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THE ENZYMATIC FORMATION OF THE VASOPRESSOR POLYPEPTIDE SUBSTANCE A BY A CRYSTALLINE ENZYME OF *BACILLUS SUBTILUS**

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Abstract—Previous reports have shown that substance A, a vasopressor polypeptide, could be produced by incubating crude α-amylase with fraction IV-4 of human plasma protein. Since the crude α-amylase was obtained primarily from *Bacillus subtilis*, we have tested other enzymes secreted by this organism and have found that a crystalline protease, BPN′ (Bacillus protease, strain N′) and commercially available as 'Nagarse' was very effective in the formation of substance A. The active material so produced stimulates guinea pig ileum and rat uterus and also produces a pressor response on dog, rat, and cat blood pressure. The active material is destroyed by trypsin, chymotrypsin, pepsin and Nagarse. Carboxymethyl cellulose column chromatography could not separate the active material from a standard preparation of substance A or Val^5-angiotensin-II but could separate it from Val^5-angiotensin-I. Chemical and pharmacological comparisons allowed the conclusion that the active material was similar to, if not identical with, a standard preparation of substance A.

PREVIOUS reports by Huggins and Walaszek[1, 2] have shown that a vasopressor polypeptide could be produced when fraction IV-4 of human plasma protein was incubated with crude α-amylase preparations. The active material, which was provisionally designated as substance A, was shown not to be bradykinin, substance P, vasopressin, oxytocin, or angiotensin decapeptide. Using a partially purified preparation of the active polypeptide,[3] Walaszek *et al.*[4] have recently reported data which classified substance A as very similar to an angiotensin octapeptide. In all of these studies[1–4] crude α-amylase was used as the enzyme; it is of interest that, commercially, this enzyme is prepared from the growth medium of *Bacillus subtilis*. Since it did not seem possible that a carbohydrate-splitting enzyme could be responsible for the apparent peptidic activity, we have assayed the crude α-amylase preparations and found them to have a high level of proteolytic activity.[5] This observation led us to test other enzymes secreted by this organism. One of these, a crystalline proteinase known as BPN′ (Bacillus protease, strain N′) and commercially available as Nagarse,[6] was very effective in the enzymatic formation of substance A. In this report we present data

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showing some of our studies with this enzyme in which the active material produced is similar to, if not identical with, our original standard substance A.

EXPERIMENTAL

Isolated smooth muscle

Preparations of guinea pig ileum and rat uterus were used. The guinea pig ileum was suspended in Tyrode's solution at 35° in a 10-ml organ bath, and rat uterus was suspended in de Jalon's solution at 27° as described previously.[7] In some experiments, promethazine HCl (10^{-6} g/ml) was present in the bath. The contact time of the polypeptide with the tissue was 60 sec for the guinea pig ileum and 90 sec for the rat uterus. The time interval between additions was 5 min.

Blood pressure

Cats were anesthetized with pentobarbital sodium (30 mg/kg), and arterial blood pressure was recorded from a cannulated carotid artery with a mercury manometer. Injections were made via a cannulated femoral vein.

Incubation mixtures

In this report the active substance produced by the crystalline enzyme 'Nagarse' (from *B. subtilis*), will be designated as substance A_n and that produced by the crude α-amylase (*B. subtilis*, NBC 7942) will be designated as substance A_s.* The formation of substance A_n was carried out by incubating Tyrode's solution containing 10 μg of Nagarse/ml and 5mg of fraction IV-4 of human plasma protein /ml at 35°. Substance A_s is a powder which was prepared several years ago[3] from crude α-amylase NBC 7942. A standard dry powder of substance A_n was prepared as reported by Huggins and Walaszek.[3] Proteolytic activity was determined by a modification of the procedure described by Herriott[5] in which 5 mg of crude α-amylase/ml was incubated with a 1% casein solution at pH 7·5 and 35°.

RESULTS

The formation of substance A_n from fraction IV-4 of human plasma protein is shown in Fig. 1. In panels A and B, guinea pig ileum was the test organ and in panel C, rat uterus. With either test system it can be seen that maximal activity occurred after approximately 16 min of incubation with a complete loss of activity after 120 min. The incubation mixture produced a vasopressor response on the blood pressure of the cat.

In our early studies[1, 2] it was shown that the formation of substance A_s was inhibited if the crude α-amylase had been preincubated with a noncrystalline amylase inhibitor. Similar results were also obtained when Nagarse was preincubated with the same

* A lyophilized crystalline bacterial proteinase (Nagarse) from *B. subtilis* N′ was obtained from Enzyme Development Corp., 64 Wall Street, New York. Crystalline preparations of trypsin, chymotrypsin, pepsin, soybean trypsin inhibitor, and noncrystalline α-amylase inhibitor were obtained from Worthington Biochemicals Corp. Crude α-amylase, proteinase-NBC 5278, protease-NBC 1382, and subtilisin-NBC 5752 were obtained from Nutritional Biochemicals Corp. HT concentrate-F9053 and HT proteolytic123 were obtained through the courtesy of Dr. L. O. Underkofler, Miles Chemical Laboratory, Elkhart, Ind. Pronase B-34045 was purchased from California Corp. for Biochemical Research. Fraction IV-4 of human plasma protein was obtained through the courtesy of the American Red Cross and E. R. Squibb & Sons. Blood protein fractions from species other than humans came from Pentex Inc., Kankakee, Ill. Val^5-angiotensin I and II were kindly supplied by Dr. Robert Schwyzer, CIBA, Basle, Switzerland.

inhibitor before incubation with the substrate. Panel B in Fig. 1 shows the data obtained under these conditions.

Prolonged incubation of the enzyme alone produced no active product. We have obtained four different batches of the enzyme 'Nagarse' and all have had the same degree of potency with similar formation and destruction curves. None of the other Cohn fractions of human plasma proteins was an active substrate. Plasma protein fractions from species other than human vary, and none was so active as the human fraction. We have obtained several other bacterial proteinases and proteases (see footnote above), but none has been found to form an active material with fraction IV-4 from human plasma protein. On the other hand, subtilisin,[8] which is also obtained from the culture medium of *B. subtilis*, is extremely active in forming a substance with properties very similar to substances A_n and A_s.

In Fig. 2 a dose–response relationship is shown for standard preparations of substance A_n and A_s. The test organ was guinea pig ileum for the data shown in panels A and B and rat uterus for those of panel C. It can be seen that comparable dose–response curves were obtained for substance A_n and A_s.

A standard preparation of substance A_n caused hypertension in the blood pressure of dog, cat, and rat. Figure 3 shows the hypertensive response obtained with substance A_n in the cat, and, in comparison, the response obtained with substance A_s. A perceptible effect on pressure is also evident in the response to 10 μg Nagarse. No attempt has been made at the present time to ascertain whether tachyphylaxis will develop in response to repeated doses of substance A_n or to Nagarse.

Standard amounts of the active material were incubated at 35° with trypsin, chymotrypsin, pepsin, and Nagarse, and their effects on substance A_n were studied. Experiments with chymotrypsin, trypsin, and Nagarse were carried out in Tyrode's solution, whereas pepsin was incubated with the substrate at pH 2·0. Under these conditions (Fig. 4) it was found that chymotrypsin and trypsin completely inactivated substance A_n within 15 min of incubation. Although it is not shown in Fig. 4, Nagarse also inactivated the standard material during a similar period of incubation. On the other hand, only 40% of the activity was destroyed by pepsin in 30 min, as seen in Fig. 4, and 80% was lost after incubation for 1 hr. These data are in accord with those reported by Walaszek and Huggins[2] concerning the inactivation of a standard preparation of substance A_s by similar enzymes. Substance A_n has been found to be completely dialyzable; it is destroyed by incubation for 20 min at 100° in 1 N NaOH; it is not destroyed under similar conditions in 1 N HCl; it is absorbed by charcoal; its activity is not abolished on the guinea pig ileum by the addition of atropine or promethazine HCl and, finally, thioglycollate treatment does not abolish the response of the activity on the guinea pig ileum.

The data obtained to the present would indicate that substances A_n and A_s are similar, if not identical. Column chromatography has been used as an added aid in establishing this similarity. Substance A_n was mixed with equipotent quantities of substance A_s, Val^5-angiotensin-I or Val^5-angiotensin II and chromatographed on a carboxymethyl cellulose column as described by Huggins and Walaszek.[3] Figure 5 presents the data obtained under these conditions; it can be seen that substance A_n could not be separated from substance A_s or Val^5-angiotensin-II, but it could be separated from Val^5-angiotensin I. These data are in accord with those reported by

Walaszek *et al.*[4] concerning the column chromatography of substance A_S and the different angiotensins and suggest the close similarity of substances A_n and A_S.

We have compared substance A_n with A_S on three different biological test systems as a further criterion in establishing their identity. Table 1 summarizes the data obtained from parallel assays using guinea pig ileum, rat uterus, and cat blood pressure. On the basis of these assays it appears that substance A_n and A_S are very similar.

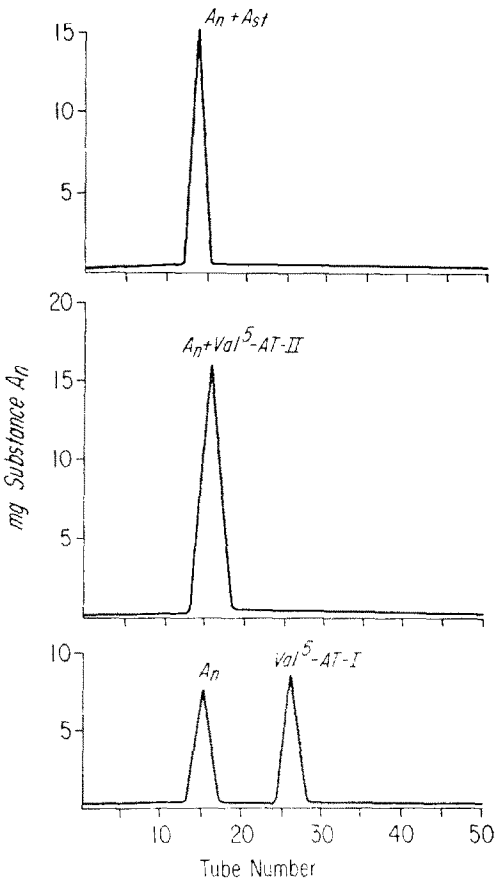

FIG. 5. Column chromatography of mixtures of substance A_n and substance A_S, Val^5-angiotensin-II or Val^5-angiotensin-I. Substance A_n (40 mg) and equiactive amounts of substance A_S, Val^5-angiotensin-I, or Val^5-angiotensin-II were dissolved in 0·02 M ammonium acetate buffer, pH 6·0, and applied to a carboxymethyl cellulose column (0·59 mEq/g). Gradient elution with 0·2 M ammonium acetate buffer, pH 7·0, through a 250-ml mixing chamber of 0·02 M ammonium acetate, pH 6·0, was accomplished in which 10-ml aliquots were collected and assayed on the guinea pig ileum. Recovery of activity from the column was approximately 90%.

Also, Table 1 lists the indices of discrimination[9] which were calculated from the data obtained with the parallel assays. It can be seen that when substance A_S is compared with A_n on cat blood pressure over guinea pig ileum, rat uterus over guinea pig ileum, and cat blood pressure over rat uterus, the index of discrimination is very nearly one. The data in Table 1 represents the average data from four different experiments and thus strongly indicate that substance A_n and A_S are identical.

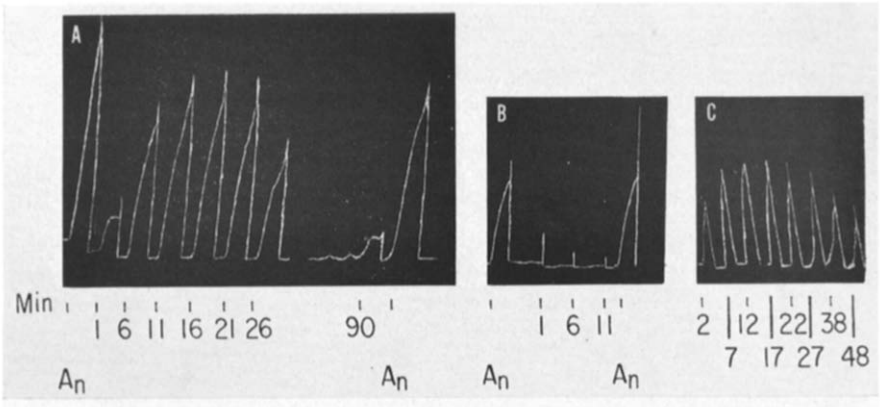

FIG. 1. Formation of substance A_n from fraction IV-4 of human plasma protein. Isolated guinea pig ileum (A and B) and rat uterus (C); A_n, standard preparation of substance A_n, 150 μg added to organ bath in panel A and 50 μg in panel B. Contractile response to 0·10-ml aliquots of an incubation mixture of Nagarse and fraction IV-4. A, Time course formation of the active material with maximal activity at approximately 16 min. In panel A the tracings were interrupted after 26 min of incubation and reinitiated at 90 min. B, Results of a duplicate experiment in which α-amylase inhibitor (5 mg/ml) was preincubated 15 min with enzyme before addition of fraction IV-4. Inhibitor had no effect alone and did not influence the response to a standard preparation of A_n. C, Time course formation of the active material as measured on the isolated rat uterus with a formation curve similar to that found with guinea pig ileum.

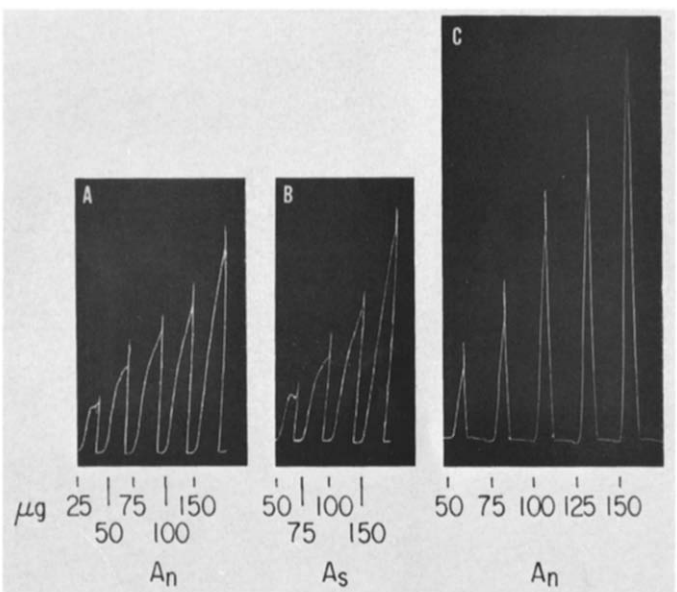

FIG. 2. Contractile response to substances A_n and A_s on guinea pig ileum (A and B) and rat uterus (C). A, Progressively increasing amounts of substance A_n on guinea pig ileum. B, Progressively increasing amounts of substance A_s on guinea pig ileum. C, Progressively increasing amounts of substance A_n on isolated rat uterus.

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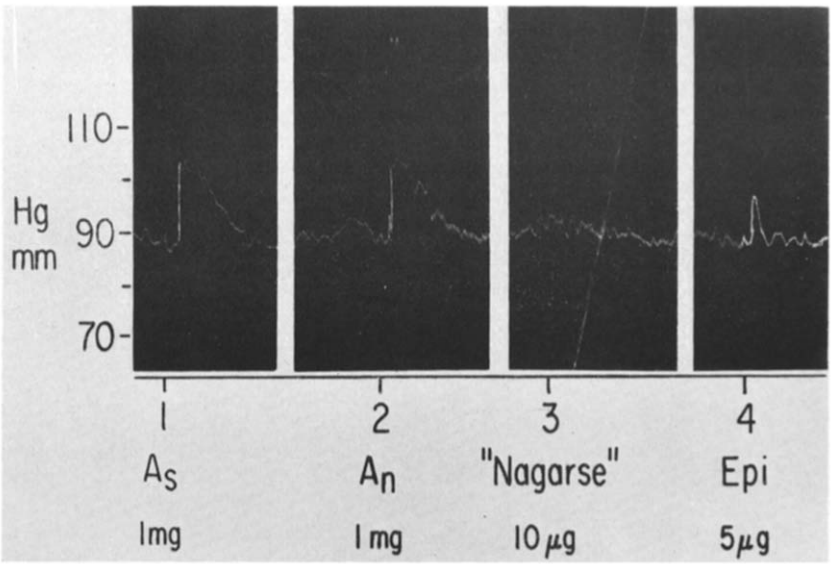

FIG. 3. Effect of substance A_n on cat's blood pressure (weight of cat, 2·2 kg). Panels 1 and 2 show the response of cat blood pressure to 1 mg of standard substance A_n and A_s. Panel 3 shows the response to 10 μg of Nagarse and panel 4 the response to 5 μg of epinephrine hydrochloride.

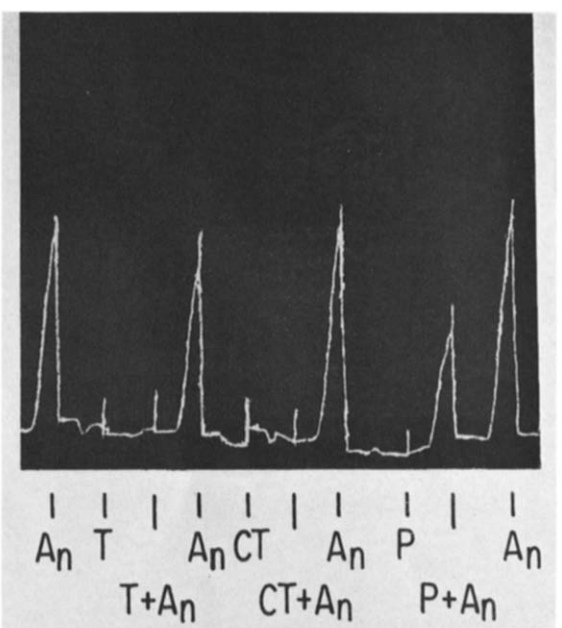

FIG. 4. Contractile response to substance A_n after incubation with proteolytic enzymes. A_n, substance A_n; T, trypsin; CT, chymotrypsin; P, pepsin. A solution containing 1 mg of substance A_n/ml was incubated with trypsin, chymotrypsin, or pepsin; final concentration of each enzyme (μg/ml) was 150, 50, 500 respectively. Incubations with trypsin and chymotrypsin were carried out at 35° for 15 min in Tyrode's solution and with pepsin as solution of 0·01 N HCl, and 30-min incubation was used. All additions represent 0·1 ml of a substance A_n solution (1 mg/ml) except P A_n in which 0·3 ml. was added.

DISCUSSION

Evidence has been presented which supports the contention that the hypertensive polypeptide substance A_n is identical with substance A_S. By means of column chromatography, further evidence was obtained concerning not only the similarity of these substances, but also the similarity of A_n to the angiotensin octapeptide. Parallel assays have also shown that substance A_n is distinct from the angiotensin decapeptides —an observation that is strengthened by the column chromatography data.

It is of interest to note that the primary source of most commercial preparations of crude α-amylase is from the growth medium for the organism *B. subtilis*. Most of our earlier studies[1–4] were carried out with this as our enzyme source. We have assayed[5] our crude α-amylase preparations and found that they possess a high degree of proteolytic activity. We therefore believe it is this proteolytic impurity in crude α-amylase preparations that is responsible for the formation of substance A. This is further strengthened by observations in our early studies[1] that only two of five crystalline α-amylase preparations possessed the ability to form the active polypeptide.

The availability of the crystalline *B. subtilis* proteinase, Nagarse, and its high degree of enzymatic activity have facilitated our study of the formation and destruction of the active polypeptide. Another enzyme, subtilisin, obtained from the growth medium of *B. subtilis*,[8] was also found to be highly active in a similar fashion. Both subtilisin and Nagarse have been well characterized as far as the physical and chemical properties and reaction kinetics are concerned. Using Lineweaver–Burk plots[10] and assaying according to contractile activity on the guinea pig ileum, we have found that the K_m for Nagarse and crude α-amylase (NBC 7942) were very similar when fraction IV-4 was used as substrate.

TABLE 1. PARALLEL ASSAYS OF THE AMOUNT OF SUBSTANCE A_n EQUIVALENT TO 1 mg OF A STANDARD PREPARATION OF SUBSTANCE A_S

Test system	Substance A_n (mg)	Indices of discrimination Test system	Index
Guinea pig ileum (GPI)	0·82	CBP/GPI	1·1
Cat blood pressure (CBP)	0·91	RU/GPI	1·03
Rat uterus (RU)	0·85	CBP/RU	1·07

Substance A_n = standard preparation obtained by incubating fraction IV-4 with the *B. subtilis* protease Nagarse.
Substance A_S = standard preparation obtained by incubation of crude α-amylase with fraction IV-4.[3]

The fact that a preparation of α-amylase inhibitor will inhibit the enzymatic formation of the active material by the crystalline enzyme Nagarse must be considered. In our early studies[1, 2] our incubation mixture contained 5 mg crude α-amylase, 5 mg fraction IV-4, and 5 mg inhibitor per ml. We have also found that the crude α-amylase enzyme contained only 1·8% protein; there was, therefore an excess of inhibitor to protein and a much larger excess of inhibitor to enzyme. When one considers that only 10 μg of Nagarse is used per ml to obtain the same degree of activity as 5 mg of crude α-amylase and that a 500-fold excess of inhibitor is present in the incubation mixture, it is possible that the inhibition observed is primarily one of competition for substrate. Support for this idea was obtained when we incubated equal amounts of inhibitor

with Nagarse, and inhibition did not occur; on the other hand, 5 mg of inhibitor produced complete inhibition. When various ratios of enzyme to inhibitor (between 1:1 and 1:500) were used, we could produce varying degrees of inhibition. However, more detailed study will be necessary in order to clarify this problem of inhibition.

There are several reports[11–14] concerning the formation of vasodepressor polypeptides by incubation of plasma globulins with enzymes from various mammalian and bacterial sources. On the other hand, there is not much available information on the formation of vasopressor polypeptides by enzymatic activity on plasma globulins. Renin,[15] which forms angiotensin decapeptide, is the best known of the latter; the pepsin and pepsitensin of Croxatto[16] is another source. Thus the crystalline enzyme from *B. subtilis* which forms an active pressor peptide may offer a convenient system for study in the area of the formation and destruction of vasoactive polypeptides.

Our studies at the present time are directed toward elucidation of the active chemical species found in this reaction and to devise systems that will enable us to study more closely the reactions involved in the enzymatic formation and destruction of active polypeptides.

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