respectively, and shifted the equivalent point to the right. Nitrophenylsulfenylation also influenced the ability to form a precipitate and had 79% of the reactivity at the maximal point. The most drastic changes could be seen with reduction and carboxymethylation. We used two types of RCM- β -

TABLE I. NUMBER OF MODIFIED RESIDUES

	Modified residue	Number of modified residues	Number of residues in intact β -Lg
CM- β -Lg	-SH	1.0	1
RCM- β -Lg (1)	-SH & S-S	4.2	5
(2)		5.0	5
NPS- β -Lg	Trp	1.7	2

No intact β -Lg was found in Su- and Gu- β -Lg samples.³⁾ We used two types of RCM- β -Lg, 84% modified and 100% modified.

FIG. 1. Immuno-double Diffusion Patterns of Modified β -Lactoglobulins.

Rabbit anti- β -lactoglobulin sera were in the center wells. 1, β -lactoglobulin; 2, carboxymethylated β -lactoglobulin; 3, guanidinated β -lactoglobulin; 4, succinylated β -lactoglobulin; 5, reduced and carboxymethylated β -lactoglobulin (84% modified); 6, reduced and carboxymethylated β -lactoglobulin (100% modified); 7, nitrophenylsulfenylated β -lactoglobulin.

FIG. 2. Determination of Valency of β -Lactoglobulin by Quantitative Immunoprecipitin Method.

The amount of precipitated antibody/amount of added β -lactoglobulin was plotted against the added β -lactoglobulin. The reaction was done at pH 8.5 (○) or pH 6.0 (△).

Lg, an 84% modified one and 100% modified one. They revealed almost the same patterns and only formed less than 30% of precipitate in comparison with native β -Lg in each case.

Radioimmunoassay

Inhibition of the reaction between native β -Lg and rabbit anti- β -Lg antibody by modified β -Lg is represented in Fig. 4. The ordinate B/B_0 is the ratio of bound radiolabeled β -Lg to antibody with competitor against without competitor. The abscissa is the amount of competitors plotted in logarithms. All binding curves became reversed sigmoidal curves with this plotting method. The addition of native β -Lg as the competitor gave a standard curve.

All modified β -Lg could completely substitute for native β -Lg, but their curves shifted to the right in comparison to native β -Lg.

It has been known that the amount of antigen at the point where $B/B_0 = 1/2$, is related to the affinity of antigen with antibody. So we used this as an index (I_A) to compare the affinity of modified β -Lg with that of the native one. I_A of native β -Lg was 6.3×10^{-4} $\mu\text{g}/\text{tube}$. Carboxymethylation, succinylation, and guanidination caused decreases of the affinity and I_A decreased to 1.4×10^{-2} , 2.0×10^{-2} , and 2.8×10^{-2} $\mu\text{g}/\text{tube}$, respectively. I_A of RCM- β -Lg (2.0×10 $\mu\text{g}/\text{tube}$) was lowest of all derivatives. I_A of NPS- β -Lg (4.0×10^{-1} $\mu\text{g}/\text{tube}$) was moderate.

DISCUSSION

Chemical modification provides us with useful information about the antigenic properties of proteins. The cysteine and cystine residues of β -Lg play an important role in the conformation and physicochemical reactions.¹⁵⁾ Amino groups have a tendency to be located on the surface of the molecule, so we suspected some amino groups might constitute the antigenic sites. Therefore, we modified the above residues to study the effects on the antigenic property of β -Lg. We also modified tryptophan residues because there are only two residues in β -Lg and so it should be easy to

FIG. 3. Quantitative Precipitin Curves of Modified β -Lactoglobulin.

A: $\circ$ — $\circ$, β -lactoglobulin; $\bullet$ --- $\bullet$, carboxymethylated β -lactoglobulin; $\bullet$ — $\bullet$, reduced and carboxymethylated β -lactoglobulin (84% modified); $\blacktriangle$ — $\blacktriangle$, reduced and carboxymethylated β -lactoglobulin (100% modified). B: $\circ$ — $\circ$, β -lactoglobulin; $\triangle$ — $\triangle$, succinylated β -lactoglobulin; $\triangle$ --- $\triangle$, guanidinated β -lactoglobulin; $\circ$ ···· $\circ$, nitrophenylsulphenylated β -lactoglobulin.

FIG. 4. Competitive Reaction for Anti- β -Lactoglobulin Antibody between ^{125}I Labelled β -Lactoglobulin and Modified β -Lactoglobulin.

$\circ$ — $\circ$, β -lactoglobulin; $\bullet$ --- $\bullet$, carboxymethylated β -lactoglobulin; $\bullet$ — $\bullet$, reduced and carboxymethylated β -lactoglobulin; $\triangle$ — $\triangle$, succinylated β -lactoglobulin; $\triangle$ --- $\triangle$, guanidinated β -lactoglobulin; $\circ$ --- $\circ$, nitrophenylsulphenylated β -lactoglobulin.

find the sites of modification.

CM-, Su-, and Gu- β -Lg were shown to keep the antigenic sites from the data that they completely fused with native β -Lg in immunodouble diffusion. However, radioimmunoassay showed that their binding affinities were lowered several hundreds of times in comparison to native one. The decrease of affinity can account for the decrease of maximal precipitate and the shift of the equivalent point of modified β -Lg in quantitative

immunoprecipitin assay.

Carboxymethylation after reduction and nitrosulphenylation seemed to delete one of the antigenic sites from the results of immunodouble diffusion. However, the quantitative immunoprecipitin assay and radioimmunoassay showed the difference between NPS- β -Lg and RCM- β -Lg. Reduction and carboxymethylation caused a more drastic change in antigenicity than nitrophenylsulphenylation did. The reactivity of RCM- β -Lg with the antibody in the quantitative immunoprecipitin assay was reduced to 24% of the native one, while NPS- β -Lg kept 79% of its reactivity. Moreover, the binding affinity of RCM- β -Lg to antibody was lowered less than 1/30,000 as compared with native β -Lg while that of NPS- β -Lg was about 1/700.

In the passive cutaneous anaphylaxis (PCA) reaction by means of IgE antibody, we concluded that NPS- β -Lg retained the antigenic reactivity to mouse IgE antibody against native protein. In this study, however, we observed that nitrophenylsulphenylation affected the antigenicity of β -Lg to some extent. We consider that the PCA assay system was not sensitive enough to detect the effects of nitrophenylsulphenylation on the antigenic properties of β -Lg.

It seems desirable to discuss the structure of antigenicity of proteins taking the whole data obtained by plural immunological methods into consideration.

REFERENCES

- 1) M. Z. Atassi, "Immunochemistry of proteins," Vol. 2, ed. by M. Z. Atassi, Plenum, New York, 1977, pp. 77~264.
- 2) M. Z. Atassi and J. A. Smith, *Immunochemistry*, **15**, 609 (1978).
- 3) A. S. Goldman, D. W. Anderson, Jr., W. A. Sellars, Saperstein, W. T. Kniker and S. R. Halpern, *Pediatrics*, **32**, 425 (1963).
- 4) C. Collins-Williams, *Int. Arch. Allergy*, **20**, 38 (1962).
- 5) A. S. Goldman, W. A. Sellars, S. R. Halpern and D. W. Anderson, Jr., *Pediatrics*, **32**, 572 (1963).
- 6) H. Bing and A. B. Stavisky, *Immunology*, **15**, 305 (1968).
- 7) H. Otani and F. Tokita, *Jpn. J. Zootech. Sci.*, **50**, 623 (1979).
- 8) J. Kurisaki, S. Nakamura, S. Kaminogawa and K. Yamauchi, *Agric. Biol. Chem.*, **46**, 2069 (1982).
- 9) R. Aschaffenburg and J. Drewry, *Biochem J.*, **65**, 273 (1957).
- 10) M. H. Klapper and I. M. Klotz, "Methods in Enzymology," Vol. 25, ed. by C. H. W. Hirs and S. N. Timasheff, Academic Press, New York, 1972, p. 531.
- 11) A. F. S. A. Habeeb, "Methods in Enzymology," Vol. 25, ed. by C. H. W. Hirs and S. N. Timasheff, Academic Press, New York, 1972, p. 558.
- 12) E. Scoffone, A. Fontana and R. Rocchi, *Biochemistry*, **7**, 971 (1968).
- 13) J. S. Garvey, N. E. Cremer and D. H. Sussdorf, "Methods in Immunology," 3rd Ed., W. A. Benjamin, Inc., Reading, 1977, p. 273.
- 14) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
- 15) H. A. McKenzie, G. B. Ralston and D. C. Shaw, *Biochemistry*, **11**, 4539 (1972).