Studies on the Proteolytic Action of Dairy Lactic Acid Bacteria*

Part III. Changes of the Casein Treated with *Streptococcus cremoris***

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Received June 12, 1967

Streptococcus cremoris was cultivated for 7 days at 30°C in sterilized skim milk or in the sterilized 10% solution of dry skim milk. This skim milk culture was divided into precipitate and supernatant by centrifugation. The absorbancy at 280 m μ of the supernatant prepared from the skim milk culture of *S. cremoris* was higher than that of the control supernatant.

Casein prepared from the skim milk culture of *S. cremoris* was less hydrolyzed by rennet than control casein at pH 7.0.

According to the free boundary electrophoretic analysis of the treated casein in M/10 veronal buffer of pH 8.5 containing urea, α -casein seemed to be hydrolyzed by *S. cremoris* but β -casein did with more difficulty.

INTRODUCTION

Various lactic acid bacteria are used to produce many kinds of dairy foods. And one can recognize that there are some delicate differences on the flavor or texture of the dairy foods depending on applying methods or strains of lactic acid bacteria used.

There are many reports about the action of lactic acid bacteria on milk. Sasaki and Nakae¹⁾ reported that skim milk culture of lactic acid bacteria contained more amounts of released amino acids than the milk without inoculation of lactic acid bacteria. Yamamoto

and Yoshitake²⁾ produced two kinds of Cheddar cheese. One of them was produced using starter and rennet, and another using only rennet. They concluded that the kinds and the amounts of amino acids released from the former, during ripening, were larger than the latter. Poznanski and Rymaszewski³⁾ showed the amounts of amino type nitrogen and non-casein type nitrogen released from cheese produced by using lactic acid bacteria were larger than that released from cheese produced by using only lactic acid to control the acidity of the cheese. And they⁴⁾ also reported that some developments of cheese flavor might depend on casein hydrolysis by the proteinase of lactic acid bacteria. Knaut et al.⁵⁾ revealed

* The main titles in the series of this report were corrected from "Studies on the Proteolytic Action of Lactic Acid Bacteria" (*J. Agr. Chem. Soc. Japan*, **39**, 299 (1965)) and "Study on Protease of Dairy Bacteria" (This Journal, **30**, 731 (1966)) to "Studies on the Proteolytic Action of Dairy Lactic Acid Bacteria"

** This report was presented at the Annual Meeting of the Agricultural Chemical Society of Japan in April of 1966.

1) R. Sasaki and T. Nakae, *Jap. J. Zootech. Sci.*, **29**, (6) 357, 362 (1959).

2) T. Yamamoto and M. Yoshitake, *Proc. 16th Dairy Congr.*, **B**, 395 (1962).

3) S. Poznanski and J. Rymaszewski, *Milchwissenschaft*, **20**(1), 14 (1965).

4) S. Poznanski et al., *XVII Int. Dairy Cong.*, **D**, 555 (1965).

5) T. Knaut et al., *ibid.*, **D**, 515 (1966).

the existence of some proportional relationship between the amounts of free amino acids and proteolytic activity of lactic acid lactobacilli in skim milk. Maeno and Oheda⁶⁾ reported that *S. lactis* and *L. casei* were not able to hydrolyze curd but *L. helveticus* could hydrolyze the curd to produce viscous one. But Williamson and Speck⁷⁾ denied the linear proportion between the hardness of the curd in which lactic acid bacteria were included and its hydrolyzed amounts. The authors reported about the action of intracellular protease of lactic acid bacteria on native casein or paracasein.⁸⁻⁹⁾ On the other hand Williamson et al.¹⁰⁾ gave some proofs about the production of extracellular protease from *S. cremoris*. Annibaldi¹¹⁾ reported that *S. faecalis* seemed to hydrolyze β -fraction of casein, and *L. helveticus* to hydrolyze both α - and β -fractions of casein but α -fraction could be hydrolyzed easier than β -fraction.

Judging from these reports it is estimated that intra- or extra-cellular protease of lactic acid bacteria hydrolyzed some parts of casein and changed its properties. But to reveal the detail, i.e., the effects of the protease of lactic acid bacteria on casein, it is desired to have more detailed studies which have to be fulfilled from many viewpoints.

This study was carried out to obtain some details about effects of one of the lactic acid bacteria, *S. cremoris*, on casein.

EXPERIMENT

1. Materials

a) **Bovine milk.** To prepare various treated caseins, raw fresh skim milk, which was separated from

cream by separator, or 10% aqueous solution of dry skim milk was used.

b) **Lactic acid bacteria.** *Streptococcus cremoris* (H-61) used was given from National Institute of Animal Industry, Chiba.

c) **Enzyme. Rennet:** Powder from Hansen's cheese rennet tablets manufactured by Chr. Hansen's Laboratory, Inc. **Trypsin:** Crystalline trypsin manufactured by Mochida Pharmaceutical Co., Ltd.

d) **Whole casein.** Raw native casein (RNC) was prepared from raw fresh skim milk according to the method of acid casein preparation.¹²⁾ From aqueous solution of dry skim milk, acid casein (DNC) was also prepared.

2. Analytical methods

a) **Cultivation of *S. cremoris*.** Skim milk medium was prepared by sterilizing raw fresh skim milk or 10% aqueous solution of dry skim milk at 120°C for 15 min. One loop from the stock culture of *S. cremoris* was inoculated to 10 ml of the medium and then the culture was incubated for 24 hours at 30°C. *S. cremoris* was precultured repeatedly until the culture was coagulated within 24 hours at 30°C to activate the growth of *S. cremoris*. Ten ml of the preculture was added to 1,500 ml of the skim milk medium and incubated at 30°C for 7 days.

b) **Measurements of pH change and hydrolysis of milk protein in the culture during cultivating *S. cremoris*.** pH Change was measured with pH meter equipped with glass electrode (Towa Denpa Co.) and the measurement was repeated several times. Finishing the cultivation for the given hours, the culture was centrifuged at 3,000 r.p.m. for 30 min. to gain completely clear supernatant, and the optical absorbancy of the supernatant at 280 m μ was measured with Hitachi-Parkinmer 139 UV-VIS Spectrophotometer. In this measurement the absorbancy stood for degree of hydrolysis of milk protein and the values shown in this report were differences of A and B.

A: Supernatant prepared from the culture in which *S. cremoris* was cultivated for 7 days at 30°C.

B: Supernatant prepared from the culture whose pH was adjusted about 3.5 with lactic acid and which was incubated at 30°C for 7 days.

c) **Preparation of various treated caseins.** The culture of *S. cremoris* was centrifuged at 3,000 r.p.m.

6) T. Maeno and M. Oheda, "Gyunnyu Kako Ho" Asakura press, 1954, p. 266, p. 269, p. 271.

7) W. T. Williamson and M. L. Speck, *J. Dairy Sci.*, **45**, 164 (1962).

8) Y. Sato and I. Nakajima, *J. Agr. Chem. Soc. Japan*, **39**, 299 (1965).

9) Y. Sato and K. Ohmiya, *This Journal*, **30**, 731 (1966).

10) W. T. Williamson et al., *J. Bact.*, **87**, 49 (1964).

11) S. Annibaldi, *Proc. 16th Int. Dairy Cong.*, **B**, 545 (1962).

12) T. Maeno and M. Oheda, "Gyunnyu Kako Ho" Asakura press, 1954, p. 397.

(about 1,500 g) for 30 min., the precipitate was suspended in vast volume of water and then the pH of the suspension was brought to near 8.0 with 1N NaOH to dissolve the precipitate, casein. From this dissolved casein solution, bacterial cells were removed by precipitating with centrifugal condition as described above.**

The pH of the supernatant was brought down to 4.7 with 1N HCl to precipitate casein. This precipitate was washed with distilled water. Such dissolving, precipitating and washing were repeated three times. Then precipitated casein was dehydrated with ethanol and defatted with ethyl ether. This defatted casein was dried under the high vacuum. The casein prepared from sterilized skim milk, according to the procedure described above, is denoted as RB and that prepared from dry skim milk is denoted as DB.

Control casein was prepared by the same procedure as described above excepting the inoculation of

S. cremoris. Instead of this exception, pH changes in the culture, which were accompanied by the growth of *S. cremoris*, were traced by adding lactic acid to the medium aseptically. Control caseins prepared from sterilized skim milk and dry skim milk were described as RA and DA, respectively.

α - and β -Casein were prepared from RNC by the method of Hipp et al.¹³⁾ α - and β -Casein and the mixture of both ($\alpha : \beta = 3 : 1$) were described as α , β , and ($\alpha + \beta$), respectively. And each casein was dissolved in M/15 phosphate buffer at pH 7.0 to make 1.5% solution and sterilized. These were described as H- α , H- β and H($\alpha + \beta$), respectively.

The mixture of glucose (10 g), yeast extract (2 g), Polypepton (5 g) and Na-succinate (7 g) was dissolved in 200 ml of water. This mixed solution was dialyzed against 800 ml of water. Dialyzate was sterilized and used as a synthetic culture. Ten ml of this synthetic culture and 5 ml of H- α or H- β were mixed, inoculated with *S. cremoris* and were cultivated at

TABLE I. TREATMENT CONDITIONS OF VARIOUS CASEINS AND THEIR ABBREVIATIONS

Abbreviation	Skim milk or casein	Sterilization	Inoculated bacteria or added matter	Incubation
RA	Raw fresh	120°C, 15 min.	Lactic acid	30°C, 7 days
RB	Raw fresh	120°C, 15 min.	<i>S. cremoris</i>	30°C, 7 days
DA	Dry	120°C, 15 min.	Lactic acid	30°C, 7 days
DB	Dry	120°C, 15 min.	<i>S. cremoris</i>	30°C, 7 days
α -A	α -Casein	120°C, 15 min.	Lactic acid	30°C, 7 days
α -B	α -Casein	120°C, 15 min.	<i>S. cremoris</i>	30°C, 7 days
β -A	β -Casein	120°C, 15 min.	Lactic acid	30°C, 7 days
β -B	β -Casein	120°C, 15 min.	<i>S. cremoris</i>	30°C, 7 days
DAW	Whey prepared from dry skim milk in which lactic acid was added and incubated at 30°C for 7 days.			
DBW	Whey prepared from dry skim milk in which <i>S. cremoris</i> was inoculated and incubated at 30°C for 7 days.			
RNC	Native casein prepared from raw fresh milk.			
α	α -Casein prepared from RNC.			
β	β -Casein prepared from RNC.			
($\alpha + \beta$)	The mixture of α - and β -casein ($\alpha : \beta = 3 : 1$).			
RHC	Casein prepared from sterilized skim milk.			
H- α	Heated α -casein at 120°C for 15 min.			
H- β	Heated β -casein at 120°C for 15 min.			
H($\alpha + \beta$)	Heated casein of ($\alpha + \beta$) at 120°C for 15 min.			
DNC	Native casein prepared from dry skim milk.			
DHC	Casein prepared from sterilized 10% solution of dry skim milk.			

** Skim milk culture of *S. cremoris* was centrifuged at 3,000 r.p.m. (about 1,500 g) for 30 min. The turbidity of the supernatant at 660 m μ was the same to that of control.

13) N. J. Hipp et al., *J. Dairy Sci.*, **35**, 272 (1952).

30°C for 7 days. To this culture 1 N HCl was added to bring its pH to 7.0. After removing the bacterial cell this culture was dialyzed against a plenty of water and lyophilized. This α - or β -casein under the influence of *S. cremoris* was denoted as α -B or β -B respectively.

Raw fresh skim milk or 10% aqueous solution of dry skim milk was heated at 120°C for 15 min. Then casein under the influence of heating was prepared according to the preparation procedure of acid casein. Casein prepared from heated skim milk or from heated 10% aqueous solution of dry skim milk was denoted as RHC or DHC, respectively.

Treatment condition and abbreviation of each casein are altogether shown in Table I.

d) Hydrolysis of various treated casein by enzyme. RA, RB, DA or DB was dissolved in M/15 phosphate buffer to make substrate of each 0.6% solution at pH 7.0. Five ml of each substrate and one ml of aqueous solution of rennet (0.05 g/ml) or of crystalline trypsin (0.05 mg/ml) were mixed and incubated at 37°C for the given time. After reaction time was over, absorbancy at 280 m μ of deproteinized filtrate¹⁴⁾ was measured with one cm cuvette to indicate the grade of casein hydrolysis.

e) Free boundary electrophoresis. Referring to the method by Sullivan et al.¹⁵⁾ or Nakanishi and Itoh,¹⁶⁾ M/15 veronal buffer of pH 8.5 containing 420 g of urea per liter was prepared. Each treated casein was dissolved in the buffer containing urea to 1.5% solution and this protein solution was dialyzed against a vast volume of the same buffer at 3°C overnight. Free boundary electrophoresis was carried out at 3°C, 5 mA for 60 min. using Tiselius apparatus (Hitachi HTD-1 Type). Whenever taking a photograph of the electrophoretic pattern, diagonal slit of the apparatus was fixed at 60°.

RESULTS

1) Changes of the skim milk culture accompanied by the growth of *S. cremoris*

pH Change of the skim milk culture in which *S. cremoris* was cultivated is shown in Fig. 1. This skim milk culture was coagulated

within 24 hours. At that time pH of the culture was about 4.7. The more cultivating time passes, the more decreasing rate of pH gets small. Thus pH of the culture became about 4.0 after 7 days incubation, though pH values had some deviation.

Absorbancy at 280 m μ of the supernatant separated from the culture is listed in Table II.

FIG. 1. pH Changes of Skim Milk Culture during Growth of *S. cremoris* at 30°C.

TABLE II. DIFFERENCE OPTICAL DENSITY OF WHEY AT 280 m μ

Difference optical density at 280 m μ

Whey	I	II	III
DAW	0.000	0.000	0.000
DBW	0.040	0.105	0.150

I, II and III are replicate numbers of experiments.

TABLE III. HYDROLYSIS OF CASEINS BY RENNET OR TRYPSIN IN M/15 PHOSPHATE BUFFER AT pH 7.0, 37°C FOR ONE HOUR

Caseins	Optical density at 280 m μ			
	Rennet		Trypsin	
	I	II	I	II
DA	0.110	0.120	0.900	0.545
DB	0.035	0.060	0.885	0.520
RA	0.090	0.095	0.815	0.395
RB	0.027	0.085	0.820	0.375

I and II are replicate numbers of experiments.

14) S. Akabori, "Koso Kenkyu Ho", Asakura press, 1956, Vol. 2, p. 242.

15) R. A. Sullivan et al., *Arch. Biochem. Biophys.*, **55**, 455 (1955).

16) T. Nakanishi and T. Itoh, *J. Agr. Chem. Soc. Japan*, **39**, 484 (1965).

FIG. 2. Boundary Electrophoretic Patterns of Raw Native Caseins in M/10 Veronal Buffer at pH 8.5.

Urea (420 g per liter) was added to the buffer. Electrophoresis was carried out at the current of 5 mA for 60 min. at about 3°C. Protein concentration was 15 mg of casein per ml. The angle of diagonal slit was fixed at 60°. All the patterns show ascending ones only.

- A: RNC—Electrophoresis was carried out for 45 min.
 B: RNC, C: α -Casein, α . D: β -Casein, β . E: Mixture of α - and β -casein—The mixture ratio of α - and β -casein was 3 to 1, ($\alpha + \beta$).

2) Proteolysis of casein by enzymes

The grades of hydrolysis of caseins (RA, RB, DA and DB) by rennet or crystalline trypsin are listed in Table III.

3) Free boundary electrophoresis

Free boundary electrophoresis of whole, α -, β -, and various treated caseins was carried out. Electrophoretic patterns of these caseins are shown in Figs. 2, 3, 4 and 5. Area ratio and relative mobility (moving distance of B peak of RNC, Fig. 2-B, was set at 100) of

FIG. 3. Boundary Electrophoretic Patterns of Casein Heated at 120°C for 15 min. in M/10 Veronal Buffer at pH 8.5.

Electrophoretic conditions are described in Fig. 2.

- A: RHC B: H- α C: H- β D: H($\alpha + \beta$)

TABLE IV. THE AREA RATIO AND RELATIVE MOBILITY OF EACH PEAK OF NATIVE AND HEATED CASEINS MEASURED FROM ELECTROPHORETIC PATTERNS

Abbreviation of caseins	Peak A		Peak B		Peak C	
	Area ratio (%)	Mobility	Area ratio (%)	Mobility	Area ratio (%)	Mobility
RNC	(1) 30	(1) 107	(2) 20	(2) 100	(3) 50	(3) 80
α	(4) 80	(4) 117	(5) 20	(5) 96	—	—
β	—	—	—	—	(6) 100	(6) 70
($\alpha + \beta$)	(7) 40	(7) 108	(8) 10	(8) 100	(9) 50	(9) 85
RHC	(10) 30	(10) 107	(11) 20	(11) 103	(12) 50	(12) 85
H- α	(13) 60	(13) 105	(14) 40	(14) 80	—	—
H- β	—	—	—	—	(15) 100	(15) 81
H($\alpha + \beta$)	(16) 35	(16) 107	(17) 20	(17) 99	(18) 45	(18) 87

The numbers in the parentheses are the peak numbers in Figs. 2 and 3.

TABLE V. THE AREA RATIO AND RELATIVE MOBILITY OF EACH PEAK OF CASEINS TREATED WITH *S. cremoris*

Abbreviation of caseins	Peak A		Peak B		Peak C	
	Area ratio (%)	Mobility	Area ratio (%)	Mobility	Area ratio (%)	Mobility
RNC	(1) 30	(1) 107	(2) 20	(2) 100	(3) 50	(3) 80
RHC	(10) 30	(10) 107	(11) 20	(11) 103	(12) 50	(12) 85
RA	(31) 30	(31) 102	(32) 40	(32) 95	(33) 30	(33) 88
RB	(34) 25	(34) 100	(35) 50	(35) 93	(36) 25	(36) 88
α -A	(37) 55	(37) 103	(38) 45	(38) 93	—	—
α -B	(39) 30	(39) 103	(40) 70	(40) 95	—	—
β -A	—	—	—	—	(41) 100	(41) 80
β -B	—	—	—	—	(42) 100	(42) 90
DNC	(19) 30	(19) 110	(20) 25	(20) 99	(21) 45	(21) 86
DHC	(22) 30	(22) 102	(23) 25	(23) 100	(24) 45	(24) 85
DA	(25) 30	(25) 102	(26) 30	(26) 95	(27) 40	(27) 85
DB	(28) 20	(28) 95	(29) 45	(29) 90	(30) 35	(30) 85

The numbers in the parentheses are the peak numbers in Figs. 2, 3, 4 and 5.

FIG. 4. Boundary Electrophoretic Patterns of Various Treated Caseins.

Electrophoretic conditions are described in Fig. 2.
A: DNC B: DHC C: DA D: DB E: RA F: RB

FIG. 5. Boundary Electrophoretic Patterns of α - and β -Casein.

Electrophoretic conditions are described in Fig. 2.
A: α -A B: α -B C: β -A D: β -B.

each peak, which were calculated from the electrophoretic patterns, of native or treated caseins are listed in Tables IV and V.

DISCUSSION

1. Hydrolysis of milk protein during the cultivation of *S. cremoris*

It is reported that the increase of lactic acid

bacterial cell number is proportional to the increase of lactic acid production.¹⁷⁾ Therefore it is estimated that rapid increase of the bacterial number occurred after one day incubation. But the increasing rate began to get small after 2 days incubation and the increasing continued slightly even after 7 days incubation (Fig. 1).

Optical density at 280 m μ of the DBW was higher than that of DAW. Therefore it is estimated that some kinds of protein included in skim milk were hydrolyzed by *S. cremoris* during its growing (Table II). Sasaki and Nakae¹¹⁾ showed the existence of protein-hydrolyzing activity of lactic acid bacterial enzyme in fermented milk and the authors⁸⁻⁹⁾ could also show the same results as described previously and in this report. These results indicate that lactic acid bacteria hydrolyze milk protein, whey protein or casein. Especially in our previous report, it might well be insisted that casein was hydrolyzed by lactic acid bacterial enzyme.

2. Free boundary electrophoresis of various treated caseins

A) Native casein. When electrophoresis of RNC was carried out for 45 min. (Fig. 2-A), its electrophoretic pattern resembles to that reported by Nakanishi and Itoh.¹⁶⁾ The pattern of RNC after 60 min. electrophoresis showed the existence of three peaks and it was estimated that peaks (i), (ii) and (iii) correspond to (1), (2) and (3), respectively. In this report the authors adopted the pattern of Fig. 2-B as a standard electrophoretic pattern of RNC and also expressed three peaks (1), (2) and (3) as A peak, B peak and C peak, respectively. In the pattern of ($\alpha + \beta$), shown in Fig. 2-E, the area ratio of each peak to the sum of all peaks was (7):(8):(9)=40:10:50 (Table IV). Relative mobilities of these three peaks, (7), (8) and (9), were almost the same as those of the peaks (1), (2) and (3), respec-

tively. In Fig. 2-E, peaks (7) and (8) were separated from each other but not so obvious. It is estimated that (7) and (8) in Fig. 2-E seemed to belong to α -casein and (9) to β -casein. Judging from the results obtained from Fig. 2-C and Fig. 2-D, the area ratios of α - and β -casein were 50 and 50, respectively. As the mixture ratio of α - and β -casein, however, was three to one, it was estimated that about one third of α -casein had the same mobility with β -casein and the part of α - and β -casein formed a single peak (9) altogether. Nakanishi and Itoh¹⁸⁾ also insisted that the peaks of β - and k -casein had the same mobilities. In Fig. 2-E, ($\alpha + \beta$), a part of α -casein was accompanied with β -casein, as described before, so that the relative mobility of the peak, which seemed to contain β -casein mainly and small parts of α -casein, was larger than that of pure- β -casein peak.

From these results it is estimated that peaks (1) and (2) belong to α -casein complex, and peak (3) is mainly β -casein.

B) Heated casein. In electrophoretic pattern of heated α -casein, H- α (Fig. 3-B), peak (13) got more flat than (4) in the pattern of α -casein (Fig. 2-C) and its area ratio and relative mobility were decreased but the area ratio of (14) was larger than that of (5). These results may support that heating caused the changes in mobilities of some components. The shape of (15) in the pattern of H- β (Fig. 3-C) got more flat than that of (6) in Fig. 2-D and relative mobility of (15) was larger than that of (6). The heating makes each peak of casein flat and it is also described in the report by Yamauchi and Tsugo.¹⁹⁾ In this report both H- α and H- β showed the same phenomena as described above, but heating decreased the relative mobility of α -casein and increased that of β -casein.

Fig. 3-D is the pattern of H($\alpha + \beta$). As the

17) R. Luedeking and E. L. Piret, *J. Biochem. Microbiol. Tech. & Eng.*, **1**, 393 (1959).

18) T. Nakanishi and T. Itoh, *This Journal*, **29**, 1099 (1965).

19) K. Yamauchi and T. Tsugo, *Jap. Zootech. Sci.*, **32**, 311 (1961).

results of heating, area ratio of (18) got a little decrease compared with that of (9) and it can be also admitted that (16) and (17) were separated from each other more obviously than that of (7) and (8). RHC and RNC showed little changes of area ratio of each peak but the mobility of each peak of RHC (Fig. 3-A) or DHC (Fig. 4-B) got close each other compared with that of RNC or DNC (Table V).

Judging from all results described above, it is estimated that heat effects on whole casein are as follows: (A) Each peak of casein got flat. (B) Relative mobility of α -casein peaks got smaller but that of β -casein peak got larger. (C) Two peaks of α -casein seemed to be separated from each other with more difficulty.

C) **Casein affected by heating and lactic acid.** The effects of lactic acid on heated skim milk or heated aqueous solution of dry skim milk were measured electrophoretically. In that case the patterns of Fig. 3-A or Fig. 4-B were changed to those of Fig. 4-E or Fig. 4-C, respectively. From these patterns, it may be considered that each peak in the pattern of Fig. 3-A, (10), (11) and (12), or each peak in the pattern of Fig. 4-B, (22), (23) and (24), seems to correspond to each peak in the pattern of Fig. 4-E, (31), (32) and (33), or of Fig. 4-C, (25), (26) and (27), respectively. The effect of lactic acid on sterilized skim milk was hardly recognized because the area ratio of (31), one peak of RA, was negligibly changed compared with that of (10), one peak of RHC (Tables IV and V). Area ratio of C peak (33) which contains β -casein mainly got smaller than that of (12) and that of B peak (32) larger than that of (11). It is estimated that some parts of the components removed from C to B peak as a result of addition of lactic acid.

By the effects of lactic acid, relative mobilities of A and B peaks, (31) and (32), got smaller than those of (10) and (11), respectively. On the other hand, C peak (33) had

a little larger mobility compared with that of (12). From these results it is estimated that relative mobilities of A and B peaks were decreased but that of C peak was increased by the effects of lactic acid. So that the differences of relative mobility between these peaks seemed to get small and that the area ratio of C peak decreased.

These phenomena could be found out in both patterns of caseins which were prepared from sterilized skim milk and sterilized 10% solution of dry skim milk.

The effects of heating and lactic acid on α - or β -casein were as follows: The area ratios of A and B peaks of α -A were 55 and 45, respectively. But only a single peak appeared in the patterns of both H- β (Fig. 3-C) and β -A (Fig. 5-C). The area ratios of A and B peaks of H- α were 60 and 40, respectively. This means that some parts of the component removed from A to B peak.

D) **Casein treated with *S. cremoris*.** The area ratios of A peak (34) and C peak (36) in Fig. 4-F were smaller than those of (31) and (33) in Fig. 4-E, respectively, but area ratio of B peak (35) was larger than that of (32). Relative mobilities of (34) and (35) decreased slightly compared with those of (31) and (32), but the relative mobility of (36) was the same to that of (33). This tendency could also be admitted in the case of dry skim milk.

α -Casein hydrolyzed by *S. cremoris* (α -B in Fig. 5-B) had also A and B peaks, both of which had the area ratio of 30 and 70, respectively. This indicates the increase of the area ratio of B peak. The pattern of β -B hardly showed any changes compared with that of H- β .

Judging from the results obtained from the free boundary electrophoretic experiment, it might be admitted that α -casein, but not β -casein, was changed by the action of *S. cremoris*.

3. Hydrolysis of casein, treated with *S. cremoris*, by enzyme

Hydrolysis of RB or DB by rennet was more difficult than that of RA or DA (Table

III). In the previous report⁹⁾ paracasein which was hydrolyzed 3~7% nitrogen against total nitrogen by rennet was hydrolyzed with more difficulty by intracellular protease of *S. cremoris* than native casein did. This might lead us to the estimation that paracasein, which was produced by hydrolysis of α -casein by rennet in the early stage of rennet action, was difficult to be hydrolyzed by intracellular protease of *S. cremoris* because this protease might hydrolyze α -casein, the similar part of which might have been hydrolyzed by rennet.

In this report, the order of enzyme action on casein was opposite, i.e., rennet was acted on casein after intracellular protease of *S.*

cremoris did, but the similar result was obtained; casein treated with *S. cremoris* was less hydrolyzed by rennet than control casein described above. This result also might support that *S. cremoris* hydrolyzed α -casein easily. This experimental fact might be supported also by the results of electrophoresis shown above.

Acknowledgements. The authors express their gratitude to National Institute of Animal Industry for supplying lactic acid bacteria.

Thanks are due to Professor S. Doi, Professor I. Uritani and Assistant Professor Y. Kaneko of Nagoya University for their interests.