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Rapid Screening of Antigenically Reactive Fragments of αs_1-Casein Using HPLC and ELISA

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Screening of antigenically reactive fragments of αs_1-casein (αs_1-CN), the major casein in bovine milk, was done by using HPLC and enzyme-linked immunosorbent assay (ELISA). BALB/c mice (6-week-old) were injected intraperitoneally with αs_1-CN and complete Freund's adjuvant, and 14 days later, all the mice were boosted with αs_1-CN and incomplete Freund's adjuvant. Twenty-one days after the 1st immunization, the mice were bled and antiserum was separated. Anti αs_1-CN antibody fraction was obtained by precipitation from the antiserum with 50% saturated ammonium sulfate. αs_1-CN was digested with trypsin and chymotrypsin, and 35 peptides were purified from the digests by reversed-phase HPLC with ODS (octadecylsilica) columns. Reactivity of peptides with the antibody were examined by ELISA. The solid phase in the wells of the polystyrene microtiter plate was coated with peptides, and the plate was successively incubated with anti αs_1-CN antibody, conjugate of anti mouse immunoglobulin with alkaline phosphatase (ALP) and substrate of ALP. Two tryptic fragments (the residues 104–119 and 133–151) and three chymotryptic fragments (33–54, 105–121, and 174–199) were positive in an ELISA test. These five fragments would correspond to four antigenic sites. We could thus find antigenically reactive fragments of αs_1-CN by the direct and simple detection of specific antigen-antibody interaction.

The antiserum against a protein antigen contains a group of antibodies which can specifically bind the protein antigen at some definite positions, the antigenic determinants. It takes many years to complete studies on the antigenic structures of a protein, *e.g.*, 11 years have been required in the case of myoglobin (*1*). So far, the antigenic residues in only a small number of proteins have been determined (*2*). The investigation of antigenic structures is expected to be useful in connection with studies of protein-protein interaction, protein folding, and topographical structure (*3–6*), as well as vaccine synthesis, detecting gene products and isolating proteins (*7*). Although there have been recent advances in protein chemistry, it remains difficult to determine antigenic residues with any precision. Therefore, methods to determine the

Abbreviations: ELISA, enzyme-linked immunosorbent assay; αs_1-CN, αs_1-casein; PBS, phosphate-buffered saline; ODS, octadecylsilica; ALP, alkaline phosphatase; Ig, immunoglobulin.

antigenic structures of many proteins simply and rapidly should be developed. We attempted to identify antigenically reactive peptides of bovine αs_1-casein (αs_1-CN), since caseins in bovine milk are known to have high allergenicity (*8*). αs_1-CN, which is the major casein in bovine milk and is absent in human milk, may be one of the main allergens. Although studies on the antigenic structures of αs_1-CN have already been started (*9–11*), the antigenic determinants have still not been revealed in detail. We describe here the identification of four antigenically reactive regions as a result of the separation of proteolytic peptides by reversed-phase high-performance liquid chromatography (reversed-phase HPLC) and enzyme-linked immunosorbent assay (ELISA). A smaller amount of protein antigen, and less time and effort are necessary with our method than with other methods such as an assay using immunoprecipitation or synthesized peptides. Our method can detect specific antigen-antibody interaction directly and simply.

MATERIALS AND METHODS

Preparation of Peptides Derived from αs_1-Casein by Proteinases—Bovine αs_1-casein-B was prepared according to the method of Zittle *et al.* (*12*) and purified by column chromatography on DEAE-Cellulofine AM (Seikagaku Kogyo Co., Ltd.). A solution of 10 mg of αs_1-CN in 0.1 M NH_4OH/HCOOH buffer (pH 8.5) at a concentration of 0.2% was treated with 0.1 mg of L-(1-tosylamide-2-phenyl)ethyl chloromethyl ketone-treated trypsin (Cooper Biomedical). Further, 10 mg of αs_1-CN dissolved in 0.1 M NH_4OH/HCOOH buffer (pH 8.0) at a concentration of 0.2% was treated with 0.1 mg of *N-p*-tosyl-L-lysine chloromethyl ketone-treated chymotrypsin (Sigma Chemical Co.). Both solutions were incubated for 24 h at 37°C.

Each digest was subjected to HPLC using LiChrosorb RP-18 (Merck), Vydac C18 (the Separations Group), and Finepak SIL C18 (Japan Spectroscopic Co., Ltd.). The amino acid composition of each peptide purified by HPLC was analyzed after acid hydrolysis.

Preparation of Mouse Anti-αs_1-CN Antibody—Six-week-old female mice (BALB/c, Charles River Japan) were injected intraperitoneally with 100 μg of αs_1-CN as a 100 μl of emulsion consisting of equal volumes of protein solution dissolved in 0.11 M phosphate buffer (pH 7.1) containing 0.04 M NaCl (PBS) and complete Freund's adjuvant (Difco Laboratories). Fourteen days later, all the mice were boosted with 100 μg of αs_1-CN as an emulsion in incomplete Freund's adjuvant (Difco Laboratories). Twenty-one days after the first immunization, all the mice were bled from the tail and the antisera were separated and pooled. Portions of 1 ml of the antiserum were each mixed with 1 ml of saturated ammonium sulfate solution. The precipitates were dissolved in 1 ml of PBS and the solution was dialyzed against PBS.

ELISA—The peptides were dissolved in 500 μl of PBS. One hundred microliters of the antigen solution was added to each well of a Petra plastic microtiter plate (Sanko Junyaku Co., Ltd.), and the plate was incubated for 2 h at room temperature. After removal of the antigen solution, the wells were washed three times with 125 μl of PBS containing 0.05% Tween-20 (PBS-Tween). Then 100 μl of the antibody diluted 100 times with PBS-Tween was added, and the plate incubated for 2 h. Following removal of the solution and triple washing, 100 μl of bovine intestinal mucosa alkaline phosphatase (Sigma)-labeled goat anti-mouse Ig (Cappel) diluted with PBS-Tween was added, and the plate incubated for another 2 h. After removal of the solution and washing, 100 μl of a solution of *p*-nitrophenylphosphate, disodium salt in 1 M diethanolamine-HCl buffer (pH 9.8) containing 0.01% $MgCl_2 \cdot 6H_2O$, and 0.02% NaN_3 was added. Thirty minutes later, the reaction was stopped by adding 20 μl of 6 M NaOH and the absorbance of each well was determined at 405 nm.

RESULTS

Purification of Fragments of αs_1-CN—αs_1-CN was exhaustively digested by trypsin or chymotrypsin, and the digests were fractionated by HPLC using an ODS column of LiChrosorb RP-18 (Figs. 1 and 2). Each fraction was chromatographed on the Vydac C18 column and subjected to amino acid analysis. The amino acid compositions of the peptides in each fraction were compared with those of fragments which were expected to be derived from the αs_1-CN sequence by considering

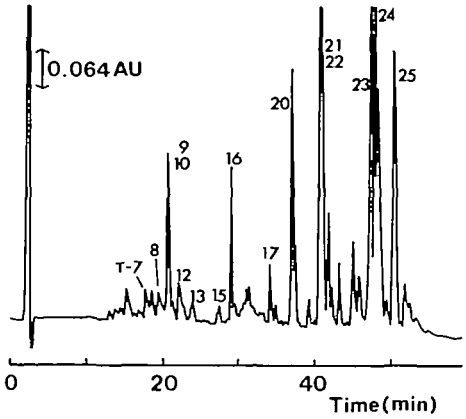

Fig. 1. The first step of purification of tryptic peptides by HPLC. One milligram of peptides derived from αs_1-CN was applied per run. Mobile phase, 0.1% trifluoroacetic acid/95% CH_3CN; column, LiChrosorb RP-18 (4.6 mm i.d. × 25 cm); detection, ultraviolet absorbance at 230 nm. After rechromatography with Vydac C18, peptides were identified by amino acid analysis (see Fig. 3).

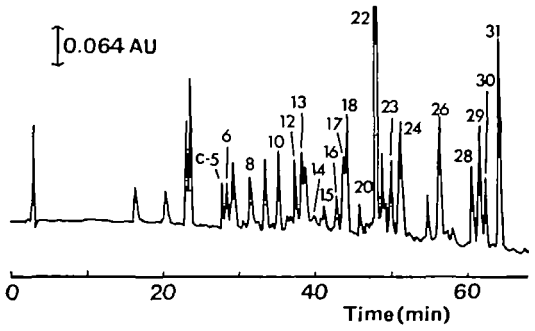

Fig. 2. The first step of purification of chymotryptic peptides by HPLC. Two milligrams of peptides derived from αs_1-CN was applied per run. Mobile phase, 0.1% trifluoroacetic acid/95% CH_3CN; column, LiChrosorb RP-18; detection, ultraviolet absorbance at 230 nm. Peptides C-23 and C-24 (rechromatographed with Vydac C18 and Finepak C18) and other peptides (rechromatographed with Vydac C18) were identified by amino acid analysis (see Fig. 3).

the substrate specificities of the proteinases. The amino acid analyses showed that the fractions corresponding to the peaks of C-23 and C-24 were not pure, and consequently rechromatography for further purification was run on Finepak SIL C18. Thirty-five peptides were purified. The amount

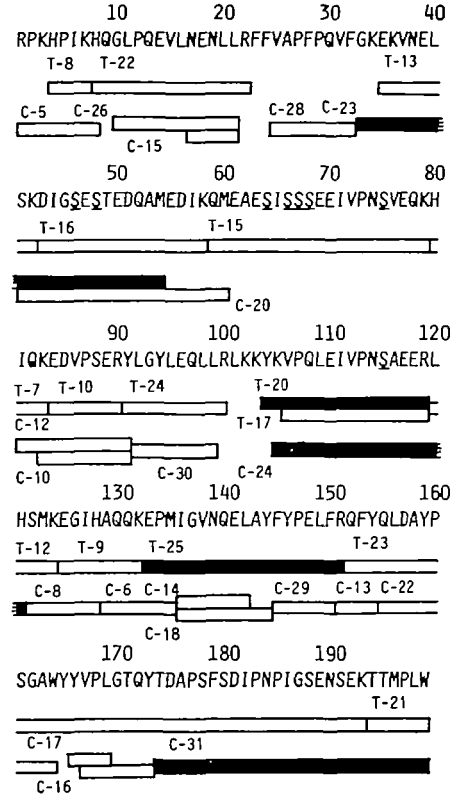

Fig. 3. Primary structure of αs_1-CN and location of peptides tested for antigenic reactivities. White and black squares show peptides negative and positive for reaction with anti αs_1-CN antibody by ELISA, respectively. "S" indicates a phosphoserine residue.

of each peptide obtained from 10 mg of αs_1-CN ranged from 10 to 200 μg. The identities of the peptides are shown in Fig. 3.

ELISA—The solid phase in the wells of the microtiter plate was coated with peptides dissolved at a concentration of 10 to 100 μg/ml in PBS. An ovalbumin solution in PBS was added to the wells for blocking after washing the peptide-coated wells, and the plate was successively incubated with the antibody, conjugate of anti-Ig with alkaline phosphatase (ALP) and substrate of ALP. In this experiment, absorbance data which were expected to show the same value varied widely from well to well and the reproducibility was poor. If the antibody solution diluted with PBS was added to uncoated wells, color was developed in the wells at the last stage of ELISA. The control experi-

ment, in which the antibody solution was diluted with PBS-Tween and added to the uncoated wells, resulted in no coloration. This indicates that in the case of no antigen coating and no blocking, the antibody in PBS was adsorbed on the solid phase, but that the antibody in PBS-Tween was not adsorbed. Reliable data were obtained by a procedure which involved incubation with an antibody solution diluted 100 times with PBS-Tween without blocking after the peptide coating. Thirty-five peptides derived from αs_1-CN were examined for reactivity with the antibody in mouse sera. No coloration was observed in wells coated with any peptide and αs_1-CN in an ELISA test using sera before immunization. Two tryptic fragments (T-20 (residues 104–119) and T-25 (133–151)), and three chymotryptic fragments (C-23 (33–54), C-24 (105–121), and C-31 (174–199)) were positive in an ELISA test (Fig. 3) using anti-αs_1-CN antibody solution. The absorbance of the well coated with αs_1-CN at 405 nm was outside the scale range, and those of these five peptides ranged from 0.1 to 0.5. The ELISA in which αs_1-CN was added to the antibody solution showed that bindings of anti-αs_1-CN antibody with αs_1-CN and these five peptides coated to the wells were inhibited by αs_1-CN dissolved in the solution.

DISCUSSION

Proteolytic fragments were purified by HPLC. The amino acid analysis showed that purification on two or three columns could exclude peptide contaminants. One tryptic fragment of ^{23}Phe-^{34}Lys and some chymotryptic fragments were missing in the elution system of trifluoroacetic acid/CH_3CN and LiChrosorb RP-18. To avoid loss of these peptides, another elution system and/or column could be adopted.

The proteolytic fragments purified by HPLC were coated on the solid phase in the wells of microtiter plates. Blocking by a method such as incubating with ovalbumin solution is generally done after antigen coating, but blocking was unnecessary to obtain reliable data in our ELISA procedure, for peptide antigens containing about ten to twenty residues. When the plate was incubated with the ovalbumin solution, the ovalbumin was presumed to have been adsorbed on peptide-free regions of the solid phase and to have influenced the specific interaction between the antigen and the antibody. The antibody was diluted with PBS containing the nonionic detergent, Tween-20, which is known to inhibit nonspecific interaction between the solid phase and immunoglobulin (*13*).

Our results by ELISA revealed some of the antigenic structures of αs_1-CN. The antibody against native αs_1-CN reacted specifically with five peptides derived from native αs_1-CN by protease treatment and adsorbed on the solid phase. Those peptides that were negative in the ELISA test either were not adsorbed on the solid phase or did not contain antigenic determinants. The sequences of some peptides which were negative in the ELISA test overlap with those of the peptides which were positive (Fig. 3). T-8, T-21, and C-15 are regarded as not being adsorbed on the solid phase, whereas T-23 is regarded as not containing a whole antigenic determinant. The C-terminal residues of T-23 seem to be significant for the antigenic determinant. If T-20 and C-24 are considered to contain the same antigenic sites, the five fragments would correspond to four antigenic sites, although there is a possibility that other antigenic sites exist. Louvard *et al.* found a linear relationship between the logarithm of molecular weight of globular proteins and the logarithm of the number of antigenic determinants (*14*). From their relationship and the molecular weight, the number of determinants of αs_1-CN was estimated to be four. In the present experiment, 20 mg of αs_1-CN was hydrolyzed for ELISA, but the experiment could be performed with a smaller amount of protein antigen. This procedure using HPLC and ELISA should be applicable to identify the antigenic regions in other proteins.

Otani *et al.* have studied the localization of the antigenic determinants of αs_1-CN (*9–11*). They found one antigenic site in each region of residues 1 to 54, 124 to 135, and 136 to 196, and two sites in the region of 61 to 123, using six fragments obtained by cyanogen bromide treatment and separated by open column chromatography. The positions of these sites were thus defined rather broadly, and a more detailed study is required to clarify whether their results agree precisely with ours. They used outbred rabbit antiserum and mainly employed a quantitative immunoprecipitation inhibition test. We used inbred mouse antiserum and detected binding of the antibody to peptides

by using ELISA with direct coating of the peptides. At present, the reason for the differences between the results is not clear. There have also been differences between the results of studies on myoglobin antigenic determinants by Atassi and Twining (*1*, *15*) and by Rodda *et al.* (*16*). Atassi and Twining used inhibition of immunoprecipitation and absorption of the antibody, whereas Rodda *et al.* used the ELISA procedure, in which the peptides were still bound to the solid phase used for peptide synthesis. Rodda *et al.* suggested that the method of Atassi may detect low-affinity interactions which occur under conditions of peptide excess.

The molecular nature of the antigenic structure of proteins, especially globular proteins having a compact structure, has been widely discussed (*17–20*), but without general agreement. Two kinds of predictions of antigenic determinants from amino acid sequences of globular proteins have been applied to αs_1-CN: one was by Welling *et al.* (*2*) and the other was by Hopp and Woods (*21*). The former prediction was that T-20 (C-24) contained short regions with relatively high values, but that T-25 and C-31 did not contain such regions. The latter prediction was that C-23, T-20 (C-24), and C-31 contained regions with high antigenicity values, but that T-25 did not contain such regions. The antigenic regions thus predicted do not coincide with all the positions of the antigenic determinants that we have deduced from the ELISA results. It is known that αs_1-CN has a rather unstable structure, which may approach a random coil behavior (*22*). Elucidation of the antigenic structures of αs_1-CN with precision may give a new insight into the nature of protein antigenicity.

REFERENCES

1. Atassi, M.Z. (1975) *Immunochemistry* **12**, 423–438
2. Welling, G.W., Weijer, W.J., van der Zee, R., & Welling-Wester, S. (1985) *FEBS Lett.* **188**, 215–218
3. Mendz, G.L. & Moore, W.J. (1985) *Biochem. J.* **228**, 305–313
4. Tainer, J.A., Getzoff, E.D., Alexander, H., Houghten, R.A., Olson, A.J., Lerner, R.A., & Hendrickson, W.A. (1984) *Nature* **312**, 127–134
5. Ghelis, C. & Yon, J. (1982) *Protein Folding* pp. 419–446, Academic Press, New York
6. Louvard, D., Maroux, S., & Desnuelle, P. (1975) *Biochim. Biophys. Acta* **389**, 389–400
7. Lerner, R.A. (1982) *Nature* **299**, 592–596
8. Bahna, S.L. & Heiner, D.C. (1980) *Allergies to Milk* pp. 11–22, Grune & Stratton, New York
9. Otani, H., Takayama, K., & Tokita, F. (1985) *Milchwissenschaft* **40**, 24–26
10. Otani, H., Takayama, K., & Tokita, F. (1985) *Milchwissenschaft* **40**, 69–71
11. Otani, H., Takahashi, F., & Tokita, F. (1986) *Agric. Biol. Chem.* **50**, 607–613
12. Zittle, C.A., Cerbulis, J., Pepper, L., & Della Monica, E.S. (1959) *J. Dairy Sci.* **42**, 1897–1902
13. Engvall, E. (1980) *Methods Enzymol.* **70**, 419–439
14. Louvard, D., Vannier, C., Maroux, S., Pages, J.-M., & Lazdunski, C. (1976) *Anal. Biochem.* **76**, 83–94
15. Twining, S.S. & Atassi, M.Z. (1980) *Biochem. J.* **191**, 681–697
16. Rodda, S.J., Geysen, H.M., Mason, T.J., & Schoofs, P.G. (1986) *Mol. Immunol.* **23**, 603–610
17. Atassi, M.Z. (1984) *Eur. J. Biochem.* **145**, 1–20
18. Berzofsky, J.A. (1985) *Science* **229**, 932–940
19. Van Regenmortel, M.H.V. (1986) *Trends Biochem. Sci.* **11**, 36–39
20. Novotony, J., Handschumacher, M., & Haber, E. (1986) *J. Mol. Biol.* **189**, 715–721
21. Hopp, T.P. & Woods, K.R. (1983) *Mol. Immunol.* **20**, 483–489
22. Swaisgood, H.E. (1982) in *Developments in Dairy Chemistry-1* (Fox, P.F., ed.) pp. 1–59, Applied Science Publishers, Barking