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ELASTASE INHIBITORS AS IMPURITIES IN COMMERCIAL PREPARATIONS OF KUNITZ SOYBEAN TRYPSIN INHIBITOR

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SUMMARY

Commercial preparations of Kunitz soybean trypsin inhibitor differ widely in their ability to inhibit porcine elastase and their purity on polyacrylamide gel electrophoresis. The inhibition of elastase is not the property of pure Kunitz inhibitor but of accompanying protein impurities. Four chromatographically distinct elastase inhibitors have been separated by DEAE-cellulose fractionation of a commercial inhibitor preparation.

INTRODUCTION

The inhibition of trypsin and chymotrypsin by the so-called Kunitz soybean inhibitor is well documented [1]. Studies on its activity on elastase have, however, yielded ambiguous and conflicting results. Walford and Kickhofen [2] and Bagdy et al. [3] found that this inhibitor was much less effective when the substrate used to test elastase was elastin than when it was hemoglobin or casein. More recently, Marshall et al. [4] reported that soybean inhibitor inhibited elastase with an apparent K_i of 1.7 μM, the substrate used being Cbz-glycine-*p*-nitrophenylester. During the course of an investigation of the enzymatic activity of α_2-macroglobulin-bound elastase [5], we have shown independently that free and bound elastase were inhibited by this protein: the apparent K_i for free elastase was 0.66 μM and the substrate Boc-Ala-ONp. This led us to investigate more extensively the elastase–soybean trypsin inhibitor interaction. We were, however, unable to duplicate our previous results by using an inhibitor preparation from a different commercial source. We came to the conclusion that pure Kunitz soybean trypsin inhibitor is inactive on elastase, the observed inhibition being due to protein impurities present in some commercial preparations.

EXPERIMENTAL SECTION

Materials

Porcine pancreatic elastase was isolated from Trypsin 1-300 (Nutritional Bio-

Abbreviations: Boc-Ala-ONp: *N*-terbutyloxycarbonyl-L-alanine-*p*-nitrophenylester, Cbz, benzyloxycarbonyl.

chemicals Corporation) by the method of Baumstark et al. [6] and stored at −20 °C as a 0.425 mM aqueous solution. Bovine trypsin (lot TRL-OGC) came from Worthington Biochemicals. The various commercial preparations of Kunitz soybean trypsin inhibitor are listed in Table I. α-*N*-Benzoyl-DL-arginine-*p*-nitroanilide was purchased from Boehringer and BOC-Ala-ONp from Mann laboratories. Elastin was from Worthington and Remazol Brilliant Blue from Hoechst.

DEAE-cellulose was a Schleicher–Schuell product (standard type, lot 2560, 0,95 mequiv/g). The various reagents for polyacrylamide gel electrophoresis were purchased from Fluka.

Polyacrylamide gel electrophoresis. The procedure was essentially that described by Davis [7] except that the concentration gel was omitted. The gels were formed and run in 0.5 cm × 7 cm glass tubes. Protein samples were dissolved in electrophoresis buffer containing 20% sucrose and bromophenol-blue as a tracer dye. Electrophoresis was performed with a laboratory-made apparatus. The gels were stained with amido-black and destained electrophoretically. The gels were scanned using a Vernon apparatus.

Column chromatography. The DEAE-cellulose was stirred for 20 min with 0.1 M HCl and washed with water. This procedure was repeated successively with 0.1 M NaOH and 0.1 M NaCl. The exchanger was then equilibrated with 0.25 M ammonium acetate, pH 6.5. After removal of the fines and degassing, the adsorbent was poured into the column and equilibrated with the starting buffer (0.05 M ammonium acetate pH 6.5). After a chromatographic run, the contents of selected tubes were pooled and lyophilized or concentrated by ultra-filtration in an Amicon Diaflo apparatus (P.M. 10 membranes). All chromatographic operations were carried out at 4 °C. Fractions were collected with a Gilson apparatus.

Enzyme inhibition assays. Elastase inhibitory activity of soybean trypsin inhibitor or chromatographic fractions was assayed with Boc-Ala-ONp [8]. A 0.5 ml aliquot of an enzyme (and inhibitor) mixture in 0.05 M triethanolamine buffer (pH 6.8) was added to 2.5 ml of substrate dissolved in the same buffer (in the presence of acetonitrile). The absorbance was recorded at 347.5 nm for 2 min in a Zeiss PM Q II spectrophotometer equipped with a thermostated cell holder (25 °C). The rates were corrected for substrate autolysis.

Inhibition of elastolysis was measured with Remazol–Brilliant Blue–Elastin. The procedure of Rinderknecht et al. [9] was slightly modified to give a zero order release of soluble peptides from the substrate. 20 mg of labelled elastin were added carefully to 5 ml of an elastase (and inhibitor) solution in 0.05 M triethanolamine buffer (pH 8.6) with or without 0.1 M NaCl. The medium was magnetically stirred for 15 min at 25 °C, cooled to −2 °C and centrifuged at 15 000 × *g* for 15 min. The absorbance was measured at 595 nm.

Trypsin inhibition was assayed as described for serum [10]. Proteins were determined by the method of Lowry et al. [11]. Human serum albumin (Sigma) served as a standard.

RESULTS

When the influence of increasing amounts of soybean trypsin inhibitor on the activity of constant amounts of elastase was tested, the patterns shown in Figs 1 and 2

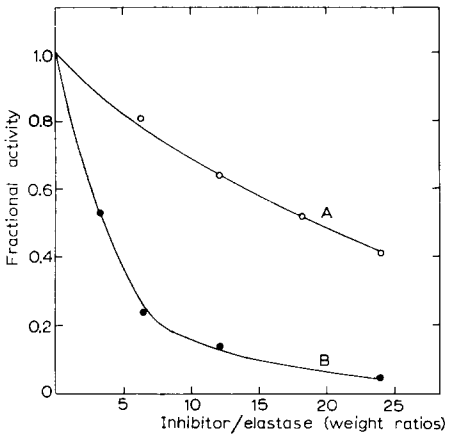

Fig. 1. Inhibition of the esterolytic activity of elastase by soybean trypsin inhibitors from Worthington (Curve A) and Calbiochem lot 000492 (Curve B). Conditions: 0.22 μM elastase, 0.2 mM Boc-Ala-ONp, 0.66% (v/v) acetonitrile (final concentrations), pH 6.8, 25 °C.

were obtained. The inhibitor from Worthington is less potent when the elastase activity is tested with elastin (Fig. 2) than in the case of Boc-Ala-ONp (Fig. 1). The inhibition of elastolysis may however be markedly increased by increasing the ionic strength of the medium (Fig. 2).

However, the most intriguing result of these experiments is that the inhibitors from Worthington and Calbiochem, when tested with the same assay system, show considerable differences in their ability to inhibit elastase (Fig. 1). This surprising finding led us to test a few other commercial soybean trypsin inhibitors. As shown in Table I, the five preparations exhibit wide differences in their inhibitory power against elastase. Variations in activity exist even between two lots from the same commercial source. Of considerable importance is the finding that the Novo preparation does not inhibit elastase at all. Dialysis was without effect on the inhibition.

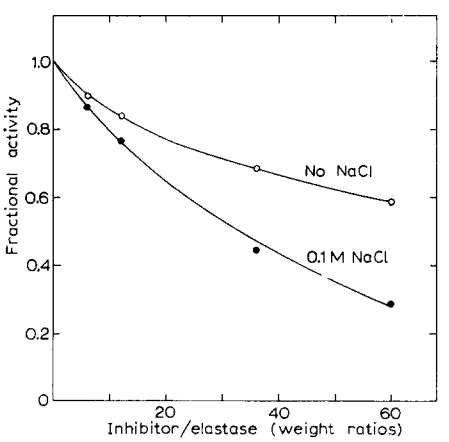

Fig. 2. Inhibition of the elastolytic activity of elastase by the soybean trypsin inhibitor from Worthington. Conditions: 1.7 μM elastase, 4 mg/ml Remazol–Brilliant Blue–Elastin and the indicated NaCl concentration (final concentrations), pH 8.6, 25 °C.

TABLE I

ELASTASE INHIBITORY ACTIVITY AND HETEROGENEITY OF SOME COMMERCIAL PREPARATIONS OF KUNITZ SOYBEAN TRYPSIN INHIBITOR

Elastase inhibition was measured as described in the legend to Fig. 1. Polyacrylamide gel electrophoresis was conducted in the following conditions: 7.5% acrylamide, pH 8.3, 100 μg of protein per gel, 4 mA per gel, 1 h.

Commercial preparations of Kunitz soybean trypsin inhibitor	Wt concn ratios of inhibitor to elastase at 50% inhibition	Polyacrylamide gel electrophoresis	
		No. of bands	% staining material migrating as true Kunitz inhibitor
Novo (lot 42)	no inhibition	2	91
Worthington (lot SI-OIA)	20	3	65
Calbiochem (lot 000492)	3	—	—
Calbiochem (lot 100946)	6.5	12	25
Sigma (lot III C-8080)	5.5	2	99
Nutritional Biochem. Corp. (lot 9418)	2	14	50

In contrast, no such large differences were found in the trypsin inhibitory activity of the various preparations. Moreover, the Novo inhibitor which does not inhibit elastase, is one of the most active preparation on trypsin. From these observations it was thought that pure soybean trypsin inhibitor is not an elastase inhibitor, i.e. the observed inhibition is the property of accompanying impurities.

The possible presence of protein impurities was explored by polyacrylamide gel electrophoresis which showed that almost all of the five commercial inhibitors tested are highly heterogeneous. The R_F value (0.85) of the protein band of the purest preparation (Sigma) was used to identify the position of Kunitz soybean trypsin inhibitor on each gel. The relative content of true Kunitz inhibitor in each preparation could thus be estimated from the densitometer tracings assuming that staining is quantitative. These results are reported in Table I together with the number of visible bands. It can be seen that the compound from Novo, which is inactive on elastase, is fairly pure whereas the preparation from Calbiochem which inhibits elastase is heavily contaminated by protein impurities. The fact that the inhibitor from Sigma is active on elastase although it is highly pure suggest that the protein impurities responsible for the inhibition of elastase by this preparation occur as trace amounts.

In an attempt to provide direct evidence for the presence of elastase inhibiting impurities in commercial soybean trypsin inhibitor, some preparations were subjected to chromatography on DEAE-cellulose. A typical elution pattern obtained with the Calbiochem inhibitor is shown in Fig. 3. This pattern compares well with the heterogeneity detected by polyacrylamide gel electrophoresis (Table I). The pooled peaks were labelled Fractions I to VII as indicated in Fig. 3. The elution behavior and electrophoretic analysis of a purer soybean trypsin inhibitor preparation indicated that Fraction VI represents true Kunitz inhibitor. This fraction showed a slight elastase inhibitory activity which disappeared on rechromatography. Fraction VII contained dialyzable material and was not analyzed further since dialysis did not reduce the elastase inhibitory activity of the inhibitor from Calbiochem.

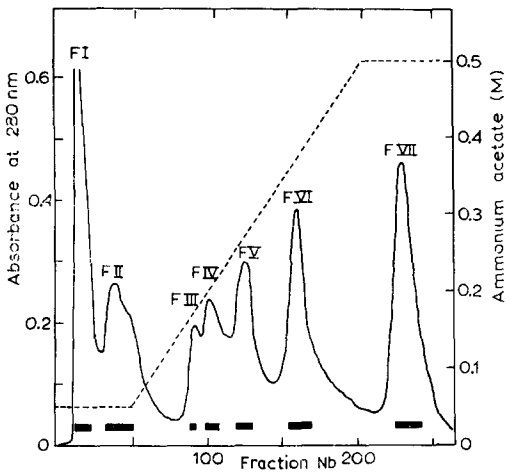

Fig. 3. DEAE-cellulose chromatography of 200 mg of soybean trypsin inhibitor from Calbiochem (lot 000 492). Column dimensions: 1.6 cm × 50 cm, fraction size: 5 ml, flow rate 0.8 ml/min. ———, absorbance at 280 nm; — — — ammonium acetate concentration of the eluting buffer (pH 6.5 throughout). Tubes indicated by black bars were pooled and treated as indicated in the experimental section.

Fractions I, II, IV and V had an appreciable activity on elastase; the weight concentration ratios of inhibitor to enzyme at 50% inhibition (determined as indicated in the legend to Fig. 1) were 50, 8.3, 6.2 and 1.9, respectively. The inhibition of elastase by the Calbiochem inhibitor (Fig. 1) is thus due to the combined effects of four chromatographically distinct protein impurities. All these elastase inhibiting fractions were also active on trypsin: this explains why the specific trypsin inhibitory activity of the Calbiochem inhibitor is higher than that expected from its low content of true Kunitz trypsin inhibitor.

DISCUSSION

Studies on the inhibition of elastase by soybean trypsin inhibitor have yielded conflicting results. Bagdy et al. [3] and Walford and Kickhofen [2] reported that the inhibition was strongly dependent upon the nature of the protein substrate used to test elastase. With synthetic substrates, Marshall et al. [4] and Bieth et al. [5] found soybean trypsin inhibitor to be moderately potent on elastase, whereas, Gertler and Feinstein [12] experienced no inhibition et all. These controversial findings may now be explained in the light of the present results. First, we have made clear that pure Kunitz soybean trypsin inhibitor is inactive on elastase, the inhibitory action of some commercial preparations being the property of accompanying protein impurities. The differences in the reported potencies of soybean inhibitor toward elastase [4, 5, 12] may thus, at first sight, be ascribed to the use of preparations more or less heavily contaminated by elastase inhibitors. Another point is however worth emphasizing; none of the commercial inhibitors did inhibit elastase stoichiometrically with our assay system: this is interpreted to mean that none of these preparations contained a very potent elastase inhibitor (i.e. $K_i \leqslant 1$ nM). Such low potencies explain why the

elastase inhibitory property of a given impure soybean trypsin inhibitor depends drastically upon the enzyme's assay conditions. This is illustrated in Figs 1 and 2: the Worthington inhibitor is more potent with the esterolytic assay than with the elastolytic one although the higher reactant concentration in the later assay system should have favoured inhibition. This behavior is well accounted for by the high affinity of elastase for elastin compared to its affinity for Boc-Ala-ONp. As a matter of fact, when the binding capacity of elastin is reduced by an increase in ionic strength [13] the inhibition is markedly enhanced (Fig. 2). The substrate dependent potency of soybean trypsin inhibitor reported by Bagdy et al. [3] and Walford and Kickhofen [2] is of course perfectly well accounted for by the above-mentioned low elastase-inhibitor affinity.

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