[CONTRIBUTION FROM THE VENEREAL DISEASE EXPERIMENTAL LABORATORY, U. S. PUBLIC HEALTH SERVICE, UNIVERSITY OF NORTH CAROLINA, SCHOOL OF PUBLIC HEALTH]

Synthesis of Protein-like Substances by Chymotrypsin from Dilute Peptic Digests and Their Electrophoretic Patterns*

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The present electrophoretic studies and our other investigations show that the high molecular weight protein-like substances produced by chymotrypsin from peptic digests of proteins are of a well-defined chemical nature. Such substances have so far been synthesized only by chymotrypsin. The mechanism of the present synthesis, and that of protein synthesis in the living organism are not known.

Introduction

In recent communications[1-3] the enzymic synthesis of insoluble protein-like substances of high molecular weight (250,000–500,000) was reported. These compounds were prepared by the addition of a small quantity of chymotrypsin to neutralized protein-free concentrates of peptic digests at *p*H 7.3 and a temperature of 37°. The digests were obtained from various proteins, and contained about 45% total solids on an ash-free basis.

It has now been observed that similar substances are formed from dilute protein digests containing only about 4% total solids if a 26% solution of sodium chloride is used as the diluting agent. The synthetic substances have been subjected to electrophoretic analysis and their patterns have been recorded. The effect of sodium chloride could be explained by assuming that sodium chloride inhibits the hydrolytic action of this enzyme; an alternative explanation is that the synthetic substances are very insoluble in the salt solution. In view of these results the influence of some physiologically important salts on the hydrolytic action on chymotrypsin has been investigated.

Experimental

Methods for the preparation of peptic digest concentrates and for the synthetic protein-like substances have been described previously.[3] The following principles were employed. Commercial egg albumin powder, serum albumin (Fraction V, Armour), blood fibrin and zein were digested at *p*H 1.5 to 1.8 with pepsin in the presence of toluene. The filtrates were adjusted to *p*H 7.3 and concentrated on the steam-bath to contain 310 to 460 mg. solids per ml. of ash free basis. From such digest concentrates, synthetic protein-like compounds were prepared by adding a small amount of crystalline chymotrypsin (Worthington Biochemical Laboratory). Sodium fluoride was used to control contaminants. The insoluble synthetic products were washed with water, dissolved in 0.02 *N* sodium hydroxide and dialyzed against distilled water which had been adjusted to about *p*H 10 with sodium hydroxide. It was found unnecessary to dialyze the synthetic products prior to drying, since in preparing the reconstituted dried products for electrophoresis, extensive dialyses against an acid or alkaline buffer of definite ionic strength were required.

Synthesis of Protein-like Substances from Dilute Peptic Digests.—To 100 ml. of the clear peptic digest concentrate of commercial egg albumin having a *p*H of 7.3 and containing 48 mg. of Kjeldahl nitrogen, 454 mg. of total solids (including ash) and 1 mg. of sodium fluoride per ml., 900 ml. of a 26% sodium chloride solution was added. A very faint precipitate formed which was removed by centrifuging. To the supernatant, having a *p*H of 7.3, 50 mg. of crystalline chymotrypsin (Worthington Biochemical Laboratory) dissolved in 5 ml. of distilled water was added. After 3 hours at 37° the solution became very turbid, and within 24 hours a large precipitate formed. Another sample contained 100 ml. of the peptic digest concentrate, without previous dilution and 50 mg. of chymotrypsin in 5 ml. of distilled water. The *p*H of this mixture was 7.3. This solution turned into a solid gel in 3 hours at 37°. After 24 hours the gel was suspended in 400 ml. of distilled water, and the synthetic product was collected by centrifuging at 2500 r.p.m. at 5°. The synthetic material was washed 3 times with a total of 1200 ml. of distilled water. The precipitate of the sample which was diluted with the sodium chloride solution, was collected by centrifuging at 5°, and was washed 3 times with a total of 1200 ml. of distilled water. The yields were 1.85 g. for the product which was obtained from the dilute digest and 3.30 g. for the product obtained from the concentrated digest. Electrophoretically and chemically both products were identical. They contained 14.3% nitrogen and 0.1% ash. Their other properties were similar to those of the products previously described.[3] The lower yield obtained from the dilute digest was due probably to the salting-in effect caused by the sodium chloride as a result of the washing. It is well known that water insoluble proteins produce such salting-in effects. Thus low concentrations of salts increase their solubility, while high concentrations decrease it, bringing about their precipitation. The suspended proteins may be removed on the addition of ethanol, acetone and other protein precipitants. Similar results were obtained when bovine albumin was the starting material. The bovine albumin peptic digest concentrate contained 43.9 mg. of Kjeldahl nitrogen and 470 mg. of total solids (including ash) per ml.

Electrophoretic Patterns of Synthetic Protein-like Substances Produced by Chymotrypsin.—The electrophoretic analyses were carried out at 0° in a modified compact Tiselius apparatus[4] (Perkin–Elmer, model 38). The Tiselius electrophoresis cell had a capacity of 2 ml., the optic channels had dimensions of 2 mm. wide, 15 mm. deep (along the optic path), and the center section was 50 mm. tall. The apparatus was operated at 2 watts.

The glycine–sodium chloride buffers (*p*H 3 and *p*H 12) had an ionic strength of 0.2.[5] The test materials (60 mg. per 6 ml.) were dissolved in *N*/50 HCl and *N*/50 NaOH. The bovine albumin fraction V (Armour) was dissolved in distilled water. All solutions were dialyzed two times (4 hours each time) against 200-ml. portions of buffer and for 24 hours against 1.5 liters of buffer, with stirring at 5°. The synthetic products were insoluble between *p*H 4 and 10. The zein product was insoluble between *p*H 3 and 10.

The Bovine Albumin Product.—The mobilities of this product (Fig. 1, A and I) and that of the original bovine albumin fraction V (Fig. 1, B and II) are fairly similar. The peaks of their patterns, however, have different contours. Pattern I indicates the presence of heterogeneous material of lower mobilities than the main component. The electrophoretic pattern produced by a mixture of equal parts of bovine albumin synthetic product and the original bovine albumin fraction V (Fig. 1, IV) differs as to the contour of its peak from that produced by those materials before they were mixed. The bovine albumin product was prepared from a concentrated peptic digest of bovine albumin.

The Egg Albumin Product.—This product (Fig. 1, C and III) shows no obvious differences from the bovine albumin fraction V product. This product was prepared from a

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(1) H. Tauber, THIS JOURNAL, **71**, 2952 (1949).

(2) H. Tauber, *Federation Proc.*, **9**, 237 (1950).

(3) H. Tauber, THIS JOURNAL, **73**, 1288 (1951).

(4) D. H. Moore and J. U. White, *Rev. Sci. Instr.*, **19**, 700 (1948).

(5) G. L. Miller and R. H. Golder, *Arch. Biochem.*, **29**, 420 (1951).

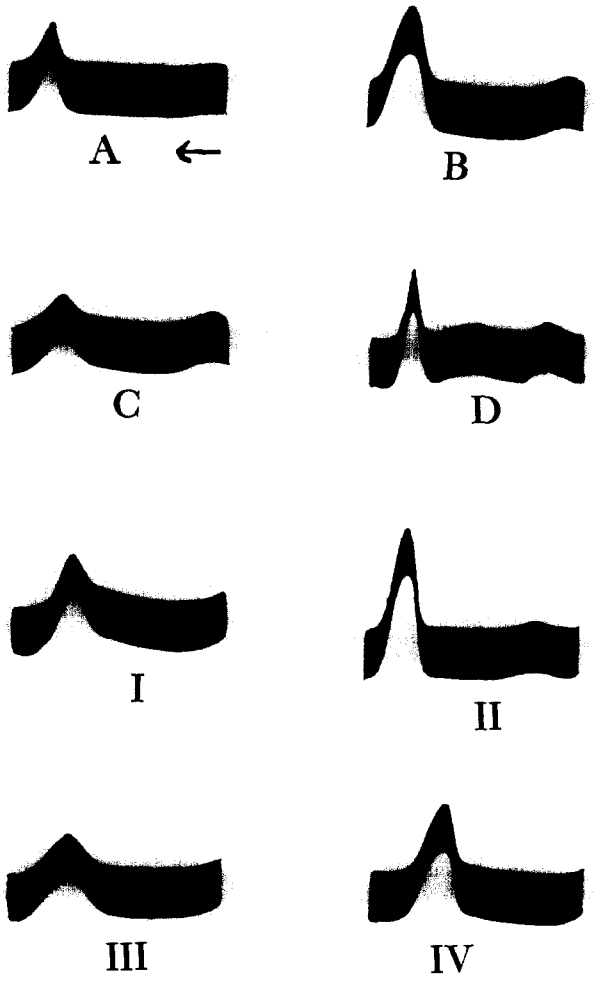

Fig. 1.—Electrophoretic patterns (descending) of synthetic protein-like substances. A through D represent the patterns obtained at *p*H 12, and I through IV those obtained at *p*H 3. A and I represent the bovine albumin synthetic product patterns. B and II are those of the original bovine albumin fraction V; and C and III are those of the egg albumin synthetic product. D is the pattern of the zein synthetic product. The zein product was insoluble at *p*H 3. Pattern IV represents a mixture of equal parts of I (bovine albumin synthetic product) and II (original bovine albumin fraction V).

dilute peptic digest of egg albumin. The electrophoretic patterns are similar to those shown by the product obtained from the concentrated peptic digest of egg albumin (not shown in Fig. 1).

The Zein Product.—The pattern of this product (Fig. 1, D) is different from the other products shown in Fig. 1. It shows more than two components, one of which, however, is very sharply defined. This product was prepared from a concentrated peptic digest of zein.

Inhibition of the Hydrolytic Action of Chymotrypsin by Neutral Salts.—In Table I are recorded results showing the inhibitory action of a series of physiologically important salts on the milk clotting action of chymotrypsin. It appears that under the conditions of the experiments all salts inhibit the milk-clotting action of chymotrypsin. Potassium iodide, sodium iodide, calcium chloride and magnesium chloride are very strong inhibitors. When acetate buffer of *p*H 7 was employed, milk clotting by chymotrypsin was much slower and the fine precipitate that formed was difficult to recognize. From Table II it may be seen that casein digestion is also inhibited by sodium chloride and by ammonium sulfate adjusted to *p*H 7.6.

TABLE I

SALT INHIBITION OF MILK-CLOTTING BY CHYMOTRYPSIN

To 0.5 ml. salt solution were added 1 mg. salt-free chymotrypsin in 0.5 ml. distilled water and 3 ml. of buffered milk. The buffered milk was prepared by adding an equal volume of homogenized milk to 1 *M* acetate buffer of *p*H 5.0. The milk clotting test was carried out at 20°.

Experiment number	Type of salt	Final molarity	Total clotting time in minutes	Delay in clotting time in minutes
1	None	...	4	0
2	NaCl	*M*/4	19	15
3	NaCl	*M*/8	9	5
4	NaI	*M*/8	25	21
5	Na_2SO_4	*M*/8	6	2
6	$NaNO_3$	*M*/8	11	7
7	KCl	*M*/8	8	4
8	KI	*M*/8	17	13
9	LiCl	*M*/8	8	4
10	NH_4Cl	*M*/8	10	6
11	$(NH_4)_2SO_4$	*M*/8	10	6
12	$CaCl_2$	*M*/8	26	22
13	$CaCl_2$	*M*/80	5	1
14	$MgCl_2$	*M*/8	22	18

TABLE II

CASEIN DIGESTION BY CHYMOTRYPSIN IN THE PRESENCE OF SALTS

To 0.1 mg. of salt-free chymotrypsin in 1 ml. distilled water, 2 ml. of salt solution and 3 ml. of 1% casein in 1 *M* acetate buffer at *p*H 7.6 were added. The digest was kept for 20 minutes at 37°. For the estimation of tryptic activity the Folin–Ciocalteu phenol reagent was employed using tyrosine as the standard. Color intensities were measured with the aid of a Cenco–Sheard–Sanford photelometer.

Experiment number	Sodium chloride, *M*	Inhibition, %	Experiment number	Ammonium sulfate, *M*	Inhibition, %
1	1.2	42	3	1.0	43
2	0.6	20	4	0.5	20

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