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An Electrophoretic Investigation of the Degradation of β-Casein by Crystalline Rennin

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An attempt was made to chart the primary course of the degradation of β-casein by crystalline rennet. The degradation follows distinctly different paths depending on the temperature, pH and other factors. Under conditions different from those under which the main experiments were made, the electrophoresis diagram shows that substances with mobilities in the range $u \times 10^5 = 0.5-11.5$ can be formed. In some cases only 2 or 3 of these substances with apparently arbitrary u-values may represent almost all of the degradation product.

In the profusion of degradation products derived from it and in the wide differences in the mobility of these products, β-casein differs considerably from α-casein. NPN formation during the degradation proceeds at only a third of the rate at which it is formed from α-casein and can almost be disregarded.

The enzymatic degradation of β-casein has been studied by Christensen[1], using trypsin, chymotrypsin and plasmin for the proteolysis and following the degradation by viscosimetry and NPN determinations. Lillevik *et al.*[2] have continued these investigations using chymotrypsin and following the degradation by alcohol titration. Peterson *et al.*[3] and Groves *et al.*[4] have studied the more or less stable degradation products that can be isolated after proteolysis. They used trypsin and followed the degradation with free electrophoresis, and pepsin together with paper electrophoresis, respectively.

The present investigation was carried out in order to obtain a picture of the earliest stages of the degradation of the β-casein by rennin as shown by changes in the electrophoretic curves. Some experiments were also made using other proteolytic enzymes under conditions that would produce the maximum variation in the appearance of the electrophoresis curves. The investigation followed more or less the same lines as the corresponding investigation on α-casein described previously (Lindqvist and Storgårds [5]).

METHODS

Preparation of β-casein. β-Casein was prepared according to Hipp *et al.*[6] by fractional precipitation from urea solution. The starting material used for the fractionation was the mother liquor left after precipitation of α-casein from a solution of whole casein.

The progress of the fractional precipitation of the β-casein was followed by electrophoresis and the precipitation procedure was adjusted as required to give a β-casein as free as possible from both α-casein and γ-casein. By adding greater or lesser amounts ammonium sulphate to the solution used to precipitate the β-casein the process can be varied to give the best conditions and only three or four reprecipitations are needed to give pure β-casein. When ammonium sulphate was not added, that is, using the original procedure of Hipp *et al.*, this could not be done nearly as readily.

The β-casein thus obtained was free from fat and other opalescent impurities and also from the proteolytic enzymes present in the milk. After freeze-drying the β-casein was stored in a tightly closed jar containing silica gel as a drying agent.

Two different preparations of β-casein were used in this investigation. One of these contained small amounts of degradation products from the β-casein. The other, much smaller batch was completely pure. The batch used in a certain experiment is indicated on the diagrams and electrophoresis curves given below. The presence of these impurities does not interfere with the evaluation of the results obtained or the conclusions drawn.

Enzymes. Rennin was prepared in a crystalline state as described in the preceding paper[5]. *Pepsin* was crystallised according to Northrop[7] from a commercial preparation with an activity of 1:10 000. *Pancreatin* corresponded to the standards of B.P. 1932. Partially *heatinactivated crystalline rennin* was obtained by the following procedure:

A concentrated solution of crystalline rennin (4 000 Ru/ml) * was mixed with an equal volume of 3 % β-casein solution containing 2 % NaCl and 1/15 M NaH_2PO_4 at pH 6.7. Within a few seconds after mixing 0.2 ml was introduced into 2 ml of the same β-casein solution as above. This 2 ml of solution was already placed in a water bath at the inactivation temperature required (60° or 65°). Through this procedure momentaneous heating of the rennin solution was obtained. After the required inactivation time (5 min to 1 h) the temperature was rapidly reduced by injection of a mixture of cold 3 % β-casein solution (20 ml) having a pH of 5.3. This solution was then kept at a temperature of 17° like the other reaction mixtures. It is at present not clear whether the proteolytic action of the partially inactivated material is due to undestroyed rennin, or whether the rennin contains some heat stable modification or whether some completely different enzyme is present in traces in the rennin crystals. Whatever the case the content as measured by the activity was only about 1 %.

The proteolyses with rennin, pepsin, and pancreatin were carried out in exactly the same way as described for the α-casein investigations. The amounts of acid used to obtain a given pH in the reaction mixtures were, however, different from those used in the previous experiments because of the different buffer capacities of the α- and β-caseins. The amount of acid added varied between 0 and 33 ml of N HCl per 125 ml of buffer solutions. When the buffer was mixed with three volumes of 4 % β-casein in water adjusted to pH 9.0 with NaOH the resultant buffered solutions had a pH between 2.4 and 6.1. In the solutions a loose coagulum was formed instead of the sticky precipitate formed in the α-casein experiments. This made it easier to dissolve the samples for analysis.

Analyses. The analyses were done in the same way as in the previous investigation. The non-protein nitrogen (NPN) was determined on a filtrate obtained after the addition of trichloroacetic acid (TCA) to a concentration of 2 %. Because of the limited amounts of β-casein available the determinations of total N och soluble N had to be restricted in order to allow a full electrophoretic investigation. The electrophoresis were run with phosphate buffer of pH 7.3 and ionic strength 0.1 for 120 min at 12 mA. Standard Spinco cells were used.

The β-casein was broken down quite rapidly compared with the α-casein and as a rule the length of the experiments could be limited to 1 week.

RESULTS

A. The formation of soluble nitrogen. The amount on non-protein nitrogen liberated ($NPN_{2\%TCA}$) was low throughout and in no case was greater then

* Ru = rennin units according to Berridge, see Ref.

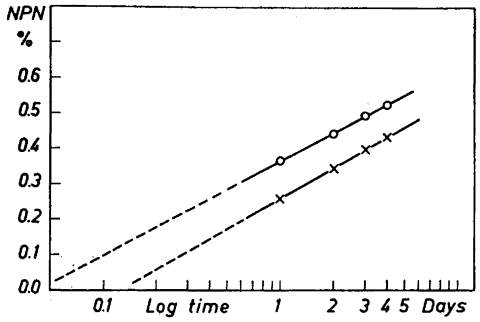

Fig. 1. Formation of NPN from β-casein in proteolysis with rennin. Mean reaction rate for the pH range 2.4—6.10.
O = β-casein containing 5 % of β-casein degradation products
× = pure β-casein

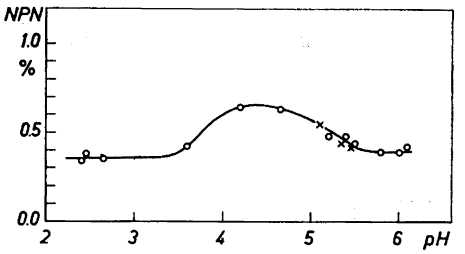

Fig. 2. Amount of NPN released from β-casein by rennin after 4 days at different pH values.

0.7 %. The values obtained showed a greater spread than the corresponding values obtained for α-casein but the tendency was the same — the NPN values plotted against log time gave straight lines starting from about 0.1 days on the time axis. This is shown in the curves in Fig. 1 for the two β-caseins preparations. The curve for the impure preparation showed a parallel displacement upwards caused by the more rapid formation of NPN from the impurities.

The points on the curves are mean values of results obtained in different experiments. Since the NPN formation was relatively independent of the pH, as is shown by Fig. 2, this should be not unreasonable. The pH optimum for NPN formation from β-casein by the action of rennin was the same as for the degradation of α-casein, pH 4.5.

B. Electrophoreses. The first stages of the β-casein degradation proceed very rapidly. It was necessary to take samples about an hour after adding the rennin but in spite of this it was not possible to obtain consecutive pictures demonstrating the details of the degradation. This would require samples to be taken perhaps every 10 min during the first few hours which was not technically feasible.

Fig. 3 gives an indication of the main lines of the degradation. The rule formulated for the degradation of α-casein also applies in this case — the primary cleavage gives only a few simple components with migration rates both higher and lower than that of the parent substance. On of these substances (βb in Fig. 6) had a migration rate of $u \times 10^5 = 0.5$ under the conditions used. Another substance with $u \times 10^5 = 6.8$ (βc) had a very short life and vanished completely within 24 h. A third main component (βa) was broken down fairly rapidly into two new parts (βa_1 and βa_2). One or both of these then underwent further degradation. The rates of degradation were very strongly dependent on the pH.

Table 1. Quantitative analysis of the electrophoresis diagrams shown in Fig. 3. Mean percentage distribution of the components of the reaction mixtures.

pH	βa_1	βa_2	βb	βc	βd	Notes
5.10	37.9	15.4	35.4	27.6	0	The values for βc are for a reaction time of 0.05 days; the values for βb are means for 0.05—4 days less the value for 0 days; other components are given as means for 1—4 days
5.35	42.0	22.5	26.8	30.6	8.6	
5.45	47.3	30.8	21.9	37.3	11.3	

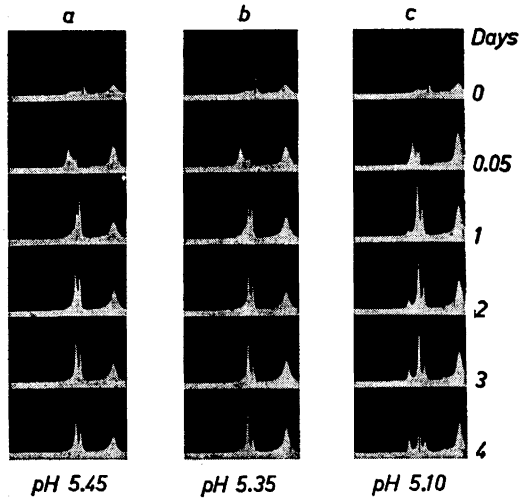

Fig. 3. Electrophoresis curves showing the course of the degradation of β-casein by rennin at three different pH values. The rate of formation and further degradation of the various components can vary widely for pH differences as small as 0.1 units. The purest β-casein was used.

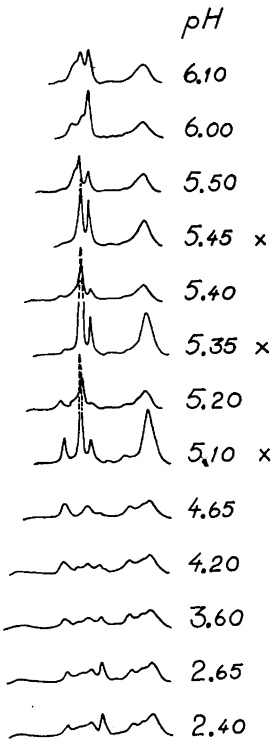

Fig. 4. Electrophoresis curves showing the final result (4 days) of the degradation of β-casein by rennin in the pH range 2.40—6.10. The maximum number of degradation products is shown at pH 4. Degradations using the pure β-casein are marked with ×.

The difficulties of quantitative estimation of the reaction products from the electrophoresis diagrams are considerable and an evaluation was possible in only a limited number of cases. Table 1 gives some measurements made on the components of the mixtures shown in Figs. 3 and 6. The values obtained confirm the subjective impression of the course of the degradation obtained by inspection of Fig. 3.

The final results of degradation varied considerably with the pH. At pH 4 and lower a large number of components are formed; at pH values above 5.4 there were only a few components. This is shown in Fig. 4. There was a certain amount of distortion of the curves when the less pure β-casein was used but this was not so large that it had to be taken into account when comparing the curves.

At pH 4.20 there were about 7—8 components, spread over the mobility range 0.5—11.5. A corresponding large spread of the components of the mixture of degradation products was also obtained at higher pH values when other enzymes were used. This is shown by a number of extreme cases given in Fig. 5. As is clear from this figure the main part of the degradation product may occur at any part of the mobility range and two or more principal components can also occur in apparently arbitrary combinations as shown in Fig. 5 B—G.

DISCUSSION

The tendency of β-casein on enzymatic degradation to give rise to substances with widely varying electrophoretic properties makes much more difficult

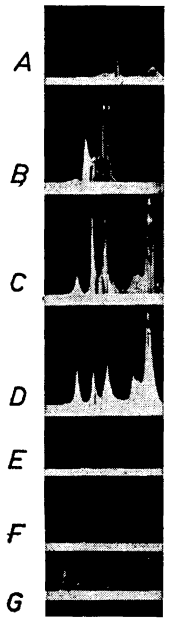

Fig. 5. Degradation of β-casein under different conditions. The electrophoresis curves shown here have been selected to show extreme variations in the distribution of the components.

A. Unaffected β-casein (starting material)
B. Degradation by partially inactivated rennin (heat-treated for 20 min at 65°. Incubated at pH 5.3 and 37° for 8 days)
C. As for B but inactivated for 20 min at 60°
D. As for B but inactivated for 5 min at 65°
E. Degradation by pepsin at pH 5.5 for 11 days at 17°
F. As for E but degradation continued for 18 days
G. Degradation by pancreatin at pH 5.5 for 8 days at 17°

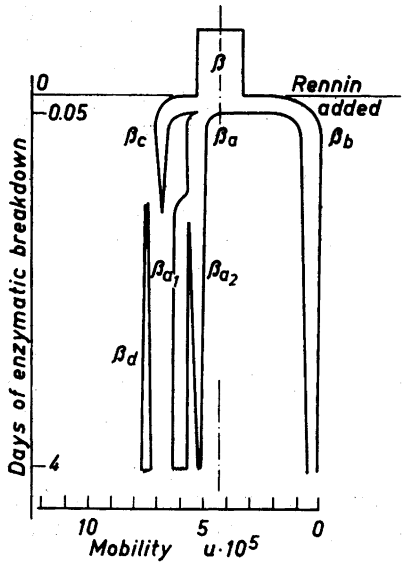

Fig. 6. Diagram of the course of the degradation of β-casein by rennin at pH 5.10 and 17°

the elucidations of the various paths of degradation under different conditions. It is not possible to give any generalised degradation curves covering the whole pH range. Fig. 6 gives a diagrammatic representation of the course of degradation as shown by the electrophoresis curves in Fig. 3 for pH 5.10. Corresponding diagrams are somewhat similar at higher pH values and during the early stages at low pH values.

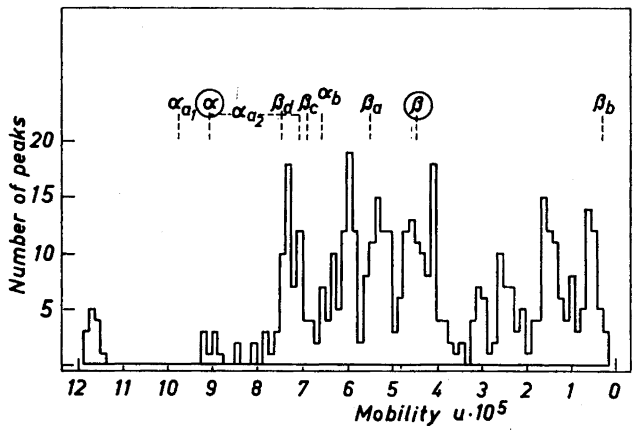

Fig. 7. Frequency of mobilities of approx. 470 peaks measured on electrophoresies from 90 degradation experiments. For comparison the mobilities of β-casein and of the degradation products observed previously för α-casein have also been marked. In this figure as in the preceding, the mobility scale has been placed with the zero point on the right so that the histogram agrees with the electrophoresis curves.

Table 2. The amino acid composition of α- and β-casein [8].

	Amino acid in g/100 g protein	
	α-casein	β-casein
Aspartic acid	8.4	4.9
Alanine	3.7	1.7
Tyrosine	8.1	3.2
Lysine	8.9	6.5
Arginine	4.3	3.4
Valine	6.3	10.2
Methionine	2.5	3.4
Leucine	7.9	11.6
Proline	8.2	16.0
Others	same within 10—15 %	

Fig. 7 gives another picture of the possible paths followed by the degradation in the form of a histogram showing the distribution of 470 peaks of various mobilities that were measured. The β-casein has a mobility in the middle of the mobilities of the group of degradation products. There was a surprisingly high content of substances that migrated very slowly at pH 7.3 and as in the case of α-casein the splitting off of a given substance usually gave a reduction in the mobility.

For comparison the positions of β-casein and its degradation products have been marked on Fig. 7. When a mixture of α- and β-casein is degraded the two sets of components partly overlap which could easily lead to misinterpretations of their origin.

The considerable differences between α- and β-casein in their reaction with proteolytic enzymes depends of course on the amino acid sequence in the two molecules. A comparison between the amino acid sequences of α- and β-casein should therefore give better information on the points of attack of the proteolytic enzymes than in proteolytic experiments with other proteins.

No investigations of this sort have as yet been published. However, a comparison of the amino acid compositions of α- and β-casein shows some points of difference. Some of the most marked of these are shown by the data in Table. 2 (Gordon *et al.*[8]).

Apart from the differences in the amino acid sequences, the major differences between α- and β-casein in potential paths of degradation on enzymatic proteolysis should be due for instance to the low content of aspartic acid and other acidic components and to the high content of proline, leucine, methionine and valine in β-casein.

In the degradation of β-casein as in that of α-casein, the formation of NPN and the cleavage of the β-casein molecule into a few simple parts have no direct connection. The formation of NPN is reasonably independent of pH and other factors but the electrophoresis curve is extremely dependent on the conditions used.

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