Similarity between γ -Casein and a Product of β -Casein Hydrolysis by Milk Protease

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A γ -casein-like fraction (FIV) was isolated from the β -casein A hydrolyzate by milk protease and compared with γ -casein. The mobility in polyacrylamide gel electrophoresis, sedimentation coefficient, molecular weight, amino acid composition and terminal amino acids of FIV were almost coincided with those of γ -casein. It is suggested that γ -casein is possibly a product of β -casein hydrolysis by milk protease.

γ -Casein is one of the minor components of bovine casein. The term, γ -casein, was first introduced by Mellander¹⁾ to designate a component of bovine casein which migrated more slowly than the major components, α - and β -caseins, in moving-boundary electrophoresis.

Subsequently, methods for the preparation of γ -casein were worked out, and its properties and composition were reported.^{2,3)} Later, Groves *et al.*⁴⁾ showed that γ -casein prepared by the method of Hipp *et al.*^{2,3)} was in likelihood a mixture of proteins by starch gel electrophoresis and that TS-, R-, and S- caseins were presented in the mixture with γ -casein. The characterization of these proteins was described in the review by Groves.⁵⁾

Recently, we suggested that TS- and R-caseins were possibly derived from β -casein by the hydrolysis with milk protease.⁶⁾ The subsequent studies have been carried out as to the origin of minor casein components and a γ -casein-like fraction was found to appear in the hydrolyzate of β -casein by milk protease. In the present report, similarity between γ -casein and the γ -casein-like fraction from the β -casein hydrolyzate by milk protease will be described.

MATERIALS AND METHODS

Caseins. β -Casein A was prepared by the urea method of Hipp *et al.*²⁾ from skim milk of a Holstein Cow. A casein fraction containing γ -casein A, TS-

casein A and R-casein was prepared according to the method of Groves *et al.*⁴⁾ from the same milk and designated MC- fraction.

Milk protease. Milk protease was separated from unfractionated casein by using dimethylformamide according to the method previously reported.⁷⁾ The specific activity of this preparation was approximately 70-fold compared with that of the original unfractionated casein.

Column chromatography on DEAE-cellulose. The method of Thompson and Kiddy⁸⁾ and Ribadeau-Dumas,⁹⁾ using 0.01 M imidazole-hydrochloride buffer containing 3.3 M urea at pH 7.1, was employed. A DEAE-cellulose column 20 by 300 mm was prepared and NaCl gradients within the range of zero to 0.05 M were utilized.

Polyacrylamide gel electrophoresis (PAGE). PAGE in urea-Tris-EDTA buffer (pH 9.1) was performed according to the method previously reported.¹⁰⁾ PAGE in urea-formic acid-acetic acid buffer (pH 3.0) was performed according to the method of Perterson and Kopfler.¹¹⁾

Ultraviolet spectrum. Ultraviolet spectrum and difference spectrum were obtained with a Cary model 14-M spectrophotometer.

Ultracentrifugal examination. A Hitachi model UCA-IA analytical centrifuge equipped with schlieren optics was used. The sedimentation velocity experiment was performed at a rotor speed of 55, 430 rpm at 7.8 and 20°C using a wedge cell.

Molecular weight. Estimation of molecular weight by chromatography of Sepharose 6B equilibrated with 6 M guanidine hydrochloride was made by the method of Fish *et al.*¹²⁾ The molecular weight was also

estimated by gel electrophoresis in the presence of sodium dodecyl sulfate according to the method of Weber and Osborn.¹³⁾

Amino acid composition. Proteins were hydrolyzed for 20 and 70 hr and analyzed automatically for amino acid content by the procedures described by Moore and Stein.¹⁴⁾ Tryptophan analyses were made by the method of Spies.¹⁵⁾ The phosphorus content of the proteins was determined by the method of Skipski and Barclay.¹⁶⁾

N- and C-Terminal amino acid. N-Terminal residue of proteins was determined with dansyl chloride according to Gray.¹⁷⁾ Dansylated protein was hydrolyzed in a sealed evacuated tube with 6 N hydrochloride at 105°C for 4 hr. The dansyl derivative was identified by the thin-layer chromatography coated with silica gel G according to the method of Morse and Horecker.¹⁸⁾ C-Terminal amino acid was determined with carboxypeptidase A by the method of Kalan *et al.*¹⁹⁾

RESULTS

DEAE-cellulose column chromatography

Five hundred mg of β -casein A was dissolved in 12 ml of 0.02 M imidazole-hydrochloride buffer (pH 7.1). To this solution, 14 mg of milk protease preparation was added and the mixture was incubated for 24 hr at 37°C with a drop of toluene as a perservative. PAGE patterns (pH 3.0) of β -casein A subjected enzymic hydrolysis were shown in Fig. 1. Three bands (A, B, C) appeared with the elapse of incubation time. The mobilities of bands A and B were equal to those of TS- and R-casein. The components corresponding to bands A and B were characterized in the previous paper⁶⁾ and their similarity to TS- and R- casein was suggested.

The mobility of band C was equal to that of γ -casein. To separate this hydrolysis product, chromatographic fractionation of the hydrolyzate of β -casein A was made with DEAE-cellulose column after the addition of equal volume of 6.6 M urea. The elution profile was shown in Fig. 2. The β -casein A hydrolyzate by 6 hr incubation with milk protease was separated into four major fractions and they were designated FI, FII, FIII and FIV. Each fraction was dialyzed against deionized water

FIG. 1. Changes in PAGE Pattern (pH 3.0, 4.5 M Urea) of β -Casein A by Milk Protease.

FIG. 2. Chromatography on DEAE-cellulose Column of Products of β -Casein A Hydrolysis (6 hr) by Milk Protease.

—, Protein (Absorbance at 280 nm); ---, NaCl concentration (M).

and lyophilized. PAGE patterns of FII, FIII and FIV were shown in Fig. 3. FIV was purified by rechromatography, lyophilized after dialysis against deionized water and used in the comparative experiments with γ -casein A. γ -Casein A was separated from MC-fraction by the same method as FIV on DEAE-cellulose column.

Polyacrylamide gel electrophoresis (PAGE)

PAGE patterns of γ -casein A and FIV at

FIG. 3. PAGE Pattern (pH 3.0, 4.5 M Urea) of Effluent Fractions of Fig. 2.

pH 9.1 and 3.0 were presented in Fig. 4. The mobility of γ -casein A was equal to that of FIV at both pH 9.1 and 3.0.

Sedimentation velocity

The results of ultracentrifugal analysis of γ -casein A and FIV were shown in Fig. 5.

At 20°C two schlieren peaks were observed in both proteins. The sedimentation coefficients ($s_{20,w}$) of leading peaks were equally 18.2S for both proteins and those of trailing peaks were equally 0.9S.

At 7.8°C, however, only one peak was ob-

FIG. 4. PAGE Pattern (pH 9.1 and 3.0, 4.5 M Urea) of γ -Casein A and FIV.

(1), (3); γ -Casein A. (2), (4); FIV.

served for γ -casein A and FIV. Their sedimentation coefficients were equally 0.9 and this value was equal to that of the trailing peak observed at 20°C.

Spectral studies

The ultraviolet spectra and difference spectrum of γ -casein A and FIV were presented in Fig. 6. The difference spectrum of both proteins showed little difference.

Molecular weight

The data presented in Fig. 7 show that the molecular weights of γ -casein A and FIV estimated by chromatography of Sepharose 6B equilibrated with 6 M guanidine hydrochloride

FIG. 5. Sedimentation Patterns of γ -Casein A and FIV.

7 mg samples were dissolved in one ml of 0.01 M imidazole-hydrochloride buffer containing 0.07 M potassium chloride (pH 7.1). Photographs were taken after reaching full speed (55,430 rpm). A, 15 min; B, 60 min; C, 120 min.

FIG. 6. Ultraviolet and Difference Spectra of γ -Casein A and FIV.

—, γ -Casein A; ---, FIV.

Both proteins were dissolved in 0.01 M imidazole-hydrochloride buffer containing 0.07M potassium chloride (pH 7.1) to give a $A_{280}=0.3$ for ultraviolet spectra. Ultraviolet spectrum was obtained in the same conditions. FIV was in reference cuvette.

FIG. 7. Estimation of Molecular Weight of γ -Casein A and FIV by Chromatography of Sepharose 6 B Equilibrated 6 M Guanidine Hydrochloride.

FIG. 8. Estimation of Molecular Weight of γ -Casein A and FIV by SDS Electrophoresis.

were equally 20,000. The molecular weight estimated based on the relative electrophoretic mobilities of sodium dodecyl sulfate complex of γ -casein A and FIV was shown in Fig. 8. The molecular weights of both proteins by this method were equally 22,000.

Amino acid composition

The relative amino acid compositions of γ -casein A and FIV were coincident as shown in Table I. The data almost agreed to those presented by Groves *et al.* for γ -casein A. Both γ -casein A and FIV contained one mole of phosphorus per one mole of protein (Table I).

TABLE I. AMINO ACID COMPOSITION AND PHOSPHOROUS CONTENT OF γ -CASEIN A AND FIV

Amino acid	γ -Casein A	FIV	γ -Casein (Groves <i>et al.</i>) ²⁰⁾
Lys	10	10	10
His	5	5	5
Arg	2	2	2
Asp	8~9	7~8	8
Thr	8	8	8
Ser	11	11	11
Glu	32	32	32
Pro	33	33	33
Gly	4	4	4
Ala	5~6	5~6	5
Val	17	17	17
Met	6	6	6
Ile	7	7	7
Leu	19	19	19
Tyr	4	4	4
Phe	8	8~9	9
Trp	1	1	1
P	1	1	1

(residue numbers/mole protein)

TABLE II. N- AND C-TERMINAL AMINO ACIDS OF γ -CASEIN A AND FIV

Proteins	N-Terminal amino acid	C-Terminal amino acid
γ -Casein A	Lys	—Ile-Ile-Val
FIV	Lys	—Ile-Ile-Val
γ -Casein (Groves <i>et al.</i>) ²⁰⁾	Lys	—Ile-Ile-Val

Terminal amino acids

The N- and C-terminal amino acids of γ -casein A and FIV were presented in Table II together with those reported by Groves and Townend.²⁰⁾ The N-terminal amino acid of both proteins was Lys and the identical sequence, -Ile-Ile-Val, was found to be C-terminal in γ -casein A and FIV.

DISCUSSION

Gordon *et al.*²¹⁾ and Ribadeau-Dumas *et al.*²²⁾ showed that γ -, TS-, R- and S-caseins are fragments of β -casein likely originating from tryptic like cleavages of the peptide chain next to the carbonyl groups of some lysyl residues. We independently showed that TS- and R-caseins were probably the decomposition products of β -casein by milk protease.⁶⁾ In addition to these two proteins, it was shown that γ -casein was probably a decomposition product of β -casein by milk protease in the present investigation.

PAGE at pH 3.0 and 9.1 show that γ -casein A and FIV have the same mobility (Fig. 4). In PAGE at pH 9.1, γ -casein A and R-casein have the same mobility and can not be distinguished each other, but these two proteins are separable at pH 3.0. In both cases, there is only one major band visible. These facts show that γ -casein A and FIV used in this experiment are almost homogeneous.

As shown in Fig. 5, the sedimentation boundaries and coefficients of γ -casein A and FIV are coincident each other. Both proteins have two schlieren peaks (18.2 S, 0.9 S) at 20°C and a single peak (0.9 S) at 7.8°C. The fast and slow moving peaks seem to represent polymer and monomer of both proteins respectively. Sedimentation coefficient ($s_{20,w}$) of γ -casein is reported by Groves *et al.*²⁾ and Tripathi and Gehrke.²³⁾ Groves *et al.*²⁾ observed one schlieren peak with an $s_{20,w}$ of 1.17 S at 0.8°C, and Tripathi and Gehrke²³⁾ did two peaks, having $s_{20,w}$ of slow moving peak of 1.35 S and of the fast moving peak of 23.20 S at 20.0°C.

Molecular weights of γ -casein A and FIV were 20,000 by chromatography of Sepharose

6B and 22,000 by sodium dodecyl sulfate electrophoresis and in both cases the molecular weight of two proteins was obviously smaller than that of β -casein A. Groves and Townend²⁴⁾ reported the molecular weight of 22,000, 23,000, 21,000 for γ -casein from the measurements of sedimentation equilibrium, sodium dodecyl sulfate electrophoresis and amino acid composition, respectively. These results were similar to our results obtained in the present experiment.

Amino acid compositions of γ -casein A and FIV are coincident each other and almost agree with the data obtained by Groves and Townend for γ -casein (Table I). The N- and C-terminals of γ -casein A coincide with those of FIV (Table II) and also agree with those of γ -casein obtained by Groves and Townend. The sequence, -Ile-Ile-Val, in C-terminal is identical with that of β -casein.¹⁹⁾

Recently, Groves *et al.*²⁵⁾ performed the disc gel electrophoresis of tryptic digestion of β -casein B and found the band corresponding in mobility to γ -casein B in the hydrolyzate. Taking into account that milk protease had similar caseinolytic activity to trypsin,¹⁰⁾ γ -casein-like fragment found by them is probably identical to F-IV described in this paper.

From these series of results, it is suggested that γ -casein is possibly a product of β -casein hydrolysis by milk protease as in the case of TS- and R-caseins.

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