An Electrophoretic Investigation of the Degradation of α-Casein by Means of Pepsin and Trypsin

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Pepsin and trypsin degrade α-casein in completely different ways. If this process (the tertiary phase) is followed by electrophoresis and by the increase of nonprotein nitrogen (NPN), it is then found that pepsin and the previously studied rennin behave similarly in certain respects. Of these three enzymes rennin attacks the α-casein structure more specifically than pepsin and the latter in its turn more specifically than trypsin. The changes in the mobilities of the degradation products which can be observed during the process are different for different pH-values in the reaction mixture. In the pepsin investigations, the changes in the mobilities were proportional to the logarithm of the time. With trypsin, an electrophoresis pattern is obtained, after a few hours, which does not then change character throughout the rest of the experiment. The peaks decrease in height but they do not move or split up into several separate peaks. In the degradation experiments using pepsin, it was found that log NPN is proportional to log time. With trypsin, the process is however more complicated.

In a previous investigation in this series [1] it was shown that the degradation of α-casein by rennin follows completely different paths for different pH-values in the reaction mixture. A similar effect has also been shown for the system β-casein — rennin [7].

In the present investigation, the work has been extended to include in addition α-casein — pepsin and α-casein — trypsin. Pepsin is of interest because of its physiological relationship to rennin. Trypsin was studied because of its ability to break certain known peptide bonds in the protein molecule [4]. For this reason, it may be possible eventually to use the results from the α-casein — trypsin investigations as a standard for judging the specific ability of other enzymes to degrade α-casein.

The action of pepsin on α-casein has been studied by several investigators. Groves and co-workers [2] have, for example, investigated phosphorus peptones which were formed as final products from the attack of pepsin on casein. Zittle and Cerbulius [3] have studied the coagulation of casein with pepsin and inves-

tigated the peptides formed during the so-called primary phase from, amongst other things, α-casein.

The action of trypsin on α-casein has been studied by Christensen [5] by means of viscosity measurements and NPN investigations. Lembke and Kaufmann [6] have investigated the effect of trypsin on total casein by means of electrophoresis. The conclusions which they have drawn concerning the changes in the α- and β-casein peaks upon electrophoresis cannot be considered to be without objection if one takes into account the very large changes in the position of the degradation products relative to the position of the starting substance which has afterwards been observed for β-casein [7].

In all of these investigations, only the degradation process occurring during the primary phase or at the end of the tertiary phase of the casein decomposition was studied while the main process occurring in the tertiary phase has not been touched at all. Just as in the investigation of the effect of rennin on α-casein, the present investigation deals with the process occurring during the whole of the tertiary phase.

EXPERIMENTAL

Casein. The same α-casein sample was used as in the previous investigation. The casein was stored in a tightly closed vessel containing silica gel. A check on the electrophoretic properties of the sample showed that it was completely unchanged at the time at which the investigations were performed.

Enzymes. Pepsin, twice crystallized, was prepared from a commercial product with an activity of 1:10 000 according to Northrop's method [8].

Trypsin, twice crystallized, with 50 % $MgSO_4$ from the Nutritional Biochemicals Corporation was employed.

Procedure and Analyses. In the preparation of the substrate and enzyme solutions as well as in the mixing of these solutions, the same procedure was used as in the previous investigation. This means, in brief, that a 4 % sodium caseinate solution was mixed with a NaCl-phosphate buffer solution containing the enzyme sample in question in such a way that the final solution was 3 % with respect to casein and had at the same time the desired pH-value. The NaCl, phosphate and enzyme concentrations were likewise automatically given the desired values. Upon mixing, the casein coagulates and thus many mixtures containing a suitable amount for one analysis of the type in question must be made. In order to perform the analysis, the whole of the content of the tube was then used.

Certain limits were applied to the experimental conditions, *viz.* the pH interval in the pepsin investigations was 4.6 — 5.7 and in the trypsin one 5.1 — 6.3. The enzyme activity of the pepsin was adjusted so that, when measured according to Berridge's method for the determination of rennin activity, it corresponded to 400 Ru/l reaction mixture. The activity of the trypsin could not be determined in this way since a calcium caseinate sol is not coagulated, but rather dissolved, by trypsin. Instead, an amount of pure trypsin was weighed out which corresponded to the amount by weight of rennin which upon solution gave 400 Ru/l, *viz.* 5 mg/l.

The nature and the extent of the analyses were the same as in the previous work, with the exception that the pepsin experiments were allowed to run for 15 — 36 days and the trypsin experiments for about 12 days.

RESULTS AND DISCUSSION

A. Formation of soluble nitrogen (NPN)

The NPN values of the reaction mixtures after various times are shown in Figs. 1 and 2. A comparison with the previously performed investigations
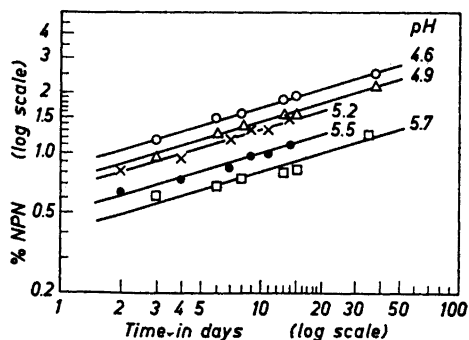
Fig. 1. Release of NPN, as a function of time, from α-casein due to the influence of pepsin. Reaction rate at different pH-values plotted using log NPN and log time scales.

on the properties of rennin shows that the NPN formation occurs in a different way. The reaction for the degradation of both α-casein and β-casein by rennin follows the empirical expression

$$\text{NPN} = k_1 \text{ log time} + C_1$$

For pepsin — α-casein, it was found on the other hand that

$$\text{log NPN} = k_2 \text{ log time} + C_2$$
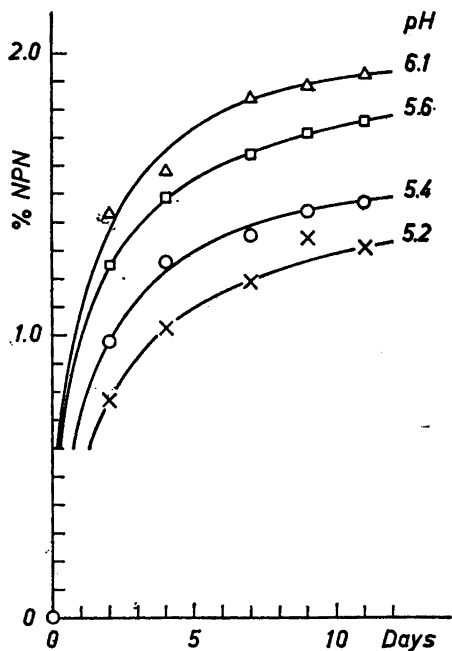
where k and C are constants.

Fig. 2. Release of NPN, as a function of time, from α-casein due to the influence of trypsin. Reaction rate at different pH-values plotted using linear scales.

Table 1. Constants in the equations for the formation of NPN from α-casein.
Rennin: % NPN $= k_1$ log time $+ C_1$
Pepsin: log % NPN $= k_2$ log time $+ C_2$

| pH | Rennin | | | | Pepsin | |
| | α-casein | | β-casein * | | α-casein | |
	k_1	C_1	k_1	C_1	k_2	C_2
4.21	1.73	0.42				
4.48	1.84	0.44				
4.6					0.306	−0.07
4.9					0.306	−0.14
5.2					0.306	−0.17
5.21	1.36	0.33	0.29	0.26		
5.45	0.98	0.23				
5.5					0.306	−0.30
5.7					0.306	−0.40
5.75	0.75	0.18				
6.4	0.45	0.11				
6.7	0.36	0.09				
7.0	0.23	0.05				

* The constants apply, approximately, for all pH-values within the interval investigated.

For trypsin — α-casein, the relationship was even more complicated. No simple transformations could be found which could make the experimental values fall on straight lines as in Fig. 1.

In Table 1, the values of the constants have been collected for several cases hitherto investigated for which calculations could be performed. It should be pointed out that the differences in NPN amounts in the cases, α-casein — rennin, α-casein — pepsin, and α-casein — trypsin, are not especially large despite the differences in the respective processes. It is of interest to compare these results with NPN determinations performed on ripening hard cheeses [9]. The casein degradation which occurs in that case is caused by rennin together with different bacterial enzymes that are formed successively. The process leads to higher NPN values and the formation follows the same mathematical expression as given above for the action of pepsin on α-casein.

B. Electrophoretic investigations

A total of about 60 electrophoretic results were obtained from each of the investigations on pepsin and trypsin. The calculation of the results has been performed in such a way that the differences caused by pH and time appear most noticeably. The results from the pepsin experiments resemble in several respects those obtained from the experiments with α-casein — rennin. The trypsin results are quite different.
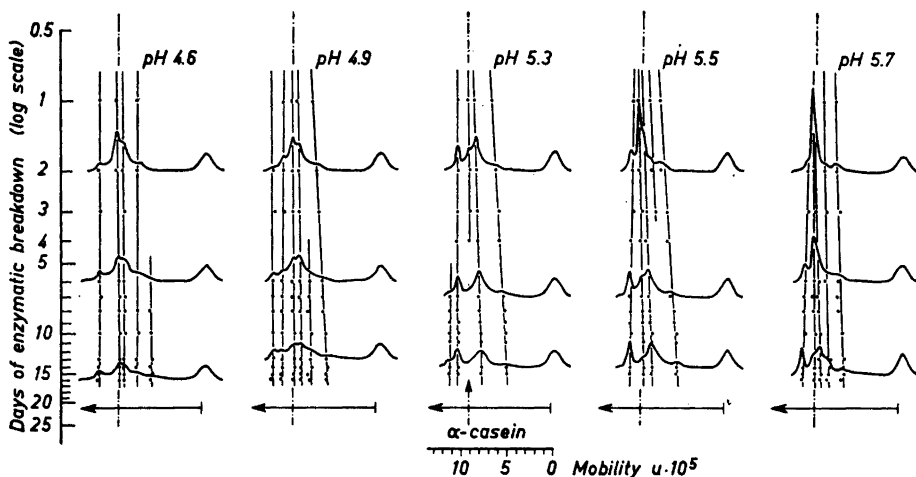
The main features of the results obtained are as follows:

Fig. 3. Summary of the changes in the electrophoresis curves obtained during the degradation of α-casein by pepsin at different pH-values. The changes in mobility are shown by the vertical lines which pass through the points for the mobility of certain peaks in the curves. (To save space, only some of the curves have been drawn in, the positions of peaks in opposite case being marked only with points corresponding to the mobility of the peak). The time scale is logarithmic.

Pepsin experiments. Fig. 3 shows the degradation process occurring in these experiments for different pH-values. The conclusions which can be drawn are:

1) With pepsin, electrophoresis curves of a flatter and more diffuse appearance than with rennin were obtained. This indicates a weakly selective attack on the α-casein molecule.

Instead of getting, as is the case with rennin, a small number of sharp electrophoretic peaks, each of which probably consisting of individual molecules, there is obtained with pepsin a main peptide in each electrophoretic peak together with a large number of other peptides lying close to it.

2) Because of the spreading of these peptides, with respect to their electrophoretic mobilities, around the main substance, the mean mobility of each peak in the electrophoretic diagram will be somewhat different from the corresponding one in the rennin experiments.

3) The difference in mobility of the substances split off from the α-casein was found as previously observed with several corresponding enzyme-substrate systems, *viz.* from a primary component with a certain mobility, various substances with higher and lower mobilities than that of the primary component are obtained. These reaction products are then degraded further, either as just described by cleavage or by a splitting off of dialysable peptides only which outwardly produce a decrease or increase of the mobility as the only effect. Whether a decrease or increase occurs depends on whether mainly

Fig. 4. Electrophoresis diagrams from the degradation of a-casein by trypsin at pH 5.6. Immediately after the start of the experiments, a certain shape for the curve is obtained which remains unchanged throughout the whole of the experiments.
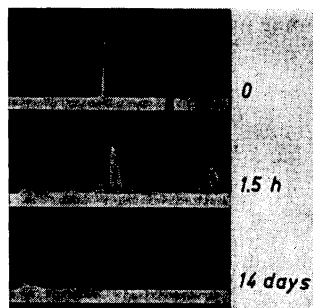
acid or mainly basic amino acids are split off in the form of oligopeptides or as free amino acids.

In the a-casein — pepsin experiments, the mobility changes linearly with log time while, in the a-casein — rennin experiments, the corresponding process was not linear, the various parts of the main processes occurring at different rates, each ebbing out at different times. A process which is linear within a limited time interval should indicate that the degradation process occurs rather independently of the amino acid sequence of the secondary products.

The conclusions which can be drawn from this work is that both the pepsin and the rennin attack a-casein at about the same points in the molecule. While the attack of the rennin is, at least in the beginning, absolutely specific, the pepsin attack occurs rather unspecifically right from the beginning of the process. The tendency towards a more general attack on the molecule increases with increasing degree of degradation of the peptide complex.

The trypsin experiments. A characteristic property for the trypsin is that the degradation products from a-casein are very similar from an electrophoretic point of view, independent of the pH during the reaction. As appears from Fig. 4, the characteristic shape of the curve is obtained within 1.5 h after the start of the experiment and thereafter retains its main features for 14 days, even if the height of the curve has then decreased somewhat. The electrophoresis curve can be described as consisting of one single main peak at the a_{a2} position [1], *i.e.* the mobility of the peak is smaller than that of the a-casein.

Judging by the shape of the curve, there are several components which make up the peak. It is only in exceptional cases that the mobilities of these components have such values that the resulting peak is markedly unsymmetrical or gets a shape differing from the normal distribution curve.

Besides this main peak, there are negligible amounts of components with high and low mobilities. In the electrophoretic diagram, one can observe amongst them a slight majority of very quickly moving substances over those with medium or low mobilities.

The electrophoresis curve does not belie the statement that trypsin splits the protein with a certain selectivity. However, if the attack of rennin on a-casein is compared with the trypsin attack studied here, then it is seen that

the rennin attack is far more specific, expecially in the early stages of the degradation. It is true that the rennin gives eventually rise to several peaks with quite different speeds, in contrast to trypsin's single peak, but on the other hand these more spread-out peaks are much more uniform.

If therefore, when comparing rennin and trypsin, we neglect any possible difference in the total number of components formed, it is nevertheless evident that the following differences in behaviour can be demonstrated:

Rennin gives a few, electrophoretically well-defined substances of about equal amounts with a certain spreading in the mobility values.

Trypsin gives a few, electrophoretically less well-defined substances with a negligible spreading in the mobility values. In addition and in contrast to rennin, trypsin gives small amounts of substances with a considerable spreading in mobility values.

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