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DETECTION OF ANTIGEN IMMUNOLOGICALLY RELATED TO BASIC PANCREATIC INHIBITOR (KUNITZ) IN PORCINE BLOOD PLASMA BY IMMUNOAFFINITY CHROMATOGRAPHY

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SUMMARY

Rabbit antiserum against trypsin—kallikrein inhibitor (TKI) was prepared. Purified immunoglobulin G (IgG) fraction was bound to Sepharose 4B. An antigen immunologically related to TKI was obtained from porcine blood plasma by adsorbing it onto the immunosorbent column. Its immunoreactivity with TKI antibodies was confirmed by immunoelectrophoresis. The antigen was an inhibitor of trypsin and acrosin.

INTRODUCTION

A number of macromolecules with biological activities are present in body fluids in trace amounts and their purification is very difficult. The selective adsorbents with biological specificity are suitable for isolation of such components. Immmunoaffinity chromatography exploits the specificity and high affinity of antibodies against specific antigens. This procedure makes it possible to obtain antigens and antibodies of high purity.

Trypsin—kallikrein inhibitor (TKI) is present in almost all bovine organs but has not been detected in porcine organs. Recently, a similar antigen was detected in boar seminal plasma and seminal vesicle fluid [1]. TKI has not been detected in porcine blood plasma [1, 2].

This paper deals with an antigen present in porcine blood plasma that is immunologically related to the proteinase inhibitor occurring in bovine organs (TKI). Immunoaffinity chromatography was used for its detection.

EXPERIMENTAL

Proteins, column supports and reagents

Blood plasma was obtained from animals killed at the abattoir. TKI was anti-

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lysin from Léčiva (Czechoslovakia). Acrosin was prepared from boar spermatozoa [3]. Trypsin was from Worthington (Freehold, NJ, U.S.A.). DEAE-cellulose DE-11 was a product of Whatman (Maidstone, U.K.). Sepharose 4B was obtained from Pharmacia (Uppsala, Sweden). N^{α}-Benzoyl-D,L-arginine-*p*-nitroanilide and agarose were purchased from Serva (Heidelberg, F.R.G.). All other chemicals were analytical grade.

Preparation of antisera and detection of antigens

Antisera specific to TKI from bovine lung tissue were prepared by immunizing rabbits [4]. Antibodies were detected by immunoelectrophoresis in 1% agarose boiled in 0.5 *M* Tris—HCl buffer, pH 8.9 [5].

Preparation of immunoadsorbent column

The immunoglobulin fraction of TKI antiserum was isolated by the batch method [6] on DEAE-cellulose suspended in 0.01 *M* sodium phosphate buffer (pH 7.5). The activation of Sepharose 4B with cyanogen bromide [7] was carried out at 4°C. Washed wet Sepharose 4B (17.6 g) was added to the freshly made solution of 3 g of cyanogen bromide in 20 ml of distilled water. The mixture was stirred and the pH was maintained at 11.0 by adding 2 *M* sodium hydroxide. The activation process was completed within 10 min. The activated Sepharose was quickly washed with cold distilled water and added to 200 mg of immunoglobulin G (IgG) dissolved in 50 ml of 0.1 *M* sodium bicarbonate (pH 9.0) and the mixture left overnight at 4°C with gentle magnetic stirring. The immunoadsorbent was packed into a column and washed with 0.16 *M* sodium chloride containing 0.01 *M* sodium phosphate buffer (pH 7.0), followed by washing with 0.1 *M* glycine · HCl (pH 2.8). Chaotropic ion elution was carried out with 2 *M* sodium chloride. Then the column was washed and equilibrated with 0.16 *M* sodium chloride containing 0.01 *M* sodium phosphate (pH 7.0).

The protein coupled to the Sepharose was determined with Folin phenol reagent [8]. Samples of 5 and 10 mg of the Sepharose—IgG were used for tests. The IgG concentration was calculated from a standard curve of the human albumin. Immunological capacity of the column was tested with TKI. Anti-lysin (4 mg) was dissolved in 10 ml of 0.1 *M* sodium phosphate (pH 7.0) and applied to the column. After washing with 0.1 *M* sodium chloride, TKI was eluted with 0.15 *M* sodium chloride adjusted to pH 2.8 with formic acid. Flow-rates were the same as described for isolation of the inhibitor from blood plasma. The fractions with absorbancy at 280 nm were pooled and in the pooled fractions the total TKI content was determined from the titration curve of the linear decrease of trypsin activity with increasing inhibitor concentration. The calculation was based on the stoichiometric ratio 1:1 for complex formation between trypsin and TKI.

Isolation of trypsin—acrosin inhibitor

Porcine blood plasma (10 ml) was mixed with an equal volume of 0.1 *M* sodium phosphate buffer (pH 7.0), centrifuged and applied to the immunosorbent in a column (4.2 × 2.75 cm I.D.), which was then washed with 20 ml of 0.1 *M* sodium phosphate buffer (flow-rate 33.6 ml/h) and 500 ml of 0.15 *M*

sodium chloride (flow-rate 53 ml/h). The inhibitor was eluted from the column with 0.15 *M* sodium chloride adjusted to pH 2.8 with formic acid (flow-rate 53 ml/h). Fractions of 4.4 ml were collected. Fractions corresponding to an absorbance peak at 280 nm were pooled, adjusted to pH 4.5 and concentrated to 0.2 mg/ml using an Amicon ultrafiltration cell equipped with a UM 05 membrane. The protein concentration was measured spectrophotometrically by absorption at 280 nm, assuming 1 mg of protein per ml exhibits an optical density at 280 nm of 1.0. Before re-using the column, 1000 ml of 0.1 *M* sodium phosphate buffer (pH 7.0) were passed through.

Anti-acrosin and anti-trypsin activity assays [9]

Inhibition of trypsin ($9.9 \cdot 10^{-8}$ *M*) and acrosin ($9.9 \cdot 10^{-9}$ *M*) was determined by measuring enzyme activities after incubating the enzymes for 30 min with the inhibitor in the absence of substrate. Residual trypsin and acrosin activities were measured by the standard photometric method at 405 nm with N^{α}-benzoyl-D,L-arginine-*p*-nitroanilide as substrate [9].

RESULTS

Preparation of the immunoadsorbent

For the preparation of immunoadsorbent, 17.6 g of Sepharose were activated and added to 200 mg of IgG. After coupling and washing, 88 mg of IgG (44%) were immobilized. The specific antigen-binding on the immunoadsorbent column was tested with TKI. A total amount of TKI inhibiting 6.3

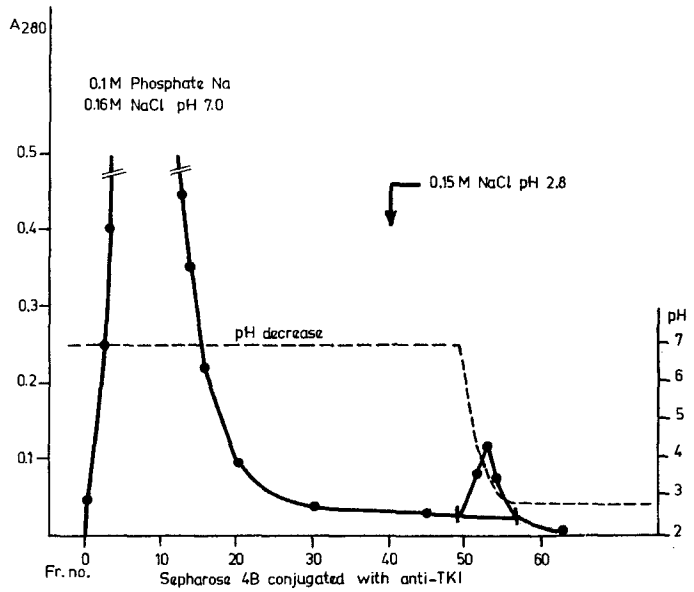

Fig. 1. Affinity chromatography of inhibitor from porcine blood plasma using anti-TKI antibodies coupled to Sepharose 4B. (•—•) Absorbance at 280 nm; (- - -) pH decrease; (——) pooled fractions. The sample (10 ml of blood plasma) was mixed with 10 ml of 0.1 *M* sodium phosphate (pH 7.0). Column size 4.2 × 2.75 cm I.D.; flow-rate of phosphate buffer 33.6 ml/h; sodium chloride adjusted to pH 2.8 with formic acid (flow-rate 53 ml/h). Fraction volumes 4.4 ml.

mg of active trypsin was adsorbed onto the column. Assuming a molecular mass 24 000 for trypsin and 6500 for TKI, it was calculated that 1.7 mg of active TKI were obtained.

Isolation of trypsin–acrosin inhibitor from porcine blood plasma

An antigen immunologically similar to TKI was obtained from porcine blood plasma by absorbing on a Sepharose—anti-TKI column. The elution profile from the column is shown in Fig. 1. The antigen was eluted as a single peak after the pH decreased to 2.8, and 1.4 mg of protein was obtained.

Immunological relation and anti-trypsin and anti-acrosin activities of the blood plasma trypsin—acrosin inhibitor

An antigen immunologically related to TKI, isolated from porcine blood plasma, inhibited both bovine trypsin and boar acrosin. It was a relatively weak inhibitor of both enzymes; trypsin and acrosin were inhibited to 50% either at 12 or 55 mass excess of inhibitor (Fig. 2).

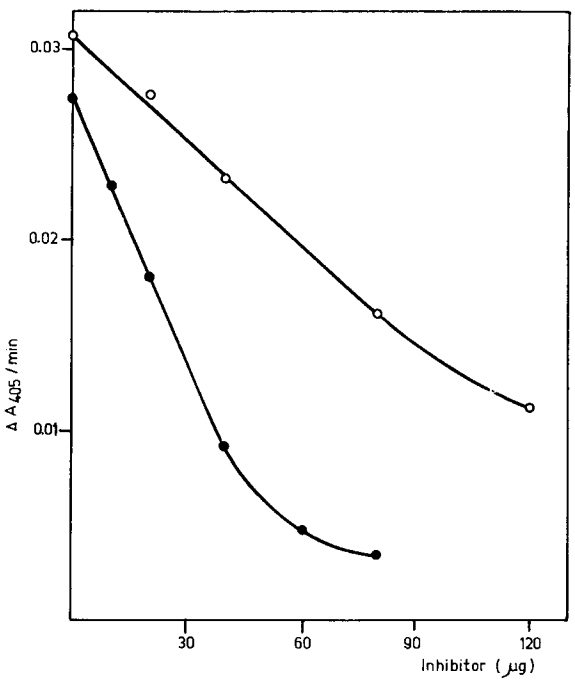

Fig. 2. Inhibition of bovine trypsin ($9.9 \cdot 10^{-8}$ *M*) and boar acrosin ($9.9 \cdot 10^{-9}$ *M*) by inhibitor from porcine blood plasma: x-axis, amount of inhibitor in μg; y-axis, residual trypsin (○) and acrosin (●) activities.

Immunoelectrophoresis revealed one precipitation zone in the reaction of TKI antiserum with a 1% TKI solution. One zone was found in the reaction of this antiserum with the trypsin—acrosin inhibitor isolated from porcine blood plasma (Fig. 3).

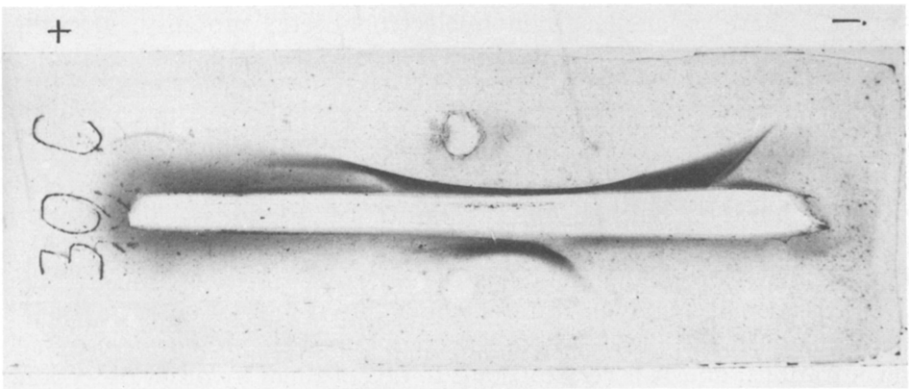

Fig. 3. Above: immunoelectrophoretic reaction of TKI antiserum with a 1% solution of TKI. Below: the reaction of TKI antiserum with porcine blood plasma inhibitor isolated on a Sepharose 4B column with absorbed TKI antibodies.

DISCUSSION

Proteinase inhibitors are proteins capable of blocking the catalytic sites of proteolytic enzymes. Proteinases are involved in most important biological processes and proteinase inhibitors serve as regulators in these systems, by limiting the action of the proteinases. The enzyme—inhibitor association is probably the best understood example of protein—protein association, and it is another motivation for the study of inhibitors. Some inhibitors are purified and characterized and become favourite objects of study for structural-protein chemists [10].

The structures of many inhibitors are reciprocally homologous. It is supposed that the ancestral gene-controlling inhibitor synthesis was duplicated during evolution and that the new inhibitors developed independently [11]. According to the inhibitor homology, it is possible to classify them into several groups. One of this group is the family of Kazal-type inhibitors. These are inhibitors homologous to pancreatic secretory inhibitors, and this family comprises proteinase inhibitors isolated from boar and bull seminal plasma [10]. The second family comprises inhibtiors homologous to the Kunitz basic pancreatic proteinase inhibitor present in mast cells of connective tissues and in many bovine organs [12].

In the present experiments, an antigen immunologically similar to the Kunitz proteinase inhibitor from bovine organs was detected and isolated from porcine blood plasma. So far, this type of proteinase inhibitor has not been detected in the blood plasma of any animal. It has been demonstrated that the inter-α-trypsin inhibitor in blood plasma is similar in structure to the Kunitz trypsin—kallikrein inhibitor [13]. However, inter-α-trypsin inhibitor and its subunits possess inhibitory properties different from those of the inhibitor described in this paper. Inter-α-trypsin inhibitor does not react with TKI antiserum [1]. For these reasons, we suppose that the Kunitz-type protease inhibitor in porcine blood plasma is different from the blood plasma proteinase inhibitors mentioned so far. Recently, a trypsin—acrosin inhibitor immunologically similar to the Kunitz inhibitor has been found in boar seminal vesicle fluid [1].

Both antigens have a similar immunoelectrophoretic mobility, but their inhibitory properties are different. Boar seminal vesicle inhibitor strongly inhibits both trypsin and acrosin, but porcine blood plasma inhibitor must be used in high concentrations to inhibit those enzymes.

The finding of a new inhibitor immunologically related to the Kunitz pancreatic inhibitor seems to show that the ancestral gene for structures of the Kunitz-type inhibitors synthetized a number of proteinase inhibitors in animals.

Immunoaffinity chromatography has the advantage of detecting immunologically related components in different body fluids and tissue extracts. It is important mainly in such cases where the compounds are present in extremely low concentrations, and it is very difficult to detect them by other procedures. Immunoaffinity chromatography enables isolation of these antigens and can be valuable for evolutionary studies of genetically related proteins.

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