Studies on Protease

IV. The cleavability of derived caseins by pepsin

(Directed by Prof. Toyoo Uchino in the Nagasaki Medical College)

By

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Introduction

In our two preceding papers (Nakamura, 1940; Uchino, 1940) we reported on the relative cleavability of various animal and vegetable proteins by trypsin, papain and pepsin. In carrying out these investigations we paid particular attention to the following two points: In the first place we measured the initial reaction velocity of the proteolysis, inasmuch as the split products, which appear in the course of the protein hydrolysis, exert, as it is well known, an inhibitory effect on the protease activity; in the second place we examined the enzymic hydrolysis at varying pH of the medium, since the optimum pH of an enzyme action is not always the same for different kinds of substrates. We thus endeavoured to get a true picture of the relative rates of enzymic hydrolysis of different kinds of proteins.

The present investigation was carried out from the same standpoint and it reports on the comparative cleavability of a number of derived caseins, such as alkali treated casein, deaminized casein, iodized casein and acetyl casein.

Materials

a. Alkali treated casein. Rimington and Kay (1926) pointed out the fact that when casein is acted upon by 0.25 N sodium hydroxide at 37°C, the whole of phosphorus, present in organic union with the protein molecule, becomes liberated as phosphoric acid within 24 hours. In preparing our alkali treated caseins, the action of alkali was effected in exactly the same way as in the case of this experiment. 10 g. casein (Merck's casein acc. to Hammarsten) were dissolved in 500 cc. distilled water with the addition of 8.0 cc. 1 N sodium hydroxide and the solution was warmed to 37°C. An equal volume of 0.5 N NaOH solution, also prewarmed to
37°C, was then added to the solution and the mixture was placed in an incubator at 37°C. After standing for 1, 2, 5, 24, 36 or 48 hours the solution was filtered from the flocculent precipitate and acidified with the careful addition of 33% acetic acid until a maximum precipitation was obtained. The supernatant liquid was removed by decantation, the gummy precipitate redissolved in sodium hydroxide and reprecipitated with acetic acid. The precipitation was repeated once more and the pasty substance was then ground up with absolute alcohol in a mortar. The fine white powder thus obtained was filtered off on a Buchner funnel, washed with alcohol and dried. These preparations of alkali treated caseins were called C\textsubscript{1}, C\textsubscript{2}, C\textsubscript{5}, C\textsubscript{24}, C\textsubscript{36} and C\textsubscript{48}, according to the duration of the alkali treatment. The nitrogen and phosphorus contents of these products are given in the following Table I.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Total nitrogen (Microkjeldahl, % of dry weight)</th>
<th>Total phosphorus (Youngburg, % of dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein acc. to Hammarsten (C\textsubscript{0})</td>
<td>14.64</td>
<td>0.858</td>
</tr>
<tr>
<td>Casein C\textsubscript{1}</td>
<td>14.66</td>
<td>0.601</td>
</tr>
<tr>
<td>Casein C\textsubscript{2}</td>
<td>14.71</td>
<td>0.468</td>
</tr>
<tr>
<td>Casein C\textsubscript{5}</td>
<td>14.75</td>
<td>0.227</td>
</tr>
<tr>
<td>Casein C\textsubscript{24}</td>
<td>13.87</td>
<td>0.0754</td>
</tr>
<tr>
<td>Casein C\textsubscript{36}</td>
<td>14.40</td>
<td>0.0358</td>
</tr>
<tr>
<td>Casein C\textsubscript{48}</td>
<td>13.96</td>
<td>0.0120</td>
</tr>
</tbody>
</table>

It will be seen from Table I, that there is a considerable decrease in the nitrogen content of alkali treated caseins, as was found by Rimington and Kay (1926). The nitrogen content of the products, obtained by the action of sodium hydroxide for less than 5 hours, was the same as that of the original casein, while the products, obtained by alkali treatment for more than 24 hours, showed distinctly lower figures.

b. Deaminized casein. Preparations of different degree of deamination were prepared according to the method of Kitajima (1935). 10 g. casein were taken in a litre flask and dissolved in 250 cc. 10% acetic acid. The flask was placed in a thermostat at 37°C and under aeration with carbon dioxide, 10% sodium nitrite solution was dropped into the solution. The amounts of the nitrite solution employed were 30, 60, 90 and 120 cc. respectively. After 20 minutes of constant stirring, 10% urea solution was added, until the solution gave no longer odour of nitric oxide. The yellow precipitate was filtered off on a Buchner funnel, thoroughly washed by repeated stirring with water and filtering, and then dehydrated by grinding up with absolute alcohol. The yellowish powder thus obtained was filtered
off by suction and dried. The preparations were called D₃₀, D₆₀, D₉₀ and D₁₂₀, according to the amount of nitrite solution used for deamination. The amino and total nitrogen contents of these deaminized caseins are shown in the following Table II.

Table II

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Total nitrogen (Microkjeldahl, % of dry weight)</th>
<th>Amino nitrogen (Van Slyke, % of dry weight)</th>
<th>Amino-N Total-N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein acc. to Hammarsten (D₃₀)</td>
<td>14.5</td>
<td>0.81</td>
<td>5.6</td>
</tr>
<tr>
<td>Casein D₆₀</td>
<td>14.2</td>
<td>0.68</td>
<td>4.7</td>
</tr>
<tr>
<td>Casein D₉₀</td>
<td>14.7</td>
<td>0.63</td>
<td>4.3</td>
</tr>
<tr>
<td>Casein D₁₂₀</td>
<td>14.8</td>
<td>0.55</td>
<td>3.7</td>
</tr>
<tr>
<td>Oasein D₁₂₀</td>
<td>13.95</td>
<td>0.25</td>
<td>1.8</td>
</tr>
</tbody>
</table>

c. Iodized casein was prepared by the method of Ludwig and Mutschenecher (1939). The product obtained was a yellowish powder. Its iodine content was determined by Prof. Uchino in the Nagasaki Medical College according to the method of Seidell (1911) and by means of Pulfrich's photometer, using a stratum thickness of 10 mm. and the filter S₅₀. It gave I, 10.74% of dry weight.

d. Acetyl casein. Acetyl casein was prepared according to the method described by Hendrix and Paquin (1938). Determination of acetyl was carried out by the method reported by the same authors. It gave acetyl, 5.06% of dry weight.

Experimental Procedure

The experimental procedure was exactly the same as mentioned in my previous paper (Uchino, 1940).

Enzyme: A 1% solution of commercial pepsin (Grübler) in 0.1 N hydrochloric acid was used.

Substrates: 2% solutions of the derived caseins were employed. The water content of each preparation was previously examined and the corresponding excess amounts were weighed in order to obtain solutions of the required concentration.

Into each of five conical flasks were pipetted 2.0 cc. of 1 M glycine-hydrochloric acid buffer of varying pH and 4.0 cc. of substrate solution. The flasks were then placed in a thermostat at 30°C. Into each of the flasks were then added 2.0 cc. of the enzyme solution, previously warmed to 30°C. One of the flasks was titrated immediately after the addition of enzyme. With three other flasks further titrations were performed after the lapse of 10, 20 and 40 minutes respectively. The titrations were carried
out by the method of Wilstätter and Waldschmidt-Leitz in 90% alcohol, by the use of N/5 KOH solution and with thymolphthalein as indicator. The solution in the remaining flask was used for the determination of pH electrometrically. At the same time two control experiments were carried out, using only buffer and substrate on the one hand and using buffer and enzyme on the other. These control experiments gave always negligible values.

**Experimental Results**

1. Experiments with casein. The activity of the enzyme solution was at first tested, using untreated casein as substrate. Fig. 1 shows the initial hydrolysis rates of casein at varying pH of the medium.

![Graph showing the action of pepsin on casein](image)

It will be seen from Fig. 1 that the highest proteolytic activity is attained at pH 0.87.

2. Experiments with alkali treated caseins. The results of the experiments with alkali treated caseins C₁, C₂, C₅, C₂₄, C₃₆ and C₄₈ are shown in Figs. 2-7 and the curves representing the highest initial peptic activities for these derived caseins, each at its own optimum pH, are summarized in Fig. 8.
Fig. 2. Action of pepsin on alkali treated casein C₁

Fig. 3. Action of pepsin on alkali treated casein C₂
Fig. 4. Action of pepsin on alkali treated casein C₁

Fig. 5. Action of pepsin on alkali treated casein C₂
Fig. 6. Action of pepsin on alkali treated casein C₉₁

Fig. 7. Action of pepsin on alkali treated casein C₉₄
Dakin and Dudley (1913) examined the behaviour of racemized casein, when subjected to the actions of pepsin and trypsin, and found that it is extremely resistant to the proteolytic enzymes. Lin, Wu and Chen (1928) pointed out, on the contrary, that racemized casein is easily digested by pepsin and trypsin, though to a slightly less extent than the natural protein. Fig. 8 shows us that the alkali treated caseins are capable of being hydrolyzed by pepsin to a considerable extent, but much slower than the original protein. The optimum pH undergoes no change and lies mostly at pH 0.9–1.0.

3. Experiments with deaminized casein. The action of pepsin on deaminized caseins $D_{30}$, $D_{60}$, $D_{90}$ and $D_{120}$ are given in Figs. 9–12. In Fig. 13 are shown the highest initial digestion rates of these derived caseins along with that of the natural one.

Nakashima (1925) observed that deaminized casein is only slowly hydrolyzed by pepsin. From Fig. 13 it follows that the initial digestion rates of the deaminized caseins by pepsin are considerably small as compared with that of the untreated casein. The optimum pH undergoes no marked change and lies at 0.9–1.0.
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Fig. 9. Action of pepsin on deaminized casein D₉₅

Fig. 10. Action of pepsin on deaminized casein D₉₆
Fig. 11. Action of pepsin on deaminized casein D₉₀

Fig. 12. Action of pepsin on deaminized casein D₁₂₀
Fig. 13. Initial rates of hydrolysis of ordinary and deaminized caseins by pepsin

Fig. 14. Action of pepsin on iodized casein
Fig. 15. Action of pepsin on acetyl casein

Fig. 16. Initial rates of hydrolysis of ordinary, iodized and acetyl caseins by pepsin
4. Experiments with iodized and acetyl casein. The initial rates of peptic hydrolysis of iodized and acetyl caseins are shown in Figs. 14 and 15. In Fig. 16 are compared the highest digestion rates of these derived proteins with that of the original protein.

From Fig. 16 it is clear that iodized casein is attacked by pepsin with slightly less speed than in the case of the natural one, while the peptic digestion of acetyl casein takes place with considerable slowness. The optimum pH lie in both cases at 0.9–1.0.

Summary

The comparative cleavability of a series of derived caseins by pepsin was examined in relation to the initial hydrolysis rates as well as to the optimum pH of the enzyme action.

1. Alkali treated casein is split by pepsin to a considerable extent, but much slower than the untreated protein.
2. The hydrolysis rate of deaminized casein by pepsin is extremely small as compared with that of the natural protein.
3. Acetyl casein is attacked by pepsin with marked slowness, while the hydrolysis of iodized casein proceeds at nearly the same rate as that of the natural one.
4. The optimum pH for the action of pepsin on these derived proteins are the same as that for untreated casein and lie mostly at 0.9–1.0.

In conclusion the author desires to thank Prof. Dr. Toyoo Uchino in the Nagasaki Medical College for his kind direction throughout this investigation. Thanks are also due to Dr. K. Kuwashima, ex-director of the Hokkaido Red Cross Hospital, for his continued interest and encouragement.

References

Nakashima (1925): J. of Biochem., 5, 293.
Uchino (1940): J. of Biochem., 31, 323.