ON THE MODE OF ENZYMIC HYDROLYSIS OF PROTEIN. PRELIMINARY. ON THE TRYPTIC DIGESTION OF WHOLE CASEIN.

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Protein, of which the molecular structure is extremely complicated, has a unique significance in biology. On the other hand, each proteinase has definite specificity of substrate, protein, and hydrolyzes such peptide bonds as are present in the substrate in a certain structural composition. Therefore the specific structure of protein is an expression of the specificity of the enzyme which hydrolyzes the substrate. It follows that, in order to define the structure of protein, it may be quite important to study the mode of enzymic hydrolysis of protein and the specificity of proteinase.

On these two points extensive studies by Bergmann and Fruton[1),(2),(3)] have yielded information about synthetic peptides, since Michaelis' concept of the enzyme-substrate compound has been proposed, it has been assumed that this combination would result in the activation, and subsequent hydrolysis, of an adjacent peptide bond of the substrate. The net charge of protein appears to be changed by this combination.

As an investigation along this line, electophoretic study of protein hydrolysis in earlier stages of digestion is reported in this paper. Some investigation of peptic digestion of egg albumin along this line has been previously made by Tiselius and Eriksson[4] at optimum temperature. But the digestion at optimum temperature seemed to have the high degree of digestion and to be not suitable for electrophoretical analysis of digestion product. Accordingly digestion at 4°C was employed in tryptic digestion of whole casein in this work.

EXPERIMENTAL

1. Materials

Whole casein was reprepared according to the method of Hammarsten. It contains three components in electrophoretic patterns.

The enzyme used was trypsin powder (made by Wako Company); it was not crystalline.

Table 1. Composition of materials.

Fraction	Total-N %	Total-NH_2-N %	Free-NH_2-N %
Whole casein	13.917	11.219	0.880
Trypsin	15.127	—	3.772

2. Measurements

Electrophoretic analysis was proceeded by HT-B type Tiselius Apparatus (Hitachi

Seisakusho Company) and the scale of cell was 2 × 15 × 50 mm. On the observation of patterns, the ascending boundary was adopted, which seemed to give stable and sharp boundary in casein.[5] Micro Kjeldahl method was used for the determination of total nitrogen, and the degree of hydrolysis was determined by Van Slyke's.

3. Samples

In electrophoretic analysis, specific refractive increment is a very important factor to decide a component according to optical method, and moreover it is affected by the thickness of cell. A small cell, 2 × 15 ×50 mm. should be used; it might be impossible even at 5.461 Å of wave length to determine any component, which has a smaller specific refractive increment than that of 3×10^{-5}.[6] As the specific refractive increment of protein is 0.0018~0.0019 / g. in ordinary, no boundary appears when the amount of protein in 15 mm. cell is less than about 0.015 g. / 100ml.

And so in this experiment, the amounts of trypsin were adjusted so as not appear in boundary.

One gm. of casein was dissolved in 90 ml. of each pH phosphate-borax buffer (μ=0.10), and casein solution was dialyzed for the same buffer for two days at 4°C. (sample : buffer=1 : 50 in volume).

0.09 g. of trypsin was dissolved in 100 ml. each pH phosphate-borax buffer (μ= 0.10) and the supernatant solution to be acquired from centrifuged trypsin solution was dialyzed for the same buffer for two days at 4°C. (sample : buffer= 1 : 50 in volume).

Composition of sample :

Table 2. Casein

Fraction	pH 7	pH 8	pH 9
Casein g./100ml.	0.968	0.990	0.996
Total-N mg./100ml.	134.78	137.81	138.67
Free-NH_2-N mg./100ml.	8.52	8.71	8.76

Table 3. Trypsin

Fraction	Trypsin g./100ml.	Total-N mg./100ml.	Free-NH_2-N mg./100ml.
Trypsin pH7,8,9	0.090	13.63	3.39

In digestion, one part of trypsin solution wes added to nine parts of casein solution, trypsin solution became 0.009 g trypsin per casein solution 100ml. and did not appear in boundary.

4. Digestion at 37°C

In order to know the optimum pH of this trypsin and the state of digestion, preliminary investigation was made at 37°C. Three ml. of trypsin solution was added to 27 ml. of casein solution and was digested at 37°C. The obttained results were as shown in Table 4.

It is evident that the optimum pH of this trypsin to casein was pH 9 and the next pH 8, as table 4 B shows.

5. Digestion at 4°C.

As the digestion at 37°C seemed to indicate a high degree of digestion and to be not suitable for electrophoretical analysis in studying the digestion of casein with trypsin, digestion at 4°C was carried through in the same manner at 37°C.

Table 4. Digestion of casein with trypsin at 37°C.

A. Free NH_2-N mg per 100 ml. sample.

Time (hour) \ Fraction	pH 7	pH 8	pH 9
0	7.67	7.84	7.89
½	10.29	14.84	16.24
1	11.75	18.06	20.15
1½	13.93	22.31	26.23
2	16.29	26.07	33.32
2½	18.65	29.51	39.83
3	21.75	33.34	43.62
3½	25.04	37.79	45.38
4	28.46	40.73	45.39
4½	31.74	42.03	45.39
5	33.92	42.05	—
5½	35.25	42.06	—
6	35.53	—	—

B. Per cent of free NH_2-N for total NH_2-N.

Time (hour) \ Fraction	pH 7	pH 8	pH 9
0	7.84	7.83	7.84
½	10.52	14.84	16.14
1	12.01	18.06	20.02
1½	14.24	22.31	26.06
2	16.65	26.07	33.11
2½	19.07	29.51	39.58
3	22.24	33.34	43.35
3½	25.60	37.79	45.10
4	29.10	40.73	45.11
4½	32.45	42.03	45.11
5	34.68	42.05	—
5½	36.04	—	—
6	36.33	—	—

The results shown in this table were the average of three digestions; free NH_2-N of trypsin was subtracted.

As table 5A and B shows, the amounts of free NH_2-N had decreased in addition of trypsin solution to casein, before it was increased by digestion. The decreasing ratio of NH_2-N was the greatest in optimum pH 9, the next at pH 8.

6. Electrophoretic analysis of tryptic digestion of casein at 4°C.

The partially digested casein solution was investigated in the HT-B type Tiselius Apparatus (Hitachi Seisakusho Company) at 4°C.

At first, dialyzed casein and trypsin solution, which were previously described under "Samples", were mixed at the ratio of 9 : 1, and digested at 4°C for the expected time. Digested casein solution was immediatly electrophoryzed in each pH phosphate-borax buffer (μ=0.10), so that some low molecular material which was produced in digestion would not dialyze out of the bag. But the accurate measurement of mobilities could not be expected. The patterns obtained were as shown in Fig. 1.

Table 5. Digestion at 4°C of casein with trypsin.

A. Free NH_2-N mg per 100ml. sample.

Fraction / Time (hour)	pH 7	pH 8	pH 9
0	7.67	7.84	7.89
Addition with trypsin	5.44	4.75	2.48
½	4.75	4.19	5.03
1	2.48	2.48	5.88
2	3.30	4.23	6.78
3	4.12	5.75	7.69
4	4.97	6.23	8.60
5	5.54	6.62	9.50
6	5.92	7.01	10.38
9	7.00	8.12	11.26
12	8.15	9.27	12.10
15	9.26	10.40	12.66
18	10.41	11.54	13.23

B. Per cent of free NH_2-N for total NH_2-N.

Fraction / Time (hour)	pH 7	pH 8	pH 9
0	7.84	7.83	7.84
Addition with trypsin	6.01	4.75	2.46
½	4.85	4.19	4.99
1	2.53	2.43	5.84
2	3.37	4.23	6.73
3	4.21	5.75	7.64
4	5.08	6.23	8.54
5	5.66	6.62	9.44
6	6.05	7.01	10.31
9	7.15	8.12	11.19
12	8.33	9.27	12.02
15	9.46	10.40	12.58
18	10.64	11.54	13.14

The results tabulated here were the average of three digestions; free NH_2-N of trypsin was subtracted.

DISCCUSION

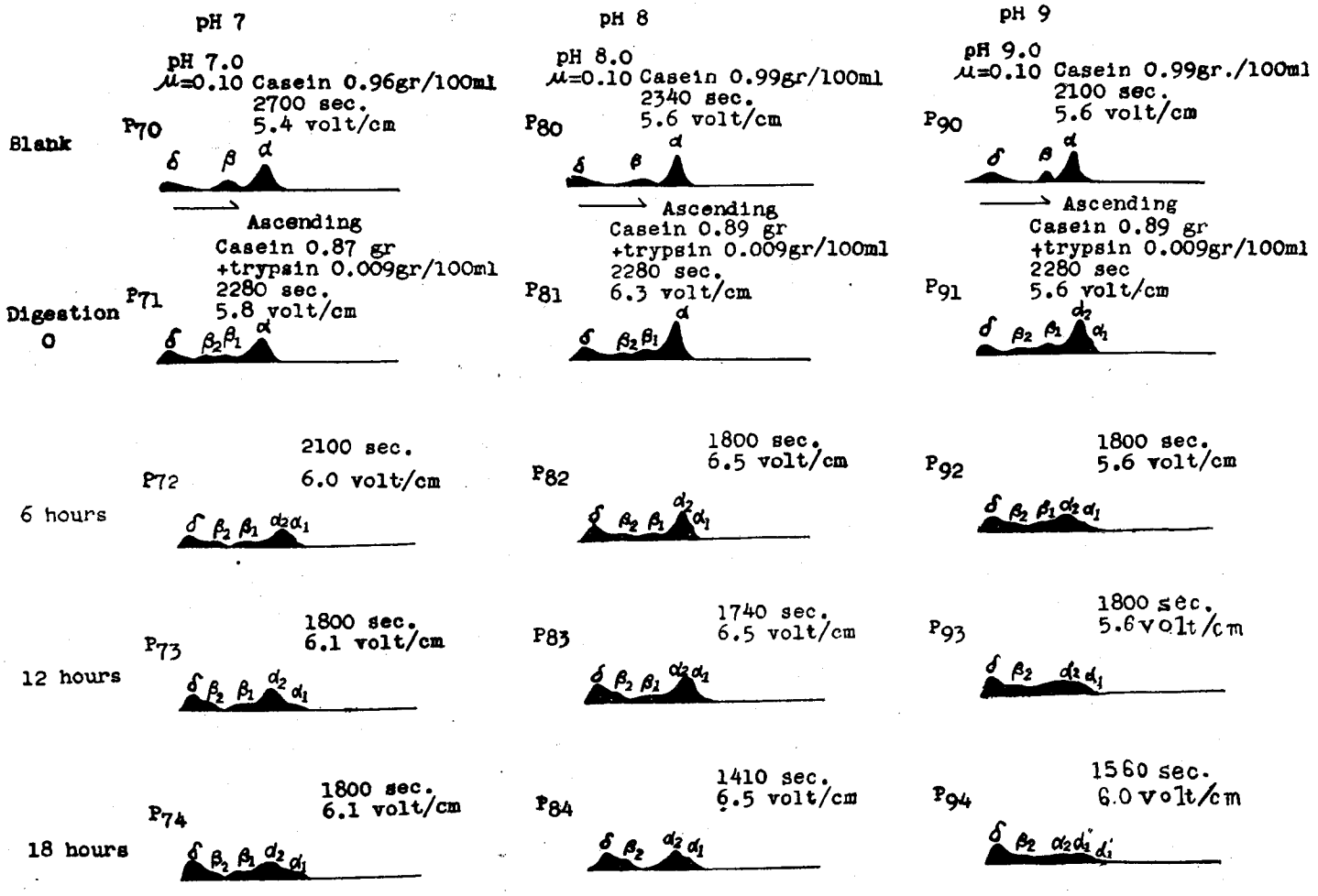
Fig. 2. and Fig. 3 are illustrated by Table 4B and Table 5B.

As shown by Fig. 2, optimum pH of this digestion at optimum temperature was pH 9, the second pH 8, and the last pH 7. The digestion at 4°C had the same tendency, but in earlier stage of digestion. it was different from the digestion at 37°C. That is, owing to the addition of trypsin. the free NH_2-N of casein solution decreased suddenly, and the speed of that decreasing tendency in respect to time was in proportion pH. Moreover, the minimum point of decreasing free NH_2-N was about the same in each pH.

It may be supposed that the decrease is due to a combination of trypsin with the substrate, casein, as in the Michaelis' concept of the enzyme-substrate compound. That is to say, trypsin combines free NH_2-groups of side chain in casein and this combination would result in the activation, and subsequent hydrolysis, of an adjacent peptide bond of the casein.

The digestion at 4° C is so slow in comparison with that at 37° C, that this decreasing

Fig. 1. Electrophoretic patterns of partially digested casein at 4°C.



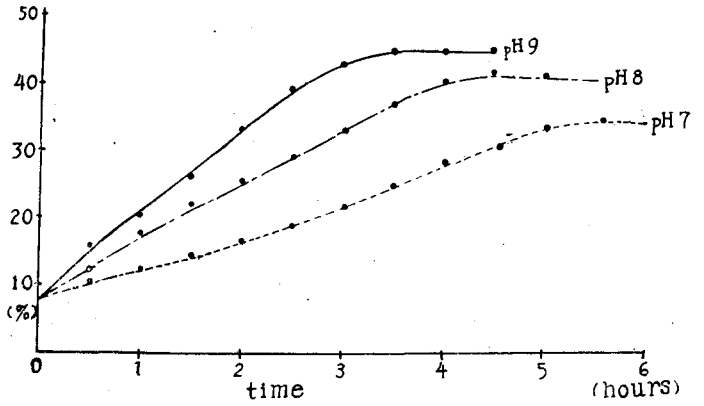


Fig. 2. Digestion at 37°C of casein with trypsin (per cent of free NH_2-N for total NH_2-N).

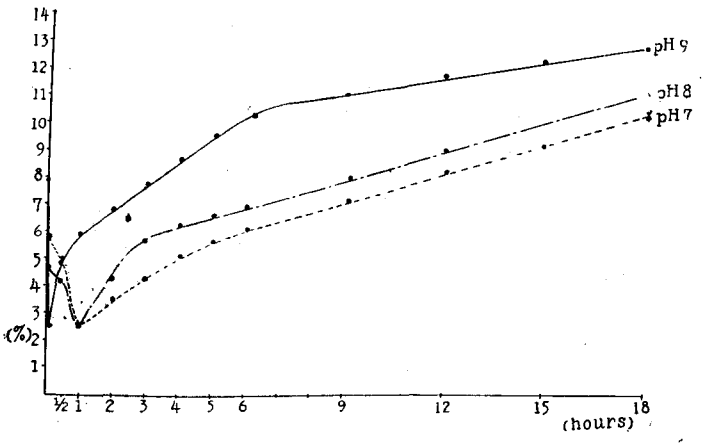


Fig. 3. Digestion at 4°C of casein with trypsin (per cent of free NH_2-N for total NH_2-N).

may be observed.

The calculated results of electrophoretic analysis were as shown in Table 6.

"Digestion 0" in Table 6 and Fig. 1 show the time of addition of trypsin, but electrophoretic time was 2280 sec. (38 minutes), then digestion may be progressing in this period. According to Fig. 3, digestion at pH 8 and pH 7 decreases in this period, but that of pH 9 increases. When trypsin had been added to casein solution "Digestion 0", a new component β_2 appeared. It is not exactly certain that β_2 may be enzyme-substrate combination, but a slight preliminary change before casein was broken down to small particles was observed and the ratio of produced β_2 was the greatest in the pH 9 sample.

According to the increase in the digestion, high mobility-components and low mobility-components which are both inhomogeneous, were produced. In addition to that, the amounts of low mobility products were greater than those of high mobility products and the ratio of these products was the greatest in pH 9 sample.

In fraction α, component α_2 did not change the mobility. It has not been broken down to small particles, which are considered unchanged α-casein. In the case of digestion of casein at 4°C, a few molecules should be attached and solwly broken down to the end-products.

According to the mechanism of enzymic proteolysis as described By Fruton, [7] it would be expected that at first trypsin combines with the side chain of substrate and whereas trypsin which can approach the peptide bond of substrate split the peptide bond.

SUMMARY

In order to learn the mode of enzymic proteolysis, the study of tryptic digestion on

Table 6. The components and mobilities of digested casein.

Digestion time (hours)	Fraction	pH 7					pH 8					pH 9				
		No.	α		β		γ	No	α		β		γ	No.	α		β		γ
Blank	ratio (%)	70	64.3		13.6		22.1	80	62.5		13.8		22.7	90	82.7		13.7		23.6
	mobility		8.4		5.1		−0.5		9.3		5.9		−0.6		9.8		7.1		1.0
0	ratio (%)	71	58.3		β_1 16.6	β_2 5.0	20.1	81	52.9		β_1 10.5	β_2 11.9	24.7	91	α_1 3.3	α_2 49.9	β_1 13.4	β_2 13.1	20.3
	mobility		8.5		5.1	2.9	−0.7		9.3		6.4	3.6	0.2		10.7	9.9	6.6	3.5	0.3
6	ratio (%)	72	α_1 10.4	α_2 36.5	β_1 13.9	β_2 11.4	27.8	82	α_1 18.3	α_2 35.3	β_1 10.6	β_2 8.2	27.6	92	α_1 7.5	α_2 33.7	β_1 12.5	β_2 18.7	27.6
	mobility		9.9	8.5	5.2	2.7	−0.8		10.0	9.8	6.2	3.5	−0.2		10.8	10.0	6.3	3.3	−0.5
12	ratio (%)	73	α_1 11.2	α_2 35.0	β_1 13.2	β_2 9.8	30.8	83	α_1 11.5	α_2 34.7	β_1 14.7	β_2 9.0	30.1	93	α_1 22.4	α_2 30.0		β_2 17.0	30.6
	mobility		10.2	8.6	5.6	2.7	−0.3		10.1	9.4	6.3	3.4	−0.7		11.4	10.0		3.4	−0.6
18	ratio (%)	74	α_1 10.8	α_2 33.3	β_1 18.0	β_2 7.3	30.6	84	α_1 17.6	α_2 32.3		β_2 8.4	41.7	94	α_1 α_1' 7.0	α_2 α_1'' 17.6 28.3		β_2 12.1	46.9
	mobility		10.6	8.7	5.2	2.8	−0.4		11.4	9.5		3.4	0.3		12.6	11.0 10.0		3.3	−0.2

mobility : 10^{-5}cm^2/volt. sec.

whole casein at 4°C was carried out by Electrophoresis and by the determination of free NH_2-N. The results obtained were as follows.

1) In early stage of digestion, when trypsin solution was added to casein solution, the free NH_2-N of casein decreased suddenly (Table 5B and Fig.3).
2) The decrease seems to be due to the fact that trypsin combines free NH_2- groups of side chain in casein.
3) With electophoretic analysis, a slight preliminary change before casein was broken down to small particles was observed and the ratio of the new component was the greatest in optimum pH (Table 6 and Fig. 1).
4) In accordance with the increase in digestion, high mobility-components and low mobility-components both inhomogeneous, were produced. (Fig. 1 and Fig. 6).

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REFERENCES

1) Bergmann, M., and Fruton, J. S. (1937): J. Biol. Chem., Vol. 117, 189.
2) Bergmann, M., and Fruton, J. S. (1938): J. Biol. Chem., Vol. 124, 321.
3) Bergmann, M., and Fruton, J. S. (1941): In Advances in Enzymology, Vol. 1. Interscience, New York.
4) Tiselius, A., and Eriksson Quensel, I-B. (1939): Biochem. J., Vol. 33, No. 11.
5) Kondo, K., Yonezawa, and Morita. (1950): *Shokuryo-Kagaku Kenkyusho Hokoku* No. 3.
6) Hess, E. L. (1951): Science, Vol. 113, 709.
7) Fruton, J. S. (1946): "In Currents in Biochemical Research", Interscience, New York, p. 123.

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