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Measurement of the Bovine Pancreatic Trypsin Inhibitors by Radioimmunoassay*

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Bovine pancreas contains two polypeptide trypsin inhibitors which are not homologous and differ in their inhibitory activity towards chymotrypsin, kallikrein, elastase and other serine proteinases [1]. The Kunitz inhibitor [2, 3] and the Kazal inhibitor [4, 5] are present in approximately equimolar concentrations in bovine pancreatic tissue [6], yet only the Kazal inhibitor is detectable in the pancreatic juice [6, 7]. The Kazal inhibitor has been named the pancreatic secretory trypsin inhibitor, PSTI [8] because its concentration in the pancreatic juice parallels that of the exocrine secretory proteins [7, 9, 10]. The Kunitz inhibitor is considered the "intracellular" inhibitor [7]. However, no direct information is available concerning the intracellular localization of these inhibitors in the pancreas. A sensitive and specific analytical method for the measurement of Kazal and Kunitz inhibitors at the picogram-nanogram level is required for intracellular distribution studies. The preparation of trace labelled ^{131}I-Kunitz inhibitor by the chloramine T method and its use in a radioimmunoassay have been described by ARNDTS *et al.* [11]. In this communication we report the preparation of ^{125}I derivatives of Kazal and Kunitz inhibitors by the lactoperoxidase method and present a radioimmunoassay for each inhibitor.

Radioiodination

The Kazal and Kunitz trypsin inhibitors were iodinated with carrier free Na^{125}I by lactoperoxidase in the presence of hydrogen peroxide [12, 13]. The experimental conditions for iodination and purification were identical for both inhibitors. The results obtained for the Kunitz inhibitor were essentially the same as those illustrated for the Kazal inhibitor in Figs. 1 and 2. Kazal inhibitor was prepared from bovine pancreas [8] and the Kunitz inhibitor, isolated from bovine lung[1], was supplied by Bayer AG.

Inhibitor, 4 μg, was iodinated by incubation with 1 mCi Na^{125}I (IMS 30, Amersham) and 1 μg lactoperoxidase (Calbiochem) in 30 μl of 0.13 M potassium phos-

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[1] The trypsin inhibitor from bovine lung, kallikrein inactivator, is identical in amino acid sequence and disulfide pairings to the Kunitz inhibitor [14].

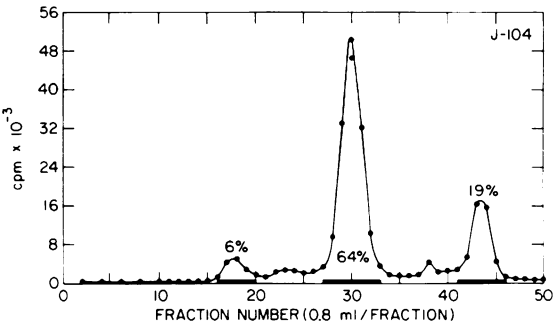

Fig. 1. Isolation of ^{125}I Kazal inhibitor by gel filtration on Sephadex G-50F. The iodination reaction mixture was applied to a Sephadex G-50F column, 0.6 x 120 cm, equilibrated with 0.05 M Tris-HCl buffer, pH 8.6, containing 0.5% bovine serum albumin, 0.5 M KCl and 0.02% NaN_3. The column was developed at 3 ml/h at 4°

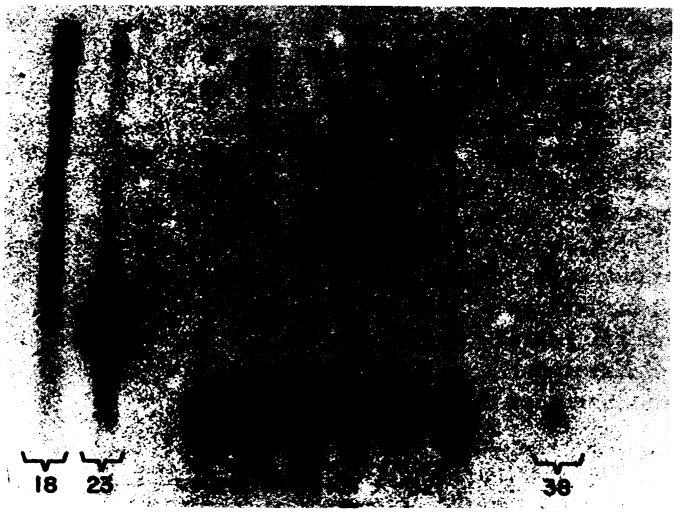

Fig. 2. SDS-polyacrylamide gel electrophoresis of iodinated Kazal inhibitor. Electrophoresis was carried out for 15 h, 21 , 9 volts/cm in Tris-glycine buffer, pH 8.3, 20% acrylamide, containing 0.1% sodium dodecyl sulfate. The samples were treated with 1% β-mercaptoethanol and 1% sodium dodecyl sulfate in Tris-HCl buffer, pH 6.8, at 100° for 2 min before electrophoresis [16]. Electrophoretic migration was from top (cathode) to bottom (anode). The samples are identified by the fraction numbers from the Sephadex G-50 elution diagram given in Fig. 1. Effluent corresponding to fractions 29, 30 + 31, and 32 were examined at two loading concentrations. Unlabelled Kazal inhibitor was also added to fraction 29. The electrophoretogram was dried and stained with coomassie blue before autoradiography

phate buffer, pH 6.1, at 21° C. Hydrogen peroxide, 2 µl of a 0.1 mg/ml solution, was added five times at 30 min intervals [15]. After 2.5 hours the reaction was stopped by the addition of 20 µl of 0.3% NaN_3, 200 µl of 0.1% NaI and 600 µl of 0.05 M Tris-HCl buffer, pH 8.6, containing 0.5% bovine serum albumin (Fraction V, Miles Laboratories), 0.5 M KCl and 0.02% NaN_3. The solution was immediately subjected to gel filtration on Sephadex G-50 (Fig. 1).

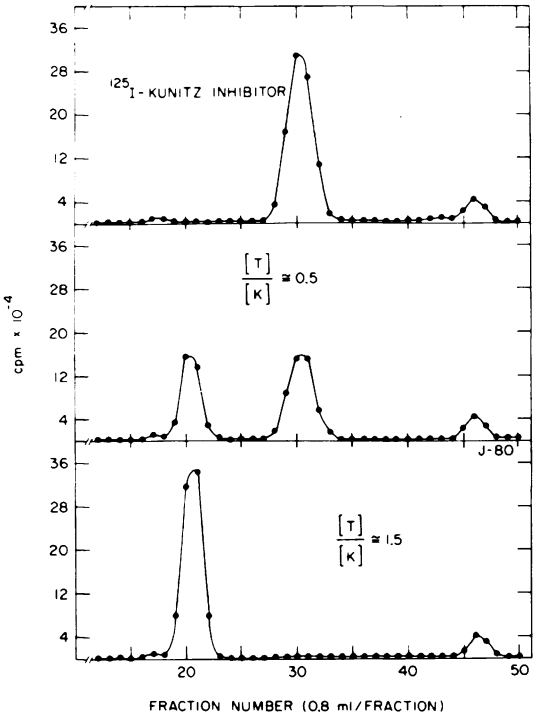

Fig. 3. Interaction of ^{125}I Kunitz inhibitor with bovine trypsin demonstrated by gel filtration. The Sephadex G-50 column was the same as described in the legend to Fig. 1. Top panel: ^{125}I Kunitz inhibitor. Middle panel: ^{125}I Kunitz, inhibitor incubated with 0.5 mole equivalents of bovine trypsin for 20 min at pH 8.0, 21°. Bottom panel: ^{125}I Kunitz inhibitor incubated with 1.5 mole equivalents of bovine trypsin for 20 min at pH 8.0, 21°

The major iodinated product, accounting for 64% of the recovered radioactivity is eluted as a symmetrical peak in fractions 27–33, the same volume in which unlabelled inhibitor is eluted. The gel filtration step effectively separated the iodinated inhibitor from small amounts of high molecular weight iodinated material (fractions 16–25) presumably derived from the lactoperoxidase preparation and from low molecular weight radioactive material eluted in fractions 41–46, which is probably free ^{125}I.

The effluent from the Sephadex G-50 column was examined by SDS-polyacrylamide gel electrophoresis [16] in order to determine the molecular weight of the ^{125}I labelled material. The autoradiogram presented in Fig. 2 shows that the major iodinated product obtained from Kazal's inhibitor (Fig. 1, fractions 29–32) contains a single, iodinated component after reduction with β-mercaptoethanol. The electrophoretic mobility of the reduced iodinated material was the same as that of reduced unlabelled Kazal inhibitor, indicating that no peptide bond cleavage had occurred during the iodination procedure or during isolation of the product. The iodinated material in fractions 18 and 23 appears to be of higher molecular weight than the inhibitor, which is consistent with their elution properties from Sephadex G-50. The behavior of the Kunitz inhibitor was essentially the same as the Kazal

inhibitor for iodination, gel filtration, and SDS-polyacrylamide gel electrophoresis.

The Kazal and Kunitz inhibitors prepared in this manner contain ~0.5–0.8 moles ^{125}I per mole peptide (~180 mCi/mg). This value may be somewhat low because it was calculated on the basis of the amount of radioactivity incorporated into the inhibitor fraction isolated by gel filtration and on the assumption of 100% recovery of inhibitor from the column. No attempt has been made to separate labelled and unlabelled forms of the inhibitors.

^{125}I labelled Kazal and Kunitz inhibitors form a complex with bovine trypsin. Figure 3 illustrates the results of a gel filtration experiment with ^{125}I Kunitz inhibitor. The top panel gives the elution diagram of the inhibitor. The middle and bottom panels show the results obtained when inhibitor was preincubated with 0.5 and 1.5 mole equivalents of trypsin, respectively. When the inhibitor is present in excess relative to trypsin, two radioactive peaks are demonstrable (middle panel). They correspond to free inhibitor (fractions 27–33) and inhibitor-trypsin complex (fractions 18–22). More than 98% of the radioactive inhibitor is eluted in the inhibitor-trypsin complex region when trypsin is in excess (bottom panel).

Iodinated preparations of Kunitz and Kazal inhibitor were stored at 4° in the Sephadex G-50 elution buffer. They were generally rechromatographed on Sephadex G-50 after 2–3 weeks storage prior to use for radioimmunoassay. Upon rechromatography most of the iodinated material was eluted in the position of the inhibitor, with 10–15% of the radioactivity in the low molecular weight region (fractions 44–49, Fig. 1).

Radioimmunoassay Procedure

Antiserum to Kunitz inhibitor was prepared from rabbits immunized with the antigen in incomplete Freund's adjuvant. The immunoglobulin fraction was isolated by ammonium sulfate precipitation and treatment with DEAE-Sephadex. Antiserum to Kazal inhibitor was prepared from rabbits with Kazal inhibitor coupled to human albumin and used without further purification. The double antibody method was employed for the radioimmunoassay using goat antirabbit IgG serum (Miles Laboratories) as the second antibody to separate bound inhibitor from free inhibitor [17].

The titration curves for the Kazal and Kunitz inhibitors are given in Fig. 4. The abscissa on the top of the figure gives the dilutions of the rabbit antiserum for the Kazal inhibitor and the abscissa on the bottom gives the dilution for the anti-Kunitz IgG. These data show that 85–90% of the radioactivity in each ^{125}I inhibitor preparation is bound by the antibody.

Standard curves for the radioimmunoassays of Kazal and Kunitz inhibitors are given in Figs. 5 and 6, respectively. The data are presented as a logit-log plot to linearize the dose response relationship [18, 19]. Y, the percent ^{125}I inhibitor bound, is expressed as $B-N/B_0-N \times 100$; B, the amount bound; B_0, the amount bound in the absence of unlabelled inhibitor; and N, the amount bound in the absence of antibody to the inhibitor. The Kazal inhibitor can be accurately assayed in the range of 50 to 2500 picograms per determination with deviations of

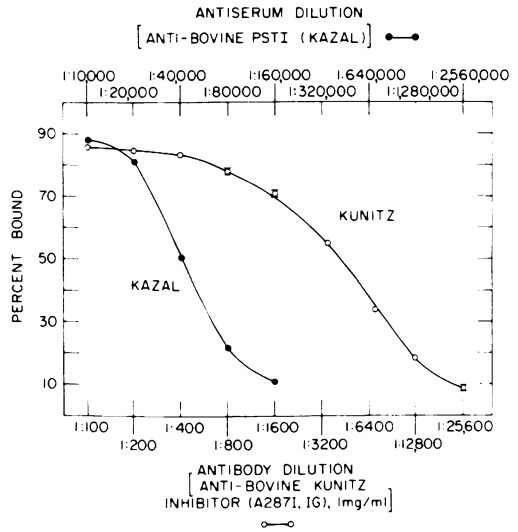

Fig. 4. Antiserum titration curves for ^{125}I Kazal and ^{125}I Kunitz inhibitors. Each tube containing 100 µl ^{125}I inhibitor (25000 c/min), 100 µl antiserum and 300 µl 0.05 M potassium phosphate buffer, pH 7.6, 0.25% bovine serum albumin and 0.02% NaN_3, was held at 21° for 48 hours. Bound inhibitor was separated by the addition of 100 µl goat antirabbit IgG serum (1:30) and 100 µl rabbit serum (1:600) followed by incubation for 24 hours at 21°. The amount of bound inhibitor was determined by counting the precipitate after centrifugation

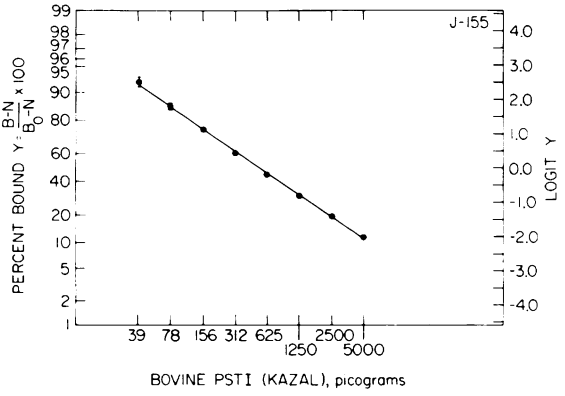

Fig. 5. Standard curve for the radioimmunoassay of Kazal inhibitor. Each tube contained 100 µl ^{125}I Kazal inhibitor (25000 c/min), 100 µl of antiserum at a dilution sufficient to bind 50% of the ^{125}I Kazal inhibitor in the absence of unlabelled inhibitor, 200 µl 0.05 M potassium phosphate buffer, pH 7.6, 0.25% bovine serum albumin and 0.25% NaN_3, and 100 µl unlabelled Kazal inhibitor in the amounts given in the ordinate. The conditions for incubation and separation of bound antibody are given in the legend to Fig. 4. The data are presented in the form of the logit-log plot [19]

replicates $\pm$ 10% or less. The Kunitz inhibitor can be determined in the range of 100 to 5000 picograms with the same precision. We have not attempted to increase the sensitivity of the assay although it is probable that sensitivity could be increased by using higher specific activity iodinated inhibitors. Since the Kazal

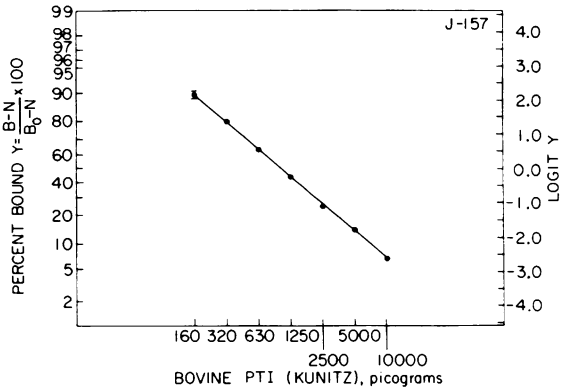

Fig. 6. Standard curve for the radioimmunoassay of Kunitz inhibitor. ^{125}I Kunitz inhibitor and antibody to Kunitz inhibitor were employed using the same volumes of reagents and incubation conditions as given in the legend to Fig. 5

and Kunitz inhibitors contain 2 and 4 moles tyrosine per mole peptide, the iodinated inhibitors used here, 0.5–0.8 moles ^{125}I per mole peptide respectively, are undersubstituted. However, increasing the extent of iodination may interfere with binding to antibody and reduce the stability of the iodinated inhibitor. The system described here is reasonably stable and provides high sensitivity.

Validation of the Radioimmunoassays

There is no detectable cross reactivity between the Kazal and Kunitz inhibitor-antiserum systems. Iodinated Kazal inhibitor was not bound by antiserum to Kunitz inhibitor nor was iodinated Kunitz inhibitor bound by antiserum to Kazal inhibitor under the conditions of the double antibody radioimmunoassay. Ten to 50-fold molar excess of one inhibitor had no effect on the quantitative determination of the second inhibitor.

Trypsin interfered with the assay of the Kunitz inhibitor and to a smaller extent with the assay of the Kazal inhibitor. This interference was reflected in a reduction of the amount of iodinated inhibitor bound to the antibody. This suggests that the inhibitor-trypsin complex is not as effectively bound to the antibody as is the inhibitor. For this reason, samples which may contain trypsin are treated with 2.5% trichloroacetic acid (TCA) to precipitate the trypsin while the inhibitors remain in solution [2, 4]. The recovery of each iodinated inhibitor added to pancreatic juice or homogenates of pancreas was 70–80% after treatment with TCA.

The Kazal and Kunitz inhibitor content of TCA extracts of pancreatic tissue and juice determined by radioimmunoassay was independent of the dilution at which the samples were assayed, i.e. the values of Y (per cent radioactivity bound) for serial dilutions defined lines which were parallel to the standard curves given in Figs. 5 and 6. This parallelism in the dose-response relationship is a necessary, though not sufficient, criterion for the demonstration of identity between the immunoreactive material present in the tissue extract and the inhibitor standard [20]. Additional support for this identity comes from gel filtration expe-

riments for the estimation of the molecular weight of the immunoreactive material present in pancreas. The TCA-soluble immunoreactive Kazal- and Kunitz-like materials from tissue were eluted from Sephadex G-50 in the same volume as the inhibitor standards. These results validate the radioimmunoassay for both the antigen interaction with the antibody and the molecular weight of the antigen. These experiments do not preclude the possibility of the existence in tissue of larger, TCA-insoluble species related to Kazal and Kunitz inhibitors.

The radioimmunoassays described here provide a method to measure picogram quantities of both the Kazal and Kunitz inhibitors in tissue extracts and physiological fluids. The sensitivity and specificity of these assays now permit the examination of the synthesis, segregation and secretion of the pancreatic trypsin inhibitors at a level which hitherto had not been possible.

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