PROTIDES OF THE BIOLOGICAL FLUIDS

PROCEEDINGS OF THE TWENTY-THIRD COLLOQUIUM
BRUGGE, 1975

Edited by

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PERGAMON PRESS
OXFORD · NEW YORK · TORONTO
SYDNEY · PARIS · FRANKFURT

U. K.	Pergamon Press Ltd., Headington Hill Hall, Oxford OX3 0BW, England
U. S. A.	Pergamon Press Inc., Maxwell House, Fairview Park, Elmsford, New York 10523, U.S.A.
CANADA	Pergamon of Canada, Ltd., P.O. Box 9600, Don Mills M3C 2T9, Ontario, Canada
AUSTRALIA	Pergamon Press (Aust.) Pty. Ltd., 19a Boundary Street, Rushcutters Bay, N.S.W. 2011, Australia
FRANCE	Pergamon Press SARL, 24 rue des Ecoles, 75240 Paris, Cedex 05, France
WEST GERMANY	Pergamon Press GmbH, 6242, Kronberg-Taunus, Pferdstrasse 1, Frankfurt-am-Main, West Germany

First edition 1976

Library of Congress Catalog Card No. 58–5908

Printed in Great Britain by A. Wheaton & Co. Exeter

0 08 019929 1 Y

0 08 019930 5 R

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*Measurement of the Proteinase Inhibitors of the Bovine Pancreas by Radioimmunoassay**

E. FINK† and L. J. GREENE††

BOVINE pancreas contains two different polypeptide trypsin inhibitors, Kunitz inhibitor[6,8] and Kazal inhibitor.[3,7] Some of their properties are summarized in Table 1. Both inhibitors are found in the bovine pancreatic tissue in approximately equimolar concentrations,[2] yet only the Kazal inhibitor is detectable in pancreatic juice.

As the first step in the study of the physiological role of these two polypeptide inhibitors we have set up a radioimmunoassay for each inhibitor, because this analytical method provides both specificity and high sensitivity. In this communication we describe the labelling of Kazal and Kunitz inhibitors with ^{125}I using the lactoperoxidase method,[9,12] present a radioimmunoassay for each inhibitor and report the application of the assays in an *in vitro* secretion study of bovine pancreas.

TABLE 1. BOVINE PANCREATIC TRYPSIN INHIBITORS

	Kazal	Kunitz
Trypsin inhibition	+	+
Acrosin inhibition	+	+
Plasmin inhibition	–	+
Chymotrypsin inhibition	–	+
Kallikrein inhibition	–	+
Amino acids/molecule	56	58
Molecular weight	6161	6518
Isoelectric point	4.8, 5.2, 5.9	10.5
Present in:		
pancreatic tissue	+ (~1.5X)	+ (~1X)
pancreatic juice	+	–

RADIOIODINATION

The procedure described by Thorell and Johansson for the iodination of polypeptide hormones using lactoperoxidase[12] was employed for the ^{125}I-labelling of Kunitz and Kazal inhibitors.[1] Iodination and purification procedures were identical for both inhibitors. Table 2 presents the experimental conditions by which the incorporation of 0.4 nmole ^{125}I per

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TABLE 2. ENZYMATIC IODINATION OF BOVINE PANCREATIC TRYPSIN INHIBITORS

(a)	$Na^{125}I$, 1 mCi, pH 8–10	10 μl
(b)	Potassium phosphate buffer, 0.4 M, pH 6.1	10 μl
(c)	Inhibitor, 4 μg in H_2O	5 μl
(d)	Lactoperoxidase (Calbiochem) 1 μg in H_2O	4 μl
(e)	H_2O_2, 1 μg in five 2 μl additions at 30 min intervals	10 μl
(f)	Reaction stopped after 2.5 hr by addition of 20 μl NaN_3 (0.3%), 200 μl NaI (0.1%) and 600 μl G-50 elution buffer	

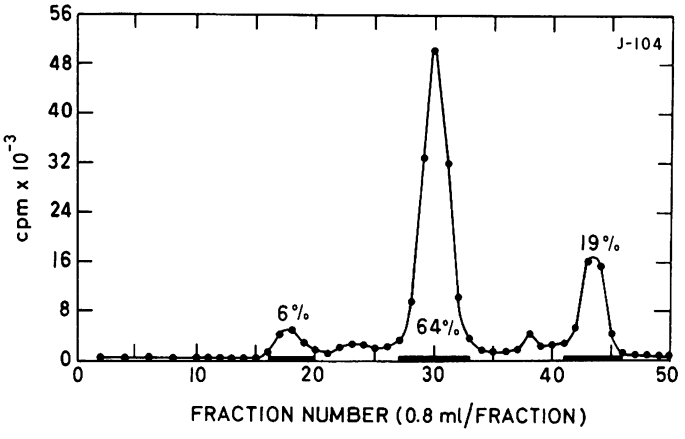

FIG. 1. Isolation of ^{125}I-Kazal inhibitor by gel filtration. The reaction mixture was applied to a Sephadex G-50 fine column, 0.6 X 120 cm, equilibrated and developed at 4°C with 0.05 M Tris-HCl buffer, pH 8.6, containing 0.5% bovine serum albumin, 0.5 M KCl and 0.02% NaN_3. The numbers indicate the percentage of recovered radioactivity in each effluent peak.

1 nmole inhibitor was achieved. The number of iodine atoms incorporated per molecule inhibitor can be controlled by altering the amount of inhibitor in the reaction mixture while keeping all other parameters unchanged.

The reaction mixture was immediately subjected to gel filtration on Sephadex G-50 fine (Fig. 1). The iodinated inhibitor was eluted as a symmetrical peak in fractions 27–33, the same volume in which unlabeled inhibitor was eluted. The gel filtration step effectively separated the radioactive inhibitor from high molecular weight iodinated material (fractions 16–25), presumably derived from the lactoperoxidase preparation, and from low molecular weight radioactive material (fractions 41–46), which is probably free ^{125}I. Iodinated Kunitz and Kazal inhibitor preparations were stored at 4°C in the Sephadex G-50 elution buffer. They were generally rechromatographed on Sephadex G-50 prior to a radioimmunoassay experiment. Upon rechromatography most of the iodinated material was eluted in the position of the inhibitor, a small amount of the radioactivity appeared in the low molecular weight region. The preparations were generally retained for up to eight weeks for use in radioimmunoassay.

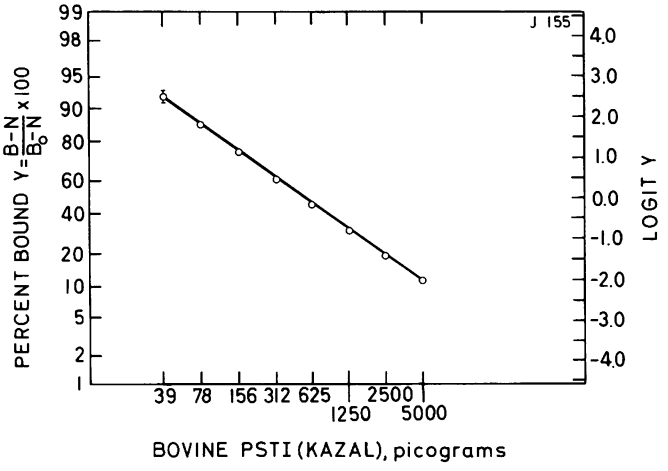

FIG. 2. Dose-response curve for the radioimmunoassay of Kazal inhibitor. Each tube contained 100 μl ^{125}I-inhibitor (25,000 cpm), 200 μl standard diluent (SD: 0.05 M potassium phosphate buffer pH 7.6, 0.25% bovine serum albumin, 0.02% NaN_3), 100 μl unlabeled inhibitor dissolved in SD, 100 μl of antiserum at a dilution sufficient to bind 50% of the labeled inhibitor. After 48 hr at 21°C the 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 at 4°C for 12 hr. The radioactivity of the precipitate was counted after centrifugation.

RADIOIMMUNOASSAY PROCEDURE

Antiserum to Kunitz inhibitor was prepared from rabbits immunized with the antigen in incomplete Freund's adjuvant; antiserum to Kazal inhibitor was prepared from rabbits with Kazal inhibitor coupled to human albumin; both antisera were used without any further purification. The double antibody method was employed for the radioimmunoassays using goat antirabbit IgG serum as the second antibody to separate bound inhibitor from free inhibitor.[4]

Studies of the binding of labeled inhibitor showed that 85–90% of the radioactivity in each ^{125}I inhibitor preparation was bound by the antibody. A standard curve for the radioimmunoassay of Kazal inhibitor is given in Fig. 2. Linearization of the dose–response curve was achieved for either antigen–antibody system by plotting the logit transform of the response versus log dose of antigen.[10,11] Both assays were sensitive to 0.04–0.08 ng inhibitor and were generally used in the range of 0.1–5 ng inhibitor per tube with deviations of replicates ±10% or less. The experimental conditions for incubation and separation of bound antigen are given in the legend to Fig. 2. We have not attempted to increase the sensitivity of the assay, although it is probable that sensitivity could be increased by using inhibitor preparations of higher specific radioactivity. The system described here is reasonably stable and provides high sensitivity.

There is no cross-reactivity detectable between the Kunitz and the Kazal inhibitor–antiserum systems. Neither iodinated inhibitor is bound by the antiserum to the other inhibitor under the conditions of the double antibody radioimmunoassay. 800-fold molar excess of one inhibitor had no effect on the quantitative determination of the second inhibitor. Trypsin interfered with the assays of both inhibitors by reducing the amount of iodinated inhibitor bound to the antibody. This suggests that the inhibitor–trypsin complex is less effectively bound to the antibody than the inhibitor. For this reason, samples which might have contained

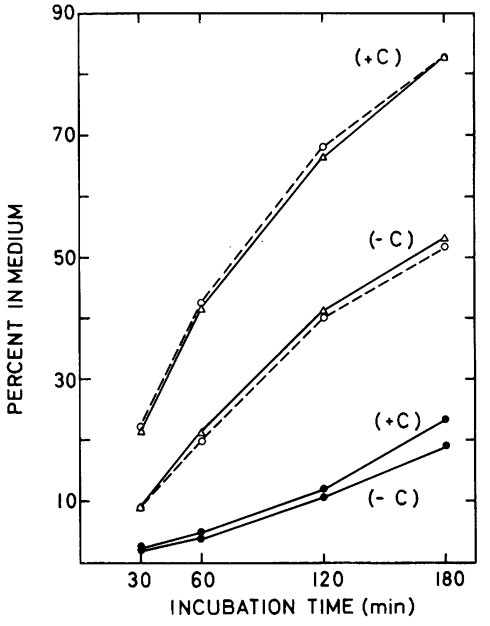

FIG. 3. Kinetics of release of amylase (△——△), and of Kazal (○——○) and Kunitz inhibitors (●——●) from bovine pancreatic slices in the presence (+ C) and absence (– C) of 10^{-6} M carbamylcholine in the incubation medium.

trypsin were treated with 2.5% trichloroacetic acid (TCA) to precipitate the trypsin while the inhibitors remain in solution.[7,8] The recovery of each iodinated inhibitor added to the pancreatic juice or homogenates of pancreas was 70–80% after treatment with TCA.

IN VITRO SECRETION STUDY OF BOVINE PANCREAS

The RIAs of Kazal and Kunitz inhibitors were employed for the study of the secretion of bovine pancreas.[5] Slices of fresh bovine pancreatic tissue were placed in Krebs-Ringer buffer containing glucose and amino acids and washed repeatedly with the same solution. Two sets of slices were incubated in the Krebs-Ringer buffer at 37°C; the medium of one set was 10^{-6} M in carbamylcholine, a secretagogue which is a choline-esterase-resistant analog of acetylcholine. At the times shown in Fig. 3 (abscissa), aliquots of the incubation media were removed and replaced by fresh medium. After the incubation the tissue slices were homogenized in water. All samples and the homogenates were assayed for their concentration in amylase by a photometric method and for their concentrations in Kazal and Kunitz inhibitors by radioimmunoassay. Figure 3 shows the kinetics of release of the three substances.

Two facts confirm the secretory nature of Kazal inhibitor. First, the secretagogue carbamylcholine stimulates its release into the medium. Second, the kinetics of release of Kazal inhibitor into the medium is identical with those of amylase, whose secretory nature is well known.

The release of Kunitz inhibitor into the medium is completely different from that of Kazal inhibitor and is probably due to cell death. By contrast with Kazal inhibitor the release of Kunitz inhibitor is not stimulated significantly by carbamylcholine, nor does it parallel that of amylase, thus demonstrating the non-secretory nature of Kunitz inhibitor.

ACKNOWLEDGEMENTS

We wish to express our appreciation to Dr. Ernst Truscheit, Biochemical Laboratories, Bayer AG, for providing us with the Kunitz inhibitor (Trasylol) and antisera to the inhibitor. We are very grateful to Dr. James D. Jamieson, Section of Cell Biology, Yale University, who supplied us with Kazal inhibitor antisera and whose collaboration made the *in vitro* secretion studies possible.

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