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# CLOSTRIDIOPEPTIDASE B INHIBITION BY PLASMA MACROGLOBULINS AND MICROBIAL ANTIPROTEASES *

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## Summary

Clostridiopeptidase B (EC 3.4.22.8) was not inhibited by stoichiometric amounts of lima bean trypsin inhibitor, ovomucoid trypsin inhibitor, Kunitz bovine trypsin inhibitor, Kunitz soybean trypsin inhibitor or ovoinhibitor. Activity was diminished at relatively high concentrations of the three latter inhibitors. Human plasma $\alpha_2$-macroglobulin inhibited both the amidase and protease activity of the enzyme. Rat and dog plasmas contained high molecular weight inhibitors, presumably macroglobulins as well. Inhibition by this component was greater in rat plasma than in dog plasma, which may be related to the observation that clostridiopeptidase B-induced generation of kinin activity is indirect in the former plasma, but direct in the latter. Leupeptin (*N*-acetyl-L-leucyl-L-leucyl-L-argininal) and antipain ([(*S*)-1-carboxy-2-phenylethyl]carbamoyl-L-arginyl-L-valyl-L-argininal) inhibited clostridiopeptidase B ($K_i$ of $2 \cdot 10^{-8}$ and $3 \cdot 10^{-8}$ M, respectively). They were potent inhibitors of clostridiopeptidase B-induced kinin release in dog plasma.

## Introduction

Clostridiopeptidase B (EC 3.4.22.8), also called clostripain, is a calcium- and sulfhydryl-dependent protease produced by *Clostridium histolyticum*. It has endopeptidase, esterase and amidase activities similar to those of trypsin, save that arginine-containing substrates are greatly preferred over those in which lysine forms the carboxyl donor of the peptide, ester or amide bond [1—5].

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Antipain: for use of this name see ref. 30.

We have added clostridiopeptidase B to rat, dog and human plasmas to generate kinin-like activity [6]. Protease inhibitors were used in that study to assess whether clostridiopeptidase B attacked directly the immediate precursor of kinin or whether it activated an earlier step of the endogenous kinin-releasing cascade in plasma. It has been reported [7] that Kunitz bovine trypsin inhibitor and ovomucoid trypsin inhibitor are without effect on clostridiopeptidase B, while Kunitz soybean trypsin inhibitor competitively inhibits the enzyme. Earlier it was reported [8] that Kunitz soybean trypsin inhibitor did not prevent generation of kinin in a mixture of clostridiopeptidase B and heat-treated horse plasma. Hence, it became important to determine effects of several widely used inhibitors of trypsin-like enzymes on clostridiopeptidase B activity.

## Materials and Methods

Three preparations of clostridiopeptidase B were used: crude *Clostridium histolyticum* collagenase (Worthington Biochemical Corp., Freehold, N.J., code CLS II, high protease activity) and two others prepared from crude collagenase by the method of Mitchell and Harrington [5] and by an affinity chromatography method (see below). Chromatographically prepared Kunitz soybean trypsin inhibitor, lima bean trypsin inhibitor, ovomucoid trypsin inhibitor, Kunitz trypsin inhibitor from bovine pancreas and ovoinhibitor were obtained from Sigma Chemical Co., St. Louis, Mo. Pepstatin, leupeptin and antipain were generously provided by Dr. H. Umezawa, Institute of Microbial Chemistry, Tokyo.

Clostridiopeptidase B was assayed in a medium of 30 mM sodium diethylbarbiturate buffer, 0.15 M NaCl, 10 mM $CaCl_2$ (pH 7.4), 1 mM dithiothreitol. Enzyme was activated at 4°C in assay buffer containing 10 mM dithiothreitol [9]. The amidase reaction was initiated by the addition of 100 $\mu$l 15.5 mM $\alpha$-*N*-benzoyl-L-arginine-*p*-nitroanilide hydrochloride (Bachem, Inc., Torrance, Calif.) in dimethylsulfoxide to 3 ml enzyme-inhibitor solution at 23°C and hydrolysis was monitored spectrophotometrically by the increase in absorption at 405 nm [10]. The molar absorptivity of *p*-nitroaniline at 405 nm was determined to be 9600. Protease activity was assessed using the same assay system, with (1 mg/ml) *N,N*-dimethylcasein [11] as substrate. The rate of exposure of new peptide amino-terminal residues was followed by the fluorescamine method [12].

Release of kinin activity was determined by the superfused organ cascade technique using cat jejunum preparations, in the presence of inhibitors of the activity of various other mediators [6]. Contractions of the isolated tissues were recorded, with pen sensitivity adjusted for a full-scale deflection in response to 10—20 ng synthetic bradykinin (Sandoz, Basle, Switzerland). Three strips were usually superfused in series. The kinin-releasing substrate was citrated dog plasma. Kininase activity was inhibited by addition of calcium EDTA (10 mM final concentration) to plasma 1 min before addition of clostridiopeptidase B. Enzyme-substrate mixtures were bioassayed after 2—4 min incubation at room temperature. Inhibitors were incubated with clostridiopeptidase B before the addition of enzyme to plasma. Controls included bioassay of citrated plasma in the presence of calcium EDTA, of bradykinin incubated with plasma in the presence and absence of calcium EDTA and of plasma

incubated with pancreatic kallikrein (Bayer, Wuppertal, Germany) in the presence of calcium EDTA.

Protein concentrations were determined by the Lowry method [13] or by the dye-binding method of Bradford [14] for samples containing dithiothreitol. Bovine serum albumin was used as a standard. Rat and dog plasmas were obtained by double centrifugation of blood collected in heparinized plastic syringes. Human $\alpha_2$-macroglobulin was prepared from pooled plasma by precipitation with polyethylene glycol, gel chromatography on Sepharose 6B and ion-exchange chromatography on DEAE-cellulose [15,16]. The preparation contained >30% functional $\alpha_2$-macroglobulin, based upon its trypsin-binding capacity [17]. An assay for human $\alpha_2$-macroglobulin-bound trypsin activity [18], modified to use $\alpha$-*N*-benzoyl-L-arginine-*p*-nitroanilide as substrate, was employed in study of rat and dog plasmas. Spectrophotometric methods were used to quantitate ceruloplasmin oxidase activity [19] and serum albumin [20]. DEAE-celluose (DE-52) was obtained from Whatman Ltd., Maidstone, U.K., gel chromatography media and epoxy-activated Sepharose 6B from Pharmacia Fine Chemicals AB, Uppsala, Sweden and trypsin from Worthington.

*Purification of clostridiopeptidase B by affinity chromatography.* L-Arginine was coupled via a hydrophilic spacer arm to Sepharose 6B using epoxy-activated gel (Pharmacia code no. 74801). The coupling procedure recommended in the Pharmacia literature was followed. The arginine-Sepharose gel was poured to form a 1 × 10 cm column and equilibrated at 4°C with 10 mM sodium diethylbarbiturate, 10 mM $CaCl_2$, 0.03 M NaCl buffer (pH 7.4). A 1 × 10 cm column of DEAE-cellulose was equilibrated with the same buffer. 200 mg crude collagenase (30 mg protein) was solubilized in 20 ml equilibrating buffer containing

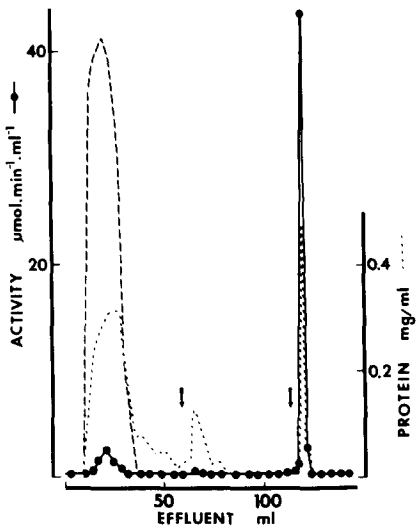


Fig. 1. Chromatography of clostridiopeptidase B on a gel column of Sepharose-linked arginine. At the position indicated by the first arrow in the elution diagram, the affinity column was eluted with pH 7.4 buffer containing 0.3 M NaCl. At the position indicated by the second arrow, the column was eluted with buffer containing 0.3 M NaCl plus 10 mM L-arginine. 2.1-ml fractions were collected. The broad peak of protein represents material which passed through both a DEAE-cellulose column, used to remove pigment, and the affinity column. Elution of clostridiopeptidase A is indicated by the line of long dashes, which illustrates hydrolytic activity (× 100) on a pentapeptide substrate.

10 mM dithiothreitol. Insoluble residue was removed by centrifugation. The clarified solution was loaded onto the DEAE-cellulose column and the eluate was directed onto the column of arginine-Sepharose gel. Eluate from the latter column was collected at a flow rate of 7 ml/h. The coupled columns were washed with equilibrating buffer containing 1 mM dithiothreitol. Under these conditions both clostridiopeptidase A (collagenase) and B pass through the DEAE-cellulose column; most of the brown pigment of the crude enzyme preparation is retained, however [2]. Clostridiopeptidase B was retained by the second column, while collagenase was eluted. Collagenase activity was determined by the method of Wünsch and Heidrich [21] using a chromophoric pentapeptide as substrate. The coupled columns were disconnected and the arginine-Sepharose column further washed with 10 mM sodium diethylbarbiturate, 10 mM $CaCl_2$, 1 mM dithiothreitol, 0.3 M NaCl (pH 7.4). Clostridiopeptidase B was eluted from the column with the same buffer, to which (10 mM) L-arginine had been added. A single fraction of 2.1 ml contained 21% of the amidase activity of the starting material (Fig. 1). Aliquots of this fraction were stored at −25°C. The specific amidase activities of the clostridiopeptidase B preparations, in μmol product formed · $min^{-1}$ · $mg^{-1}$ protein were 10.9 for the crude collagenase, 25.4 for enzyme purified by the method of Mitchell and Harrington [5] and 51.7 for enzyme prepared by affinity chromatography. Emöd and Keil [22] recently reported purification of clostridiopeptidase B on affinity columns of L-arginine linked to Sepharose 4B.

## Results

*Effects of macromolecular trypsin inhibitors*

At concentrations in the final assay mixture from $5 \cdot 10^{-9}$ to $5 \cdot 10^{-5}$ M, lima bean trypsin inhibitor and ovomucoid trypsin inhibitor did not diminish amidase activity of clostridiopeptidase B. Ovoinhibitor at a concentration of $5 \cdot 10^{-5}$ M reduced amidase activity to 55% of its original value. Kunitz soybean trypsin inhibitor at high concentrations diminished amidase activity of clostri-

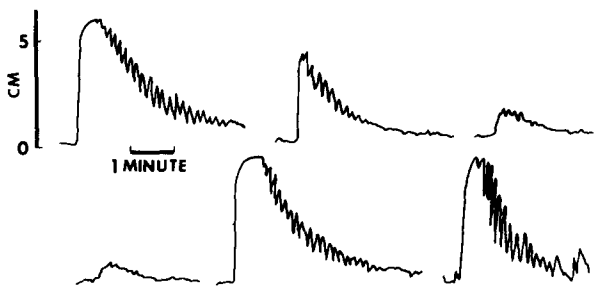


Fig. 2. Recordings of contractions of isolated cat jejunum superfused with mixtures of clostridiopeptidase B, inhibitors and citrated dog plasma. The trace at the upper left illustrates the response to kinin activity generated in a mixture of 0.4 ml citrated dog plasma containing 10 mM calcium EDTA with 40 μl clostridiopeptidase B (7 μg of enzyme purified by the method of Mitchell and Harrington [5]). Four other traces illustrate kinin activity when inhibitors were added 2 min before enzyme was mixed with plasma: antipain (1 μg), antipain (10 μg) (upper traces), leupeptin (0.2 μg) and pepstatin (lower traces), from left to right, respectively. The trace at the lower right illustrates the contraction caused by 10 ng of bradykinin.

diopeptidase B. Loss of half the original activity was observed at a concentration of inhibitor of $3 \cdot 10^{-5}$ M. Kunitz bovine trypsin inhibitor at a concentration of $4 \cdot 10^{-5}$ M reduced hydrolysis of the amide substrate by 50%.

*Effects of microbial protease inhibitors*

At concentrations up to $5 \cdot 10^{-4}$ M, pepstatin was without effect on clostridiopeptidase B activity. Leupeptin and antipain potently inhibited hydrolysis of the amide substrate. Initial rate data were collected at six or more concentrations of each inhibitor at each of three concentrations of substrate. The data were analyzed by means of weighted Dixon plots [23]. From these plots, competitive inhibition constants of $2 \cdot 10^{-8}$ and $3 \cdot 10^{-8}$ M were found for leupeptin and antipain, respectively. Incubation of leupeptin and of antipain with clostridiopeptidase B resulted in suppression of the enzyme's ability to release kinin activity from dog plasma (Fig. 2). Addition of leupeptin or of antipain to pancreatic kallikrein, or to plasma for intervals up to 20 min before addition of clostridiopeptidase B failed to interfere with release of kinin. A greater amount of pepstatin was without effect on clostridiopeptidase B cleavage of the kinin precursor (Fig. 2).

*Inhibition by constituents of blood plasma*

A sample of pooled rat plasma was chromatographed on a column of Sephacryl S-200 gel and effluent fractions were assayed for inhibition of clostridiopeptidase B. Inhibitory activity was found near the void volume of the column, indicative of a high molecular weight inhibitor (Fig. 3). Indeed, the inhibitor eluted with the highest molecular weight plasma components, as indicated by

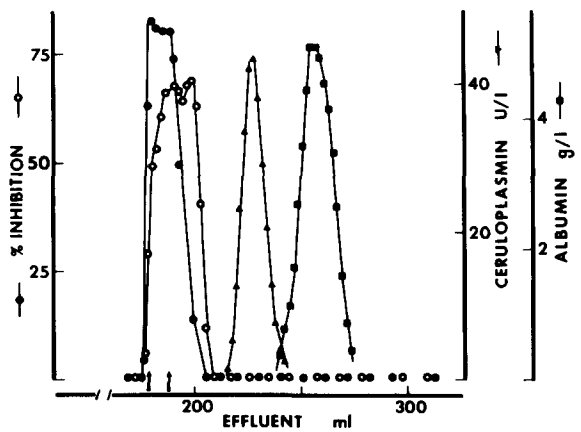


Fig. 3. Chromatography of blood plasma on Sephacryl S-200. A 2.6 × 92 cm column of gel was eluted at 4°C with a buffer of 0.14 M NaCl, 10 mM sodium diethylbarbiturate, 0.1 mM disodium EDTA, pH 7.4, using a flow rate of 15 ml/h. 3.1 ml of rat plasma was chromatographed. Effluent fractions of 2.1 ml were collected. 20-$\mu$l aliquots of those fractions (170—200 ml) eluting near the void volume of the column were assayed for inhibitor activity against clostridiopeptidase B (filled circles). 100-$\mu$l aliquots of other effluent fractions were assayed. Elution of ceruloplasmin and serum albumin is indicated as well. In a second chromatogram, 3.1 ml of dog plasma was similarly chromatographed; the elution of inhibitors of clostridiopeptidase B, assessed using 100-$\mu$l aliquots of effluent fractions, is illustrated (open circles) in superposition upon the results obtained with rat plasma. Fractions No. 86 and 91 (see Fig. 4) are indicated by arrows.

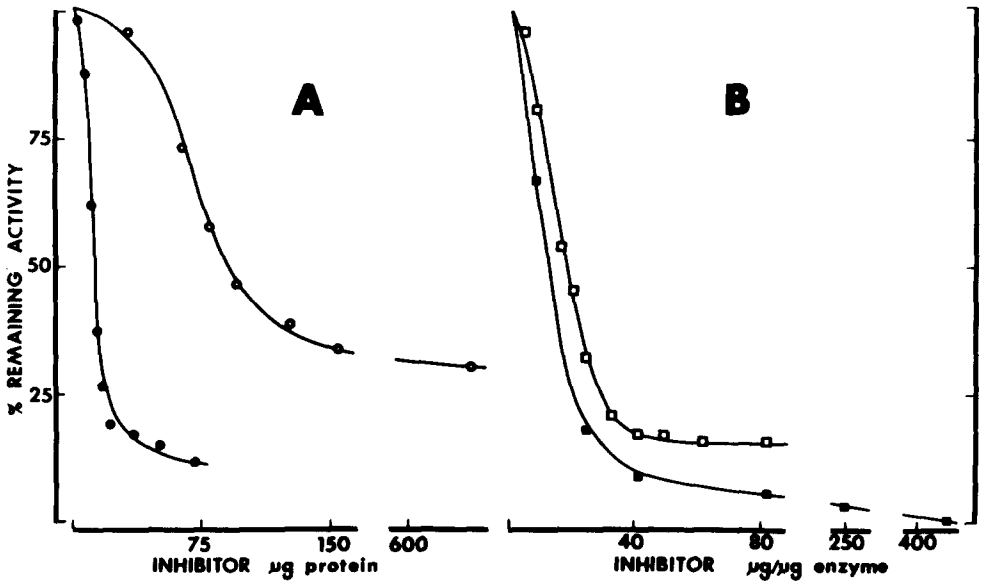


Fig. 4. (A) Extent of inhibition of amidase activity of clostridiopeptidase B by rat (filled circles) and dog (open circles) plasma macroglobulins. The rat plasma inhibitor was fraction No. 86 in the chromatogram illustrated in Fig. 3; it contained 0.71 mg protein/ml. The dog plasma inhibitor was fraction No. 91; it contained 1.59 mg protein/ml. The assay mixtures contained 0.5 µg/ml of crude collagenase protein. (B) Extent of inhibition of amidase (open squares) and protease (filled squares) activity of clostridiopeptidase B by partially purified human $\alpha_2$-macroglobulin. The two assays used different concentrations of enzyme (approx. 2 times greater for the amidase assay), so the abscissa indicates as a ratio the amounts of protein taken from the inhibitor and enzyme preparations which were incubated together. Enzyme prepared by the method of Mitchell and Harrington [5] was used in these assays.

the elution profile of total protein (not shown in Fig. 3) and well before ceruloplasmin activity and rat serum albumin. These data suggested that rat macroglobulin inhibited clostridiopeptidase B, since the equivalent human protein, plasma $\alpha_2$-macroglobulin, is known to inhibit a great variety of proteases [24]. Complete inhibition of the amidase activity of clostridiopeptidase B could not be attained by the rat plasma inhibitor; the maximum inhibition which was obtained was approx. 90% of original activity (Fig. 4).

Dog plasma was subjected to an identical chromatographic analysis. Inhibitory activity was found in the effluent containing high molecular weight constituents (Fig. 3), but the inhibitory activity was less than that found in rat plasma. 20-µl aliquots of maximally inhibitory rat plasma effluent fractions produced 80% inhibition of the assayed amount of enzyme, while 100-µl aliquots of the corresponding effluent fractions of the chromatogram of dog plasma were required to achieve 70% inhibition. 70% inhibition was about the maximum which could be obtained with the high molecular weight material in dog plasma (Fig. 4). Inhibitor activity was spread over a greater number of effluent fractions in the chromatogram of dog plasma, compared to the rat plasma chromatogram, suggesting the possibility of multiple inhibitors in the former. Of course, the chromatographic results just presented do not by themselves rule out the possibility of multiple high molecular weight inhibitors in rat plasma. Protection of trypsin against inhibition by Kunitz soybean trypsin

inhibitor, which in normal rat plasma reflects the content of $\alpha_1$-macroglobulin [25], was determined by activity assays of six rat and six dog plasma samples. The mean activity of dog plasma was 34% of that found in rat plasma.

Partially purified human $\alpha_2$-macroglobulin inhibited both the amidase and protease activity of clostridiopeptidase B (Fig. 4). The maximum inhibition of amidase activity which could be achieved was approx. 85%, while the protease activity of clostridiopeptidase B could be completely inhibited.

## Discussion

Kunitz soybean trypsin inhibitor, lima bean trypsin inhibitor, ovomucoid trypsin inhibitor, ovoinhibitor and Kunitz bovine trypsin inhibitor have been shown to affect several proteases, one or all of them inhibiting various trypsins, chymotrypsin, kallikrein, plasmin, elastase and others, although they may not inhibit some thiol proteases such as papain [26—29]. The reaction of these inhibitors with bovine trypsin is stoichiometric [26,27]. Clostridiopeptidase B was not inhibited by stoichiometric amounts of these inhibitors. These inhibition tests were carried out with enzyme prepared by affinity chromatography; the purity of this preparation and its content of active enzyme [9] were unknown, but the molar concentration of functional enzyme could not have been greater than $2 \cdot 10^{-9}$ M, based on a molecular weight of clostridiopeptidase B of 50 000 [5]. At much higher concentrations of Kunitz soybean trypsin inhibitor, Kunitz bovine trypsin inhibitor and ovoinhibitor ($>1 \cdot 10^{-5}$ M), some reduction of hydrolysis of the amide substrate was observed. Siffert et al. [7] reported that Kunitz soybean trypsin inhibitor inhibits clostridiopeptidase B with a $K_i$ of $8.8 \cdot 10^{-5}$ M. We do not feel that diminution of apparent enzyme activity at such concentrations indicates inhibition by these proteins which is similar to their inhibition of a variety of serine proteases.

Leupeptin and antipain were potent inhibitors of clostridiopeptidase B. This was demonstrated using both amide and macromolecular substrates of the enzyme. These peptides, in which a carboxy-terminal arginine is reduced to an aldehyde, inhibit among other enzymes, trypsin and the thiol-dependent enzymes papain and cathepsin B [30]. Substrate-related aldehydes were proposed [31] to inhibit papain by formation of thiohemiacetals at the active site of the enzyme. Such a mechanism may be involved in the inhibition of clostridiopeptidase B by antipain and leupeptin.

Human $\alpha_2$-macroglobulin is perhaps a universal antiprotease, as it binds and inhibits, in an equimolar ratio, a variety of serine, thiol, carboxyl and metal endoproteases [26,27,32]. Macroglobulin-bound proteases may retain hydrolytic activity, especially against low molecular weight substrates. $\alpha_2$-Macroglobulin-bound kallikrein is protected against inhibition by other plasma antiproteases and is capable of releasing kinin from both high and low molecular weight kininogen [33]. Our observations using clostridiopeptidase B are completely consistent with this description of the inhibition of proteases by human $\alpha_2$-macroglobulin. In particular, while macroglobulin-bound clostridiopeptidase B may lose nearly all its activity against *N,N*-dimethylcasein, it apparently retains enough protease activity to activate the kinin-releasing cascade in human plasma.

It has been reported [6,8] that clostridiopeptidase B directly attacked kinin precursor when added to dog or horse plasma, thus generating kinin-like activity. In human or rat plasma, the enzyme acted indirectly, as generation of kinin was blocked by Kunitz soybean and other trypsin inhibitors [6]. The present experiments were carried out to justify the use of trypsin inhibitors in such systems and to support the conclusions drawn in the previous study. We conclude that the concentrations of those inhibitors which were used to block proteases of the endogenous kinin cascade in plasma would have only marginal effect on clostridiopeptidase B activity. Many mechanisms may be speculated upon as underlying differences in clostridiopeptidase B-induced kinin generation in dog, horse, rat and human plasmas. One could be the susceptibility of the enzyme to inhibition in the various plasmas. Clostridiopeptidase B was inhibited by human, dog and rat plasmas. In each, macroglobulin appeared to be one plasma component with inhibitory activity. Inhibition by this component in dog plasma was less than the inhibition by macroglobulin fractions of rat plasma.

There was also a difference between dog and rat plasmas in the protection of trypsin against inhibition by Kunitz soybean trypsin inhibitor. Such protection probably is a function of macroglobulin in all three plasmas. The quantitative dissimilarity could be due to a reduced concentration of macroglobulin in dog plasma, or a reduced effectiveness of binding by macroglobulin in that plasma, or both possibilities may obtain. The same considerations would apply to the differences observed in inhibition of clostridiopeptidase B. The importance of macroglobulin in accounting for differences in the mechanisms of clostridiopeptidase B-induced generation of kinins in rat, dog and human plasmas may be assessed only when several other points are resolved, such as the differential susceptibility of the various kininogens and kinin cascade zymogens to free and macroglobulin-bound enzyme.

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