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THE FORMATION OF A VASOPRESSOR POLYPEPTIDE BY SUBTILISIN CARLSBERG (SUBTILISIN), A PROTEOLYTIC ENZYME OF *BACILLUS SUBTILIS**

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Abstract—Subtilisin Carlsberg (subtilisin), which has proteolytic properties similar to those of subtilisin BPN' ("Nagarse"), has been shown to produce a vasoactive polypeptide when incubated with fraction IV-4 of human plasma protein. The active material, which could not be distinguished from substance A, produced a contractile response on the isolated guinea pig ileum and rat uterus and was vasopressor on cats' blood pressure. It behaves in a fashion similar to other physiologically active polypeptides with respect to alkali lability, acid stability, dialysis, and destruction by proteolytic enzymes. Divalent ions were not required for the enzymatic formation of the active substance. However, Ni^{2+}, Co^{2+} and Cu^{2+} were partially inhibitory while phenylmethylsulfonyl fluoride was completely inhibitory. Pharmacological and physiological comparisons failed to distinguish between substance A and the active material formed by subtilisin. In addition, column chromatography with carboxymethyl cellulose failed to distinguish between the two substances. Therefore, it is proposed that the vasoactive polypeptide produced when subtilisin is incubated with fraction IV-4 of human plasma protein is similar to, if not identical with, substance A.

HUGGINS and Walaszek in 1959[1,2] reported that crude alpha-amylase from *Bacillus subtilis*, when incubated with fraction IV-4 of human plasma protein, could produce a pharmacologically active polypeptide. The active material caused contraction of the guinea pig ileum and rat uterus and produced a pressor response on dog, cat and rat blood pressure. The active substance was designated as substance A and was later shown by Walaszek *et al.*[3] to be similar in physiological activity to an angiotensin octapeptide. Since a proteolytic enzyme is more likely to be responsible for the formation of a polypeptide from protein than a carbohydrate-splitting enzyme, it was suspected that the crude alpha-amylase was contaminated with a protease. On this hypothesis, Huggins *et al.*[4] have used a crystalline protease, subtilisin BPN' (Bacillus protease, strain N' from *B. subtilis*), which is commercially available as "Nagarse" These investigators have shown the formation of an active material when Nagarse was incubated with fraction IV-4 of human plasma protein. This active material, like substance A, was formed in approximately 20 min and was destroyed in about 100 min by an enzyme or enzymes present in the incubation mixture. As far as various

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chemical and pharmacological assay methods could demonstrate, this active polypeptide could not be distinguished from standard substance A.[4]

Another enzyme, subtilisin Carlsberg,[5] which is produced by a different strain of *B. subtilis*, has been shown to have proteolytic properties similar to those of Nagarse. It was, therefore, desirable to study subtilisin Carlsberg with regard to its ability to form a pharmacologically active substance by incubation with fraction IV-4 of human plasma protein. These studies form the basis for the report which follows.

EXPERIMENTAL

Isolated smooth muscle. Preparations of guinea pig ileum were used for biological assay. The guinea pig ileum was suspended in Tyrode's solution at 35° in a 10-ml organ bath. 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 the interval between additions was 5 min.

Blood pressure. Cats were anesthetized with pentobarbital sodium (30 mg/kg). 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, subtilisin Carlsberg (obtained from *B. subtilis*), will be designated as substance A_{st}; that produced by the crystalline enzyme, Nagarse (also obtained from *B. subtilis*), will be designated as substance A_N; and the active product produced by the crude alpha-amylase from *B. subtilis* (NBC No. 7942) will be designated as standard substance A. The formation of substance A_{st} was carried out by incubating a Tyrode's solution containing 10 μg subtilisin and 5 mg fraction IV-4 of human plasma protein per ml at 35°. Standard substance A is a powder which was prepared several years ago from crude alpha-amylase, NBC 7942. Standard dry powders of substance A_N and substance A_{st} were prepared as reported by Huggins and Walaszek.[6]

Materials. The crystalline bacterial protease subtilisin BPN' (Nagarse) from *B. subtilis* strain N' was obtained from Enzyme Development Corp. (64 Wall Street, New York, N.Y.). Crystalline preparations of trypsin, chymotrypsin and pepsin were obtained from Worthington Biochemicals Corp. Subtilisin Carlsberg (NBC No. 5752) was obtained from Nutritional Biochemicals Corp. 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 Corp., Kankakee, Ill. 5-Valine-angiotensin I and II were kindly supplied by Dr. Robert Schwyzer, CIBA, Basle, Switzerland.

RESULTS

The formation of substance A_{st} by the action of subtilisin on fraction IV-4 of human plasma protein is shown in Fig. 1. It can be seen that maximal activity occurred after approximately 11 min of incubation with almost complete loss of activity after 40 min. A vasopressor response in the cat was obtained after injecting an aliquot of the mixture after 12 min of incubation. Data obtained from this laboratory have shown that when dialyzed fraction IV-4 of human plasma protein was incubated with dialyzed Nagarse in distilled water, the rate of formation of active material readily occurred but the rate of destruction of the active substance seemed to be inhibited

or prolonged.* When subtilisin was treated in a similar manner and then incubated with dialyzed substrate, comparable data with respect to rate of formation were obtained. Prolonged incubation of subtilisin or substrate alone produced no active product. Several different batches of the enzyme have been obtained from several sources and all have shown the same degree of potency.

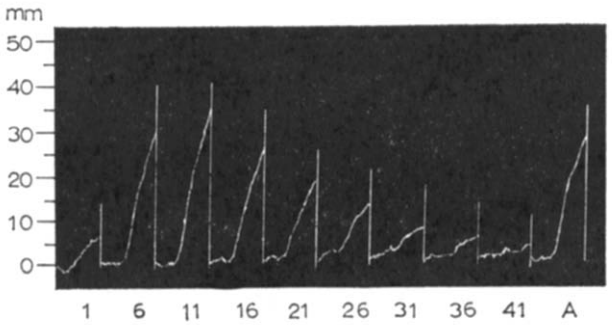

FIG. 1. The formation and destruction of substance A_{st} in an incubation mixture of fraction IV-4 of human plasma protein and subtilisin. Activity is recorded in terms of contraction of the guinea pig ileum when 0·05-ml aliquots of the incubation mixture were applied to the muscle bath. The number on the abscissa indicates time of incubation in minutes. "A" represents the contraction produced by 50 μg of standard substance A.

In Fig. 2 a dose-response relationship on the guinea pig ileum is shown for standard preparations of substance A and A_{st}. It can be seen from these data that a linear response was obtained for substance A as compared with A_{st}. Parallel assays were also carried out between substance A, a standard preparation of substance A_{st}, substance AN, and 5-valine-angiotensin II. The data obtained under these conditions show that 1 mg substance A_{st} is equivalent to 0·84 mg substance A_N, 0·79 mg standard substance A and 0·14 μg 5-valine-angiotensin II.

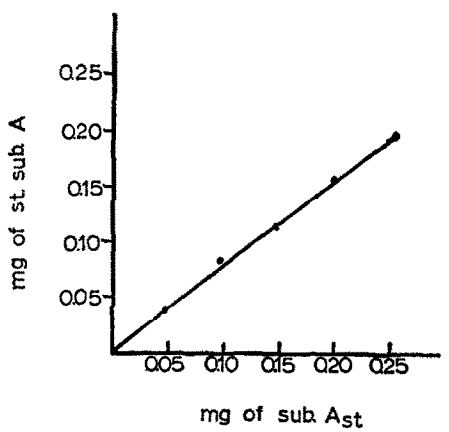

FIG. 2. Dose-response curve for substance A_{st} on guinea pig ileum. Contractile response to substance A_{st} compared with the response to the same dose of standard substance A.

* L. PAVESI and C. G. HUGGINS, *Biochem. Pharmac.* **17**, 1699 (1968).

A standard preparation of substance A_{st} caused a pressor response on the dog, cat and rabbit. Fig. 3 shows the hypertensive response obtained with substance A_{st} in the cat and, in comparison, the response obtained with substance A and substance A_N. There is no effect with small doses of subtilisin (10–50 μg) on the blood pressure of the cat; however, with larger doses in the range of 1–3 mg, a definite depressor response was obtained with subtilisin. The latter data are in agreement with those reported by Prado *et al.*[7] and by Huggins and Thampi,[8] both of whom have injected 1 mg Nagarse enzyme into recipient dogs and cats and obtained a marked depressor response.

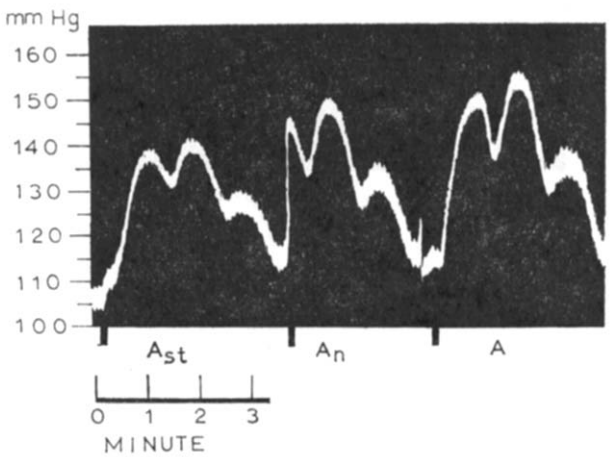

FIG. 3. Blood pressure response of a cat (2·3 kg) to 1 mg each of substance A_{st} (A_{st}), substance A_N (A_N) and standard substance A (A).

Previous results have shown that the formation of substance A_N in an incubation solution containing Nagarse and fraction IV-4 of human plasma protein was dependent upon pH and temperature. When subtilisin was incubated with fraction IV-4 of human plasma protein at different pH values and the reaction mixtures were tested for activity after 10 min of incubation, the data in Fig. 4 were obtained. Very little formation of substance A_{st} was obtained at pH 3, with maximum formation occurring at pH 7–8. Since we had shown that pH did influence the rate at which the active material was formed, it also became of interest to determine the effect of pH on the destruction of the active material. A standard preparation of substance A_{st} in a concentration of 1·0 mg/ml was incubated with subtilisin at a concentration of 10 μg/ml. The data obtained under these conditions at different pH values (see Fig. 4) show that the ability of subtilisin to inactivate the active product increased with an increase in pH, starting at pH 3 and reaching a maximum at pH 8. There is practically no destructive activity below pH 3 under these conditions. The effect of temperature was also studied on the formation and destruction of the active material, substance A_{st}. When subtilisin was incubated with fraction IV-4 at different temperatures from 0° to 100°, the data obtained under these conditions indicated that there was a slow formation of substance A_{st} at incubation temperatures from 0° to 25°. The rate then increased rapidly and reached a maximum at approximately 50°. Above this temperature the rate decreased

with increasing temperature, and formation was completely inhibited above 80°. When a standard preparation of substance A_{st} was incubated with subtilisin as described above at various temperatures, it was found that inactivation of the active material was not very appreciable below 25°. The optimum temperature was found to be approximately 45° and no enzymatic inactivation occurred above 75°.

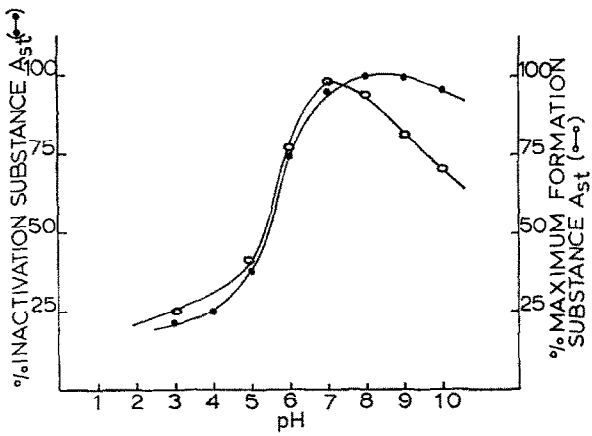

FIG. 4. The effect of pH on the formation and destruction of substance A_{st} by subtilisin. The effect of pH on inactivation (●—●) and on enzymatic formation (○—○) of the active material. Formation of substance A_{st} was carried out by incubating 5 mg fraction IV-4 with subtilisin (10 mg/ml) at different pH values for 10 min at 35°. A standard preparation of substance A_{st} (1 mg/ml) was incubated with subtilisin (10 μg/ml) at different pH values for 10 min at 35°.

TABLE 1. EFFECT OF DIVALENT IONS AND PROTEOLYTIC INHIBITORS ON THE FORMATION OF SUBSTANCE A_{st}*

Additions (10^{-3} M)	% Inhibition
Ni^{2+}	35
Cu^{2+}	19
Co^{2+}	12
Fe^{2+}	5
Ba^{2+}	5
Mn^{2+}	5
Mg^{2+}	0
Phenylmethylsulfonyl fluoride	100
Sodium iodoacetate	− 7
l-Cysteine	− 10

* A Tyrode's solution containing subtilisin (10 μg/ml) was preincubated with the above ions and inhibitors for 15 min before addition of the substrate, fraction IV-4 (5 mg/ml). Appropriate aliquots were tested for activity on the isolated guinea pig ileum and compared with an incubation mixture containing all the ingredients except the ions or inhibitors.

Several divalent ions were investigated with regard to their influence on the activity of subtilisin in the formation of substance A_{st}. Several known proteolytic activators and inhibitors were also employed. These include sodium iodoacetate, cysteine and phenylmethylsulfonylfluoride. Subtilisin and fraction IV-4 of human plasma protein, both of which had been dialyzed against EDTA, were used. The concentration of

subtilisin in the incubation mixture was 20 μg/ml and the concentration of the substrate was 5 mg/ml. The ions and inhibitors (10^{-3} M) were dissolved in a Tyrode's solution containing the enzyme and incubated for 15 min at 37°. To these solutions, substrate was added and after 15 min of incubation at 37° the solution was placed in a boiling water bath to inactivate the enzyme. Biological activity was measured on the guinea pig ileum and compared with appropriate controls. Table 1 shows the effect of these ions and inhibitors on the activity of the enzyme. It can be seen that the activity of subtilisin is partially inhibited by the heavy metals, nickel, copper and cobalt, and is completely inhibited by phenylmethylsulfonylfluoride, while the activity seemed to be enhanced by iodoacetate and cysteine. When a standard preparation of substance A_{st} was incubated at 35° with either trypsin, chymotrypsin, pepsin, Nagarse or subtilisin, the activity remaining after 15 min of incubation revealed (see Table 2) that trypsin, chymotrypsin, subtilisin and Nagarse completely inactivated substance A_{st}.

TABLE 2. EFFECT OF VARIOUS PROTEOLYTIC ENZYMES ON THE BIOLOGICAL ACTIVITY OF SUBSTANCE A_{st}*

Enzyme	Concn (μg/ml)	pH	% Destruction
Trypsin	150	7·4	100
Chymotrypsin	50	7·4	100
Pepsin	500	2·5	60
Nagarse	10	7·4	100
Subtilisin	10	7·4	100

* Substance A_{st} (1 mg/ml) was incubated for 15 min at 37° with each enzyme at the indicated concentration and pH before assay on the isolated guinea pig ileum.

The destruction by pepsin of the standard preparation of substrate A_{st} was similar to the effect of pepsin on substance AN and substance A. A standard preparation of substance A_{st} has been found to be dialyzable. It is inactivated by incubation for 20 min at 100° in 0·1 N sodium hydroxide, but is stable under similar conditions in 0·1 N HCl. It is adsorbed by charcoal. The addition of atropine, promethazine HCl, thioacetic acid (and other sulfhydryl reagents) does not affect the activity of A_{st} when assayed on either the guinea pig ileum, the rat uterus or on cat's blood pressure. The above data would tend to indicate that substance A*st* produced by the action of subtilisin on fraction IV-4 of human plasma protein is similar to, if not identical with, substance A_N and standard substance A.

Column chromatography has been used as an aid in establishing this similarity. Substance A_{st} was mixed with an equipotent quantity of substance A and chromatographed on carboxymethylcellulose columns as described by Huggins and Walaszek.[6] Fig. 5 presents the data obtained under these conditions. It can be seen that substance A_{st} could not be separated from substance A. These data are in accord with those reported by Walaszek *et al.*[3] concerning column chromatography of substance A and the angiotensins and are also in agreement with the data reported by Huggins *et al.*[4] concerning the column chromatography of substance A_N and standard substance A, and therefore support the contention of the similarity of substance A_N and A_{st}.

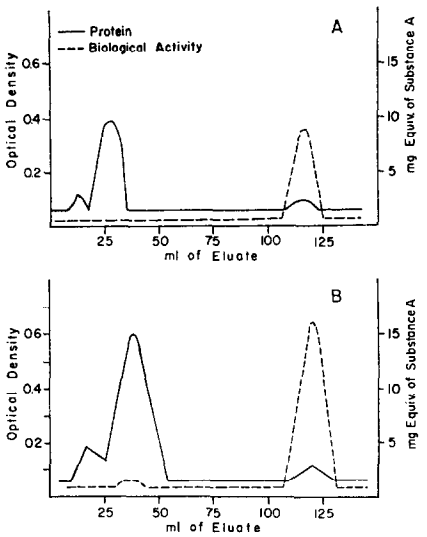

FIG. 5. Column chromatography of substance A_{st} on carboxymethylcellulose. Biologically active substances were applied to the column (1 × 30 cm) in 0·02 M ammonium acetate buffer, pH 6, with a gradient elution to 0·2 M ammonium acetate buffer, pH 7. Ten-ml fractions were collected. *A* shows the elution pattern of 15 mg substance A_{st}. *B* shows the elution pattern of 15 mg substance A_{st} plus 10 mg standard substance A. O.D. refers to absorption at 280 mμ.

DISCUSSION

Subtilisin Carlsberg, a proteinase derived from *B. subtilis*, has been shown to liberate a vasopressor smooth muscle-stimulating substance when incubated with fraction IV-4 of human plasma protein. By means of chromatography as well as by parallel assays and chemical and pharmacological comparisons, substance A_{st} was shown to be similar to, if not identical with, a substance A preparation prepared by incubation of fraction IV-4 with crude alpha-amylase or with Nagarse. Pavesi and Huggins* have shown that the Nagarse enzyme will destroy substance A_N and that this destruction occurs at pH 7 or higher. The data found for inhibitors of subtilisin activity in forming the vasopressor material are in agreement with those found by Pavesi and Huggins* with respect to the Nagarse enzyme. Both enzymes were inhibited with Cu^{2+} and Ni^{2+} ions and neither is inhibited by sulfhydryl reagents. This latter is not too surprising when one considers that cysteine has not been demonstrated to be present in either enzyme.[9,10]

In 1964, Prado *et al.*[7] reported that Nagarse (from *B. subtilis*) is a kininogenase in that an active substance was released from heat-treated horse plasma by its action. These data may be contrasted with the reports of Huggins *et al.* in 1964[4] with fraction IV-4 of human plasma protein and that of Huggins and Thampi[8] with heat-treated horse plasma, who have shown that a vasopressor rather than vasodepressor substance was released by Nagarse. This discrepancy might be explained by the varied specificity[5] of these enzymes. It would not be too surprising that several vasoactive substances might be formed from protein substrates.

* L. PAVESI and C. G. HUGGINS, *Biochem. Pharmac.* **17**, 1699 (1968).

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