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KINETIC STUDIES ON THE FORMATION AND DESTRUCTION OF THE VASOPRESSOR POLYPEPTIDE, SUBSTANCE A*

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Abstract—Kinetic studies on the enzymatic formation and destruction of substance A, a vasopressor polypeptide similar to angiotensin II, were carried out by incubating fraction IV-4 of human plasma protein with subtilisin BPN′ ("Nagarse"), a crystalline proteinase from *Bacillus subtillis*. By means of biological assay in an isometric tension apparatus, the general requirements for the formation and destruction of substance A were investigated. The optimal pH and temperature for formation were found to be 7·5 and 40°, whereas 8·5 and 40° were found to be optimal for enzymatic inactivation. Studies on formation of substance A in the absence of destruction of the active material were accomplished by incubation of the enzyme and substrate at pH 6·0. The apparent K_m for the formation of substance A by Nagarse was found to be $1{\cdot}17 \pm 0{\cdot}28 \times 10^{-5}$ M, while the apparent K_m for the same reaction with crude alpha-amylase was found to be $1{\cdot}7 \pm 0{\cdot}34 \times 10^{-5}$ M. DFP and phenylmethylsulfonyl fluoride produced 100 per cent inhibition of the enzyme. The enzymatic formation and destruction of substance A were inhibited by Cu^{2+} and Ni^{2+} and this inhibition can be reversed by histidine. Fraction IV-4 or serum from species other than human was found to serve as substrate for the enzymatic formation of a vasopressor polypeptide similar to substance A.

WE HAVE previously reported[1–5] that substance A, a vasopressor polypeptide, could be produced by incubating fraction IV-4 of human plasma protein with crude alpha-amylase, subtilisin BPN' ("Nagarse"), and subtilisin Carlsberg. Furthermore, the active material produced by these enzymes appeared to be identical in all test systems studied. Substance A was found to stimulate the isolated guinea pig ileum and rat uterus, to relax and then contract the rat duodenum, and to produce a pressor response on dog, rat and cat blood pressure. Its biological activity was destroyed by incubation with proteolytic enzymes and it could not be separated from 5-valine-angiotensin II. Finally, a neutral proteinase which required Zn^{2+} and which was also obtained from *Bacillus subtillis* was found to produce a vasoactive material when incubated with fraction IV-4 of human plasma proteins.

There are numerous reports concerning the formation of vasodepressor polypeptides by incubation of plasma globulins with enzymes from various sources. On the other hand, there is not much information available on the formation of vasopressor polypeptides from plasma globulins by enzymes other than renin. Neither of the

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mammalian enzymes, renin or plasma kallikrein, which form angiotensin I and bradykinin respectively, has been studied extensively from a biochemical standpoint due to the lack of an adequate supply of the highly purified crystalline enzyme. Thus, the crystalline enzyme Nagarse, which forms the active pressor polypeptide substance A, offers a convenient system for study in the area of the formation and destruction of vasopressor polypeptides. In this report, we are concerned with the kinetic aspects of the formation and destruction of substance A by the enzyme Nagarse.

EXPERIMENTAL

Isolated smooth muscle

(a) *Isotonic contractions.* Preparations of guinea pig ileum were suspended in Tyrode's solution at 35° in a 10-ml organ bath as described previously.[5] In some experiments, promethazine HCl (10^{-6} g/ml) was present in the bath and did not change the response of the ileum to substance A. The contact time of the polypeptide with the tissue was 60 sec and the time interval between additions was 5 min.

(b) *Isometric contraction.* Segments of guinea pig ileum were used for biological assay in an isometric contraction system patterned after those reported by Csapo in 1954[6] and by Randall *et al.* in 1961[7]. The complete system is formed by a transducer, a transducer-amplifier indicator and a recorder. The mechanical displacement of the transducer is sufficiently small so that for all practical purposes the contraction is isometric. The strength of the physical variable acting on the transducer is interpreted by the amplifier as load units and that information is recorded graphically.

The instrument is calibrated by applying known loads to the transducer and by adjusting the sensitivity controls in the amplifier circuit to obtain a desired deflection on the recorder for a given amount of load. For the data reported herein, the apparatus was calibrated to obtain a displacement of 11 mm when 0·5 g of load was applied to the transducer under maximum attenuation. A calibration graph showing the linearity in response was prepared by applying to the transducer 0·5, 1·0, 2·0 and 3·0 g of load. The height of the transducer above the muscle chamber was adjusted mechanically in order to provide and maintain a constant load of 0·5 g upon the segment of smooth muscle.

A 20-mm segment of guinea pig ileum was connected to the bottom of a 3-ml lucite muscle chamber and the other end was attached by means of a silk thread (20 mm long) to the transducer, which in turn was connected with the amplifier-recorder system. A short period of equilibration of approximately 20 min preceded the initiation of the experiment. Test substances were added at 4-min intervals and, in general, only a few spontaneous contractions occurred during the equilibration and use of the muscle for analysis. A high degree of reproducibility was obtained under these conditions and the magnitude of the response was very similar in assays performed on ileal segments obtained from different animals. In order to minimize experimental error due to variability in the response of the muscle, we calibrated the response of the smooth muscle to a standard preparation of substance A. In all cases the amplifier system was adjusted to give a response indicating 2 g of tension when 0·05 mg of standard substance A was added. Thus, it was possible to determine quantitatively the activity of an unknown solution by comparing the unknown to several different doses of the standard preparation. This procedure permitted a comparison of results obtained with ileal segments from different animals and at different time

periods. Since this system is calibrated in terms of grams of tension, most calculations and data presented herein will be in this term rather than as milligrams of a standard substance.

Blood pressure

Cats (2–3 kg) 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

Fraction IV-4 of human plasma protein was dialyzed against 0·01 M EDTA for 48 hr at 0–4°. This plasma protein was then further dialyzed against distilled water at 0–4° for 24 hr. This latter process was repeated two additional times. The dialyzed solution was collected, pooled and lyophilized to obtain a powder which was used as the substrate in all studies. The enzyme Nagarse was dialyzed under the same conditions as described for fraction IV-4 of plasma protein. It is possible that the dialysis procedures may be shortened, since the periods used were a matter of convenience in terms of technique. The formation of substance A was carried out by incubating the desired quantity of fraction IV-4 (usually 3 mg/ml) in a buffer of desired pH with the enzyme Nagarse (10 μg/ml) in a total volume of 10 ml. In this report the active material produced by the crystalline enzyme Nagarse will be designated as substance A. A standard powder of substance A was prepared as described previously[3] with Nagarse as the enzyme.

RESULTS

Our earlier studies[5] had shown that substance A was rapidly formed in an incubation mixture of Nagarse and fraction IV-4 and that on continued incubation the active material was destroyed. The data in Fig. 1 represent the results from a typical experiment using the isolated guinea pig ileum as the test system. It was obvious from these experiments that if kinetic studies were to be carried out a procedure would be necessary which would permit studies concerned only with the enzymatic formation of substance A or its enzymatic destruction. This was accomplished when we found that the pH curves for formation and destruction of substance A were quite different. In Fig. 1 the incubation mixture was Tyrode's solution at pH 7·6 showing the rapid formation and the less rapid rate of destruction of the active material. This may be contrasted with results we have obtained in which the incubation was carried out at pH 6·0 showing the same rapid rate of formation but no inactivation, even after 80 min of incubation. In addition, we found that dialyzed Nagarse had no effect on a standard preparation of substance A at pH 6·0, whereas the same material was rapidly inactivated by Nagarse in Tyrode's solution at pH 7·6. When the components of Tyrode's solution were tested for restoration of the destroying activity of the Nagarse enzyme, none was found to be effective except sodium bicarbonate and this latter was not specific, since adjusting the incubation solution to pH 7·6 restored the destroying activity completely.

The effect of pH on the formation and destruction of substance A was studied in an effort to ascertain the role of this parameter. Solutions of dialyzed fraction IV-4 (3·5 mg/ml) were prepared in distilled water and then adjusted to the desired pH.

The dialyzed enzyme (10 μg/ml) was added to each solution and the mixture was tested for substance A activity after 8 min of incubation at 37°. Numerous experiments had previously established that this time period was within the initial velocity portion of the formation curve. Similar conditions of incubation with substance A (1 mg/ml) and the dialyzed enzyme (10 μg/ml) were also carried out. The data shown in Fig. 2 were obtained under these conditions. Maximum formation of the active material

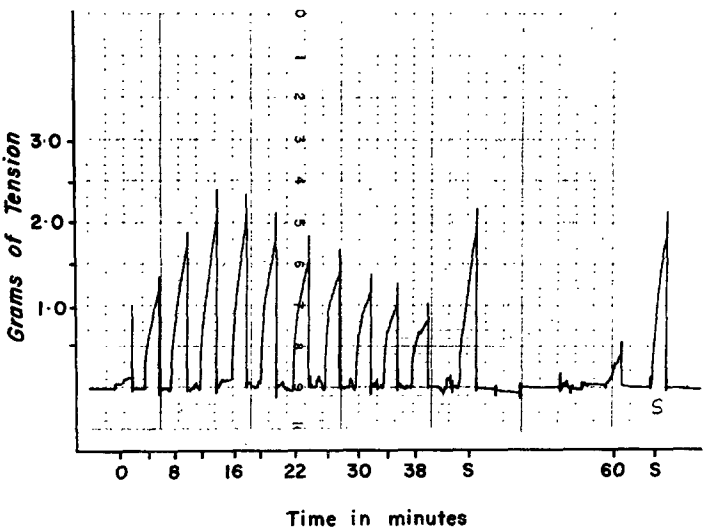

FIG. 1. Formation and destruction of substance A from an incubation mixture of fraction IV-4 and Nagarse assayed on the isolated guinea pig ileum (isometric tension system). The incubation mixture contained 35 mg fraction IV-4 of human plasma protein dissolved in 9 ml Tyrode's solution, pH 7·6, to which 1 ml Nagarse solution (0·1 mg/ml) was added. Aliquots of the incubation solution (0·05 ml) were tested at the indicated time intervals.

occurred when the reaction was carried out at pH 7·0. Furthermore, we found that only a small degree of inactivation occurred below pH 7·0, with maximum inactivation between pH 8 and pH 9. As a result of these data, subsequent experiments concerning formation of substance A were carried out at pH 6·0 and those involving inactivation were carried out at pH 8·0.

The effect of temperature on the formation and inactivation of substance A by Nagarse is shown in Fig. 3. The substrate, either fraction IV-4 or substance A powder, was preincubated at the appropriate temperature for 15 min before addition of the enzyme. Test aliquots were assayed on the guinea pig ileum after 8 min of incubation in the case of formation and after 10 min for studies on inactivation. The optimum temperature for formation and inactivation was found to be 40°. However, there was very little inactivation at 0°, whereas a considerable degree of formation occurred at this temperature; these latter data are in agreement with earlier published data with respect to the enzyme in crude alpha-amylase.[3]

The effect of substrate concentration on the velocity of the formation of substance A by Nagarse offered some problems, since a pure substrate was not available. According to Putnam,[8] fraction IV-4 contains as its major component a protein with a

molecular weight of 100,000 and we have used this for calculating the molar substrate concentration. Four concentrations of the substrate were used, e.g. 10, 20, 30 and 50 mg/ml of incubation solution. Appropriate aliquots were assayed for activity after 0, 2, 4, 6, 8, 10 and 12 min of incubation. When velocity expressed as grams of tension was plotted against time for each substrate concentration, we were able to establish

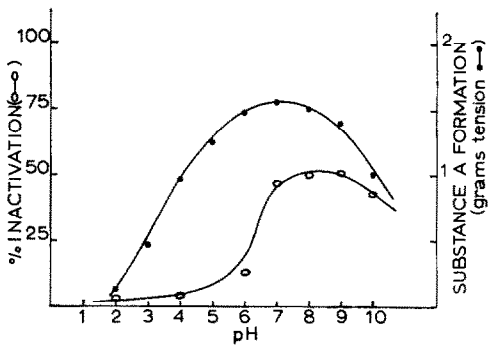

FIG. 2. The effect of pH on the formation and destruction of substance A. (●—●, formation of substance A). For studies on formation, the incubation mixture contained 35 mg fraction IV-4 and 0·05 mg Nagarse dissolved in 10 ml at the proper pH value. (○—○ inactivation of substance A). For studies on inactivation, 10 mg of a standard substance A powder and 0·05 mg Nagarse were dissolved in 10 ml at the proper pH value. The substrate, either fraction IV-4 or substance A, was dissolved in 9·9 ml of distilled water and the pH was adjusted to the desired value before the enzyme in 0·1 ml was added. Aliquots were tested for activity after 8 min of incubation at 37°.

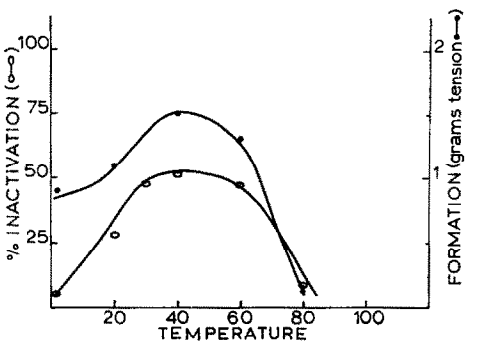

FIG. 3. The effect of temperature on the formation and destruction of substance A. (●—●, formation of substance A). For studies on formation, the incubation mixture contained 35 mg fraction IV-4 and 0·05 mg Nagarse in 10 ml, pH 6·0, at the desired temperature. (○—○, inactivation of substance A). For studies on inactivation, 10 mg substance A and 0·05 mg Nagarse were dissolved in 10 ml Tyrode's solution and incubated at the desired temperature.

that the initial velocity of the enzymatic reaction was represented by the 0–8 min portion of the formation curve. The initial velocity or slope of each curve was then used for calculation of the Michaelis constant (K_m) by means of double reciprocal plots according to Lineweaver and Burk.[9] The plot for the Nagarse enzyme is shown in Fig. 4 with an apparent K_m of $1{\cdot}17 \pm 0{\cdot}28 \times 10^{-5}$ M. The data are based on calculations from 10 different experiments using 10 guinea pig ilea. The early studies concerning the formation of substance A were performed with fraction IV-4 and crude

alpha-amylase. The commercial source of alpha-amylase was from the growth medium of *B. subtilis*, which is also the source for the Nagarse enzyme; thus, the suggestion that the Nagarse enzyme may be identical with the proteolytic enzyme in the alpha-amylase preparation. Since K_m values are considered to be physical constants for enzymes, the finding of equal or nearly equal K_m values would further support the similarity. By using procedures similar to those described above, but with crude alpha-amylase (NBC No. 7942) as the enzyme, data were obtained which allowed

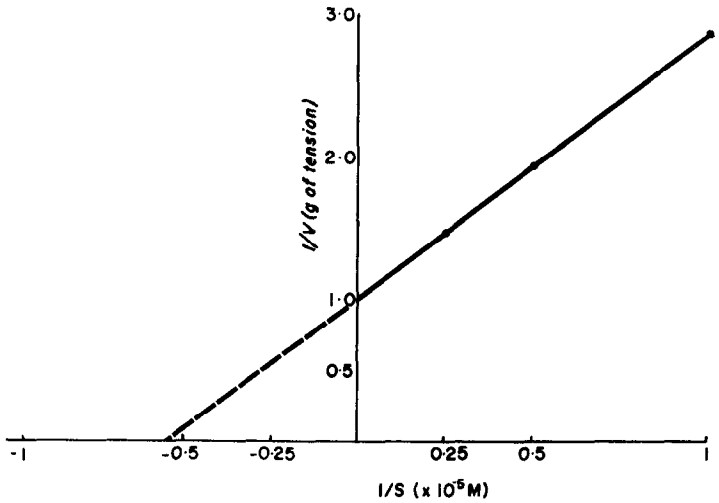

FIG. 4. Lineweaver and Burk double-reciprocal plot of the formation of substance A by incubating fraction IV-4 with Nagarse. The apparent $K_m = 1{\cdot}17 \pm 0{\cdot}25 \times 10^{-5}$ M. Since fraction IV-4 is not a pure compound, we have used the mol. wt. of 100,000 for our calculations, based on the data of the composition of this fraction by Putman.[8] The apparent K_m represents the mean value obtained from 10 different experiments. Each point on the Lineweaver–Burk plot represents the mean value of 10 experiments. The line was plotted from a least squares calculation made from the means at these points.

calculations for the apparent K_m value of $1{\cdot}7 \pm 0{\cdot}34 \times 10^{-5}$ M. These data were calculated from 6 different experiments and give strong support to the similarity of the Nagarse enzyme and the substance A-forming enzyme in crude alpha-amylase.

The effect of various commonly used enzymatic inhibitors was investigated by preincubating Nagarse with the inhibitor for 30 min and then adding this to a solution of the substrate, either fraction IV-4 or substance A. The concentration of inhibitor in the preincubation medium was 10^{-2} M, which was diluted to 10^{-3} M in the incubation solution containing the substrate. Appropriate aliquots were tested at 8 min of incubation and compared with a similar solution containing no inhibitors. The data in Table 1 were obtained under the above conditions and show that only DFP and phenylmethylsulfonyl fluoride were active inhibitors.

The effects of various divalent cations were investigated by preincubating the dialyzed enzyme with the divalent cation for 30 min before adding the dialyzed substrate. The concentration of divalent cation in the preincubation solution was 10^{-2} M and was then diluted to 10^{-3} M in the solution which contained the substrate. Appropriate aliquots were tested for activity on the guinea pig ileum after 8 min of incubation and

TABLE 1. EFFECT OF VARIOUS INHIBITORS ON THE ENZYMATIC FORMATION AND INACTIVATION OF SUBSTANCE A BY SUBTILISIN BPN' (NAGARSE)*

Inhibitor	Concn. (M)	Per cent inhibition	
		Formation	Inactivation
Monoiodoacetate	10^{-3}	0	0
p-Chloromercuribenzoate	10^{-3}	0	0
ε-aminocaproic acid	10^{-3}	0	0
EDTA-Na_2	10^{-4}	0	0
EDTA-Na_2	10^{-3}	6	5
l-Cysteine	10^{-3}	0	0
1,10-Phenanthroline	10^{-3}	0	ND†
α,α-Dipyridyl	10^{-3}	0	ND
DFP	10^{-3}	100	100
Phenylmethylsulfonyl fluoride	10^{-3}	100	100
α-Amylase inhibitor	10:1, by weight	5	ND
Soybean trypsin inhibitor	50:1, by weight	0	ND

* Nagarse was preincubated with the inhibitor for 30 min at 37° and then added to the appropriate substrate solution, which was also kept at 37°. Incubations concerning formation of substance A were carried out at pH 6 and those for inactivation at pH 8·0 in Tyrode's solution. Appropriate aliquots were tested for activity after 8 min of incubation.

† ND = not tested.

the data (see Table 2) were compared with incubation solutions which did not contain divalent cation. From the various ions used, it is of interest that Co^{2+}, Ni^{2+} and Zn^{2+} were the only ones tested which showed inhibition. Furthermore, these ions are are adjacent in the periodic table of the elements. The observation that only Cu^{2+} and Ni^{2+} are strongly inhibitory may be correlated with the known ability of histidine to form a strong complex with these two ions. When histidine in a concentration of 0·1 M was added to the preincubation solution containing enzyme and Ni^{2+} or Cu^{2+}

TABLE 2. EFFECT OF VARIOUS DIVALENT CATIONS ON THE ENZYMATIC FORMATION AND INACTIVATION OF SUBSTANCE A BY SUBTILISIN BPN (NAGARSE)*

Divalent cation	Concn. (M)	Per cent inhibition	
		Formation	Inactivation
Ca^{2+}	10^{-3}	0	0
Ba^{2+}	10^{-3}	0	0
Fe^{2+}	10^{-3}	0	ND†
Mn^{2+}	10^{-3}	0	0
Mg^{2+}	10^{-3}	0	0
Co^{2+}	10^{-3}	6	5
Ni^{2+}	10^{-3}	80	25
Cu^{2+}	10^{-3}	80	25
Zn^{2+}	10^{-3}	10	5
Ni^{2+} + histidine (10^{-2} M)	10^{-3}	20	10
Cu^{2+} + histidine (10^{-2} M)	10^{-3}	20	10

* Nagarse was preincubated with the divalent cation for 30 min at 37° and then added to the appropriate substrate solution, which was also kept at 37°. Incubations concerning formation of substance A were carried out at pH 6 and those for inactivation were in Tyrode's solution at pH 8·0. Appropriate aliquots were tested for activity on the guinea pig ileum after 8 min of incubation.

† ND = not tested.

(10^{-2} M), the inhibitory activity of these two cations was partially abolished. Histidine, 10^{-2} M, had no influence on the enzymatic activity and it is also of interest that no divalent ion was found to be required for activation of the enzyme reaction.

Plasma proteins from species other than human were tested for their ability to serve as a substrate in the formation of substance A by Nagarse. Solutions of 35 mg of fraction IV-4 or 50 mg of lyophylized serum from several species were dissolved in distilled water and Nagarse was added to produce a solution containing 10 μg/ml of enzyme. Appropriate aliquots were tested for activity on the guinea pig ileum and cats' blood pressure after 8 min of incubation. A comparison of the results of this experiment can be seen in Table 3. Human plasma appeared to serve as the best substrate for the formation of substance A.

TABLE 3. ENZYMATIC FORMATION OF SUBSTANCE A BY THE ACTION OF SUBTILISIN BPN' (NAGARSE) ON PLASMA PROTEINS FROM SEVERAL DIFFERENT SPECIES*

Substrate	Concn. (mg/ml)	Activity (Grams of tension)	Activity on Blood pressure
Human IV-4	3·5	1·2	Pressor
Pig IV-4	3·5	0·82	Pressor
Dog IV-4	3·5	0·80	Pressor
Cow IV-4	3·5	0·76	Pressor
Sheep IV-4	3·5	0·33	Pressor
Human serum	5	1·0	Pressor
Pig serum	3·5	1·0	Pressor
Guinea pig serum	3·5	0·55	Pressor
Horse serum	3·5	0·4	Pressor
Cow serum	3·5	0·35	Pressor

* Fraction IV-4 (3·5 mg/ml) or lyophylized serum (5 mg/ml) was incubated with Nagarse (10 μg/ml) at pH 6·5 and 37° for 8 min. Appropriate aliquots were tested for activity on the guinea pig ileum (grams of tension) and cats' blood pressure.

DISCUSSION

Substance A, a vasopressor polypeptide, can be shown to be formed enzymatically by a reaction between subtilisin BPN' (Nagarse) and fraction IV-4 of plasma protein. Under standard incubation conditions and above pH 7·0, there is a simultaneous inactivation of the active material as well as formation; however, the latter activity is much more rapid. These data are interpreted as evidence of sequential reactions as follows:

(1) fraction IV-4 + Nagarse → substance A;
(2) substance A + Nagarse → inactive products.

During the first 20 min of incubation, reaction 1 exceeded reaction 2 with the substrate for reaction 1 being steadily consumed, until approximately 30–35 min, when the decrease in concentration of the substrate in fraction IV-4 was reflected in a decrease in reaction 1 with respect to reaction 2, and as a consequence a decrease in the concentration of substance A was shown.

We have developed an isometric tension system using the guinea pig ileum in order to study the kinetics of formation and destruction of substance A. The problem of the isometric tension method has been discussed extensively by Csapo[6] as applied to uterine muscle and also by Randall *et al.*,[7] who applied the method to isolated guinea pig ileum. A linear response to well denfied loads applied to the transducer

and reproducible dose response curves to active polypeptides when expressed as grams of tension indicated that the isometric system was useful under properly controlled conditions.

It has been demonstrated by Hagihara[10] that the proteolytic activity of Nagarse is inhibited by DFP. In our study, only DFP, and phenylmethylsulfonyl fluoride were effective inhibitors, both producing 100 per cent inhibition at 10^{-4} M. The effect obtained by these two inhibitors suggests by analogy that serine may be involved at an active site.[11] A commentary by Oosterban and Cohen[12] is of interest in which they report that it is quite remarkable that the dipeptide sequence, dicarboxylic amino acid–serine, occurs in all the proteolytic enzymes of animal origin, whereas the bacterial and mold proteases both contain the sequence threonine–serine at their active center. This latter commentary is supported by the report of Smith *et al.*,[13,14] who have determined the amino acid sequence for Nagarse.

The effect of bromo- or iodoacetic acid on enzymes shows a rather broad specificity. In low concentrations, the highly inhibitory effect of these compounds could be mainly related to the alkylation of a free thiol group of a cysteine residue. In our studies monoiodoacetic acid and *p*-chloromercuribenzoate failed to produce inhibition, which was to be expected in view of the reports that subtilisin BPN' does not contain any cysteine.[13,14] The data with chelating agents would support the hypothesis that divalent ions are not required for the enzymatic formation or inactivation of substance A. However, it is possible that the enzyme has a stronger affinity for the divalent ion activator than do the chelating agents used in this study.

The fact that the apparent K_m value found for Nagarse and the apparent K_m value found for the crude alpha-amylase preparation (originally used[1,2] to prepare substance A) are very similar gives additional support to the similarity of identity of substance A as prepared by the two enzymes. This is in support of previous evidence reported by Huggins *et al.*[5] and based on identical behavior of substance A prepared from both sources with respect to column chromatography, destruction by trypsin, pepsin, chymotrypsin and Nagarse, and on pharmacological comparisons. These results suggest that Nagarse is the proteolytic component in crude alpha-amylase[1–3] responsible for forming substance A.

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