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Immunological Relationships of the Glandular Kallikreins

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The immunological properties of various forms of pancreatic kallikreins, *i.e.*, kallikreins A and B, and asialo-kallikreins A and B, submaxillary kallikrein, renal kallikrein and intestinal kallikrein from hog were studied by immunodiffusion and immunoelectrophoresis techniques to investigate their immunological relationships. The immunological assay showed that there is cross-reaction among the various forms of hog pancreatic kallikreins and other hog glandular kallikreins (submaxillary, renal and intestinal kallikreins) but there is no cross-reaction with either pancreatic kallikreins of other species (dog, rat and human) or human urinary kallikrein (HUK) against rabbit anti-hog pancreatic kallikrein A serum and anti-B serum. The only significant differences observed were in the electrophoretic mobilities of the various forms of hog pancreatic kallikreins and other hog glandular kallikreins under the same conditions of immunoelectrophoresis. In the case of hog pancreatic kallikreins A and B, it could be assumed that the different electrophoretic mobilities of these heterogeneous forms are not mainly due to differences in the amounts of sialic acid residues of these kallikreins. Rabbit anti-HUK serum recognized human glandular kallikreins (pancreatic, salivary and urinary kallikreins), but this antiserum did not react completely with hog pancreatic kallikrein. The implications of this are discussed.

Keywords——glandular kallikrein; human; hog; pancreatic kallikrein A; pancreatic kallikrein B; submaxillary kallikrein; renal kallikrein; intestinal kallikrein; urinary kallikrein; immunodiffusion; immunoelectrophoresis.

Introduction

Glandular kallikreins (EC 3.4.21.35) are serine proteases that generate the vasoactive decapeptide kallidin (Lys-bradykinin) from plasma α_2-globulins, the kininogens.[1,2] The enzymes are glycoproteins that are found in various tissue extracts, secretions of saliva, urine, *etc.*[3] Extensive studies on hog submaxillary,[4,5] urinary[6] and autolyzed pancreatic kallikreins[7-19] have revealed that these enzymes share the same protein moiety but differ in their carbohydrate contents. Experimental evidence for the identity of rat glandular kallikreins[20] and for the identity of human glandular kallikreins[21] has also been presented.

In this work, the antigenic relationships among the four hog glandular kallikreins (pancreatic, submaxillary, renal and intestinal kallikreins) were comprehensively surveyed by means of immunodiffusion and immunoelectrophoretic experiments. We also compared the immunological properties of three human glandular kallikreins (pancreatic, salivary and urinary kallikreins).

Materials and Methods

Materials——Hog pancreas, submaxillary gland, kidney and intestine, which were kindly supplied by Teikoku Hormone Mfg. Co., Tokyo, were separately minced and stored at −20 °C until required. The human urinary protein fraction was kindly supplied by the Green Cross Co., Osaka, Japan. *O*-(Diethylaminoethyl) (DEAE)-cellulose (0.88 meq/g) was obtained from Serva Fein-biochemica GmbH, Heidelberg; Sephadex G-50 (medium), G-75

(fine), DEAE-Sepharose CL-6B (15±2 meq/100 ml, gel) and Sephacryl S-200 superfine (40—105 μm) were from Pharmacia Fine Chemicals Inc., Sweden. Hydroxyapatite was from Seikagaku Kogyo. Co., Tokyo, Japan. Benzoyl-L-arginine ethyl ester hydrochloride (Bz-Arg-OEt), benzoyl-L-tyrosine *p*-nitroanilide (Bz-Tyr-*p*NA), succinyl-L-alanyl-L-alanyl-L-alanine *p*-nitroanilide (Suc-$(Ala)_3$-*p*NA) and tosyl-L-arginine methyl ester hydrochloride (Tos-Arg-OMe) were from the Protein Research Foundation, Osaka, Japan. Freund's complete adjuvant was from Iatron Lab., Tokyo, Japan, and Seakem agarose (ME) was from Marine Colloids Inc. Neuraminidase (*Arthrobacter ureafaciens*) and *N*-acetylneuraminic acid were from Nakarai Chemicals, Ltd., Kyoto, Japan. Trypsin from hog pancreas (Type I, 2X crystallized) and α-chymotrypsin from bovine pancreas (49 U/mg) were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. Other chemicals were of guaranteed reagent grade.

Methods

Activity Assays of Kallikreins and Other Proteases——The vasodilator activity was determined by measuring the increase in arterial blood flow following the injection of samples into the femoral artery of an anesthetized dog according to the method of Moriya *et al.*[22] The activity was expressed in terms of the kallikrein unit (KU). Esterolytic activities of pancreatic kallikreins from hog, human, dog, rat and monkey, hog submaxillary kallikrein and hog pancreatic trypsin were measured spectrophotometrically at 25 °C, pH 8.0, using Bz-Arg-OEt as the substrate (final substrate concentration, 0.5 mM),[23] while esterolytic activities of hog renal and intestinal, and human urinary and salivary kallikreins were measured colorimetrically at 30 °C, pH 8.0, using Tos-Arg-OMe as the substrate (final substrate concentration, 2.13 mM).[24] Amidolytic activities of bovine pancreatic α-chymotrypsin and hog pancreatic elastase were measured spectrophotometrically at 30 °C, pH 8.0, using Bz-Tyr-*p*NA (final substrate concentration, 1 mM) and Suc-$(Ala)_3$-*p*NA (final substrate concentration, 1 mM)[25] as substrates, respectively.

Double Immunodiffusion——Double-diffusion analysis[26] was done at 4 °C for 5—6 h in 0.8% (w/v) agarose in 0.05 M veronal buffer, pH 8.6, containing 1 mM ethylenediamine-*N,N,N',N'*-tetraacetic acid (EDTA)-2Na and 5% (w/v) sucrose. The distance between holes was 3 mm (3 mm diameter) and 7 μl each of antigens and antiserum were applied, separately. Thereafter the precipitin lines formed were observed directly.

Counterimmunoelectrophoresis——This electrophoresis was performed according to the method of Gocke *et al.*[27] in 0.8% (w/v) agarose gel (69×89 mm) for 45 min at a constant current of 1.5 mA/cm at room temperature (20 °C), using veronal buffer, pH 8.6, containing 1 mM EDTA-2Na and 5% (w/v) sucrose. Antigens and antibodies (7 μl) were separately applied to wells of 3 mm in diameter.

Immunoelectrophoresis——Samples (7 μl) were applied to the holes (3 mm diameter) in 0.8% (w/v) agarose plates (69×89 mm). The electrophoresis was carried out in 0.05 M veronal buffer, pH 8.6, containing 1 mM EDTA-2Na and 5% (w/v) sucrose for 75 min at 20 °C at a constant current of 3 mA/cm as described by Scheidegger.[28] Antiserum (70 μl) was then added to each channel and diffusion was allowed to occur over a period of 6—10 h at 4 °C.

Preparations of Glandular Kallikreins and Elastase——Highly purified hog pancreatic kallikreins A and B were obtained according to the method given in the previous paper.[16] The purified pancreatic kallikreins A and B showed activities of 1350 KU/mg and 102 EU/A_{280}, and 1400 KU/mg and 105 EU/A_{280}, respectively.

Human urinary kallikrein (HUK) (850 KU/mg) was purified according to our previously described method[29] with minor modifications from crude urinary protein fractions kindly supplied by Dr. E. Sako of the Green Cross Corp., Osaka, Japan.

The final preparations of hog pancreatic kallikreins A and B, and HUK were homogeneous in both disc electrophoresis with 12.5% (w/v) polyacrylamide gels at pH 8.9 and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis with 10% (w/v) polyacrylamide gels containing 1% (w/v) SDS at pH 7.4.

Submaxillary (810 KU/A_{280}), renal (19 KU/A_{280}) and intestinal (75 KU/A_{280}) kallikreins from hog, pancreatic (270 KU/A_{280}) and Bartter's urinary (160 KU/A_{280}) kallikreins from human, and pancreatic kallikreins from dog (910 KU/A_{280}), rat (350 KU/A_{280}) and monkey (190 KU/A_{280}) had been purified in our laboratory. Elastase from hog pancreas had also been highly purified in our laboratory (7.48 U/A_{280}; substrate, Suc-$(Ala)_3$-*p*NA).

Preparations of Anti-Hog Pancreatic Kallikrein A Serum and Anti-B Serum, and Anti-HUK Serum——Hog pancreatic kallikreins A and B (1350 KU/mg and 102 EU/A_{280}, and 1400 KU/mg and 105 EU/A_{280}, respectively) were used for the preparations of anti-hog pancreatic kallikrein A serum and anti-B serum. A solution of hog pancreatic kallikrein A or B (1 mg/ml), 1.5 ml was emulsified with an equal volume of Freund's complete adjuvant and the emulsion was injected intracutaneously into the foot pads and the backs of a rabbit. The whole blood of the rabbit was collected 13 d after the second injection. The anti-hog pancreatic kallikrein A serum or the anti-B serum was obtained by centrifugation (3000 rpm, 20 min at 4 °C), and the ammonium sulfate precipitate formed between 0 and 50% saturation was collected by centrifugation (8000 rpm, 20 min at 4 °C). The precipitate was dissolved in saline and dialyzed against 0.02 M phosphate buffer, pH 6.8. The dialysate was used as anti-hog pancreatic kallikrein A serum or anti-B serum, and stocked at −20 °C until required. The titers of anti-hog pancreatic kallikrein A serum and anti-B serum were 0.4 and 0.3 EU/ml of antigen solution, respectively, as determined by counterimmunoelectrophoresis.

Anti-HUK serum was raised in a rabbit as described by Miyaura *et al.*[30]

Sialidase Treatment——Kallikreins (10 mg) were incubated for 24 h at 35 °C with 0.1 U of sialidase in 2 ml of 10 mM phosphate buffer, pH 6.8, containing 0.5% (w/v) human serum albumin as described previously.[16]

Results

Double Immunodiffusion and Counterimmunoelectrophoresis

The immunodiffusion of the three preparations of hog pancreatic kallikreins (pancreatic extract and pure kallikreins A and B) was performed by using antisera to electrophoretically homogeneous kallikreins A and B (Fig. 1). Antiserum (7 μl) to pancreatic kallikrein A and antiserum (7 μl) to pancreatic kallikrein B (for details see Materials and Methods) were separately applied to each hole of a and b. Equal activities (7 μl, 1000 KU/ml) of hog pancreatic extract and purified hog pancreatic kallikreins A and B were separately placed in holes 1, 2 and 3, respectively. Our anti-hog pancreatic kallikrein A serum and anti-B serum both produced a single precipitin line of complete identity having no spurs with hog pancreatic kallikreins A and B, and hog pancreatic extract. No precipitin line was observed

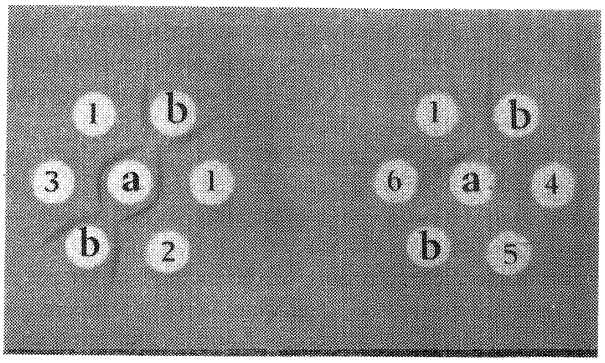


Fig. 1. Double Immunodiffusion Analysis of Hog Pancreatic Kallikreins against Rabbit Anti-Hog Pancreatic Kallikrein A Serum and Anti-B Serum

a: antiserum to pure hog pancreatic kallikrein A.
b: antiserum to pure hog pancreatic kallikrein B.
1, hog pancreatic extract (1000 KU/ml); 2, pure hog pancreatic kallikrein A (1000 KU/ml); 3, pure hog pancreatic kallikrein B (1000 KU/ml); 4, hog pancreatic trypsin (150 EU/ml); 5, bovine pancreatic α-chymotrypsin (98 U/ml); 6, hog pancreatic elastase (25 U/ml). See the text for experimental details.

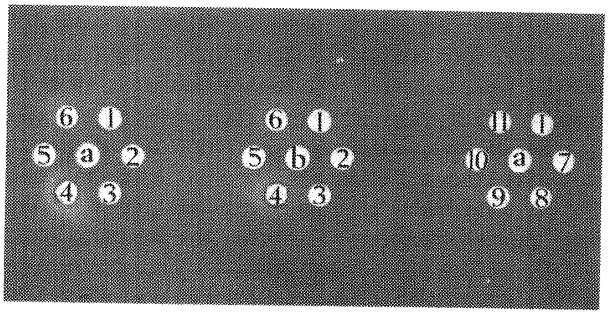


Fig. 2. Immunological Cross-reaction of Various Glandular Kallikreins against Rabbit Anti-Hog Pancreatic Kallikrein A Serum and Anti-B Serum

a: antiserum to pure hog pancreatic kallikrein A.
b: antiserum to pure hog pancreatic kallikrein B.
1, pure hog pancreatic kallikrein A; 2, pure hog submaxillary kallikrein; 3, mixture of hog pancreatic kallikreins A and B; 4, partially purified hog renal kallikrein; 5, pure hog pancreatic kallikrein B; 6, partially purified hog intestinal kallikrein; 7, partially purified human pancreatic kallikrein; 8, pure HUK; 9, partially purified dog pancreatic kallikrein; 10, partially purified rat pancreatic kallikrein; 11, saline.
Each kallikrein solution had an activity of 1000 KU/ml. See the text for experimental details.

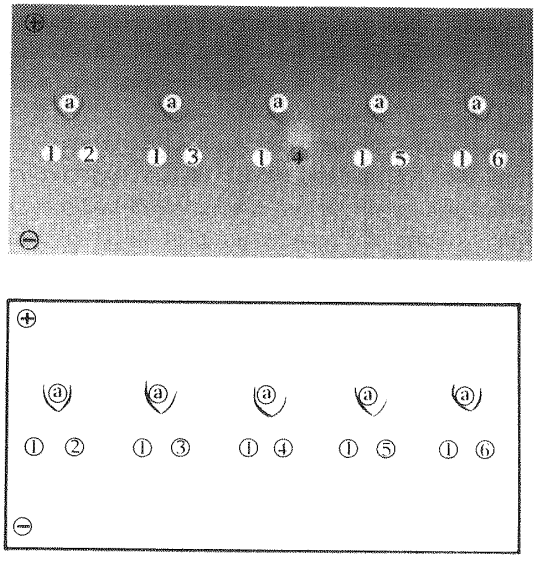


Fig. 3. Counterimmunoelectrophoresis of Hog Glandular Kallikreins against Rabbit Anti-Hog Pancreatic Kallikrein A Serum

a: antiserum to hog pancreatic kallikrein A.
1, pure hog pancreatic kallikrein A; 2, pure hog pancreatic kallikrein B; 3, pure hog submaxillary kallikrein; 4, partially purified hog renal kallikrein; 5, partially purified hog intestinal kallikrein; 6, hog pancreatic extract. Each kallikrein solution had an activity of 1000 KU/ml. See the text for experimental details.

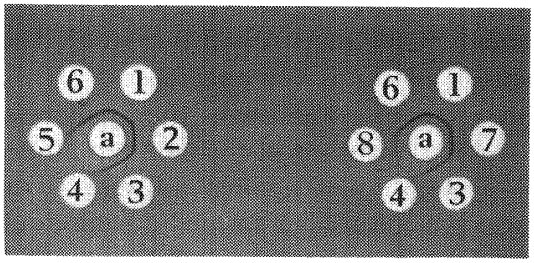
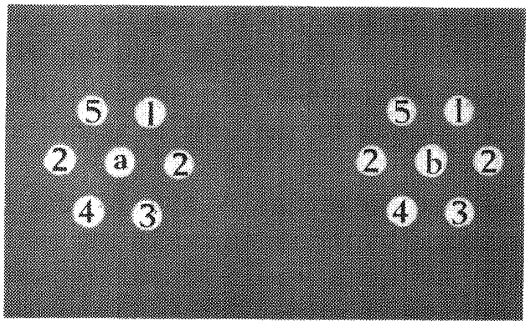


Fig. 4. Immunological Cross-Reaction of Various Glandular Kallikreins against Rabbit Anti-HUK Serum

a: antiserum to pure HUK.
1, pure HUK; 2, crude HUK; 3, partially purified human pancreatic kallikrein; 4, mixture of hog pancreatic kallikreins A and B; 5, saline solution; 6, partially purified human salivary kallikrein; 7, Bartter's HUK; 8, partially purified monkey pancreatic kallikrein. Each kallikrein solution had an activity of 1000 KU/ml. See the text for experimental details.



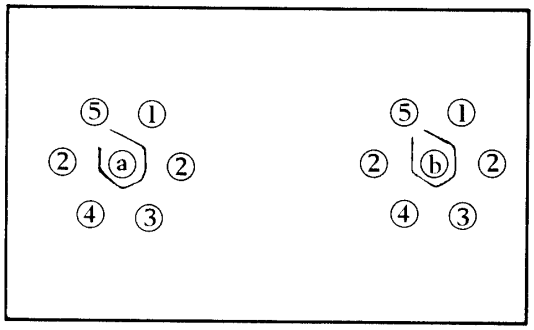


Fig. 5. Immunodiffusion Analysis of Various Forms of Hog Pancreatic Kallikreins against Rabbit Anti-Hog Pancreatic Kallikrein A Serum and Anti-B Serum

a: antiserum to pure hog pancreatic kallikrein A.
b: antiserum to pure hog pancreatic kallikrein B.
1, pure hog pancreatic kallikrein A; 2, pure hog pancreatic asialo-kallikrein A; 3, pure hog pancreatic kallikrein B; 4, pure hog pancreatic asialo-kallikrein B; 5, saline. Each kallikrein solution had an activity of 1000 KU/ml. See the text for experimental details.

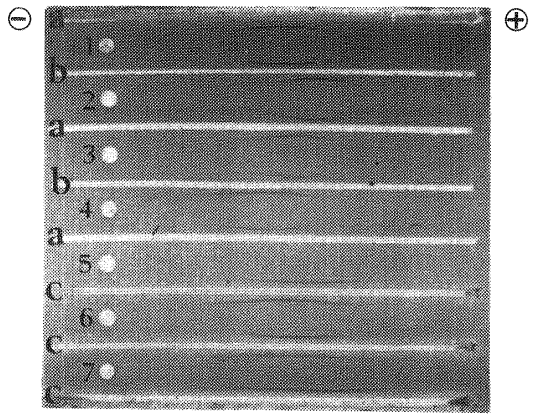


Fig. 6. Immunoelectrophoresis of Hog Glandular Kallikreins and HUK against Rabbit Anti-Hog Pancreatic Kallikrein A Serum and Anti-B Serum, and Rabbit Anti-HUK Serum

a: antiserum to pure hog pancreatic kallikrein A.
b: antiserum to pure hog pancreatic kallikrein B.
c: antiserum to pure HUK.
1, hog pancreatic extract; 2, pure hog pancreatic kallikrein A; 3, pure hog pancreatic kallikrein B; 4, pure hog submaxillary kallikrein; 5, pure normal HUK; 6, partially purified Bartter's HUK; 7, partially purified human salivary kallikrein. Each kallikrein solution had an activity of 1000 KU/ml. See the text for experimental details.

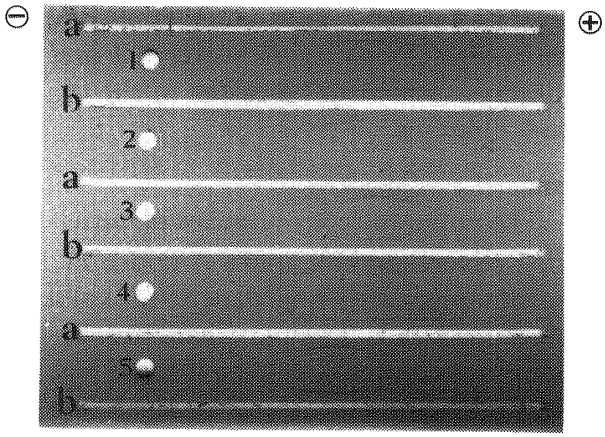


Fig. 7. Immunoelectrophoresis of Various Forms of Hog Pancreatic Kallikrein against Rabbit Anti-Hog Pancreatic Kallikrein A Serum and Anti-B Serum

a: antiserum to pure hog pancreatic kallikrein A.
b: antiserum to pure hog pancreatic kallikrein B.
1, hog pancreatic extract; 2, pure hog pancreatic kallikrein A; 3, pure hog pancreatic asialo-kallikrein A; 4, pure hog pancreatic kallikrein B; 5, pure hog pancreatic asialo-kallikrein B. Each kallikrein solution had an activity of 1000 KU/ml. See the text for experimental details.

between pure hog trypsin, pure bovine α-chymotrypsin or pure hog elastase (each 7 μl/well, 2 mg/ml) and the antisera directed against hog pancreatic kallikreins A and B (Fig. 1). These results suggest that kallikrein is the only cross-reacting protein in hog pancreas.

The antigenic relationships among the four hog glandular kallikreins (hog pancreatic, submaxillary, renal and intestinal kallikreins) were studied by means of the immunodiffusion (Fig. 2) and counterimmunoelectrophoretic (Fig. 3) techniques against antisera to hog pancreatic kallikreins A and B, separately. The single precipitin lines formed during double diffusion and counterimmuno-electrophoresis of the four hog glandular kallikreins against each rabbit antiserum were completely confluent with no spurs, indicating that all four hog glandular kallikreins are antigenically very closely related.

No cross-reaction occurred with partially purified human pancreatic kallikrein, pure HUK, rat pancreatic kallikrein or dog pancreatic kallikrein in an identical test against antiserum to hog pancreatic kallikrein (Fig. 2), suggesting an immunological species-specificity among the glandular kallikreins.

Human glandular kallikreins from different fluid (urine, saliva and pancreatic juice), and pancreatic kallikreins from different species (monkey and hog) were also studied by means of the double diffusion technique against the antiserum to HUK (Fig. 4). Rabbit anti-HUK serum gave a single precipitin line of complete identity with crude HUK, pure HUK, partially purified human salivary and pancreatic kallikreins, and monkey pancreatic kallikrein. No precipitation was obtained with pancreatic kallikreins from hog, rat and dog (not shown). Once again, identical results were obtained with the rabbit anti-HUK serum; this anti-HUK serum was specific for human glandular kallikreins and monkey pancreatic kallikrein. Furthermore, the single precipitin lines of normal HUK and Bartter's HUK against anti-HUK serum were completely confluent with no spurs.

The antigenic relationships among hog pancreatic kallikreins A and B, and hog pancreatic asialo-kallikreins A and B were next analyzed by the immunodiffusion method (Fig. 5). A single, continuous precipitin line with no spurs was produced, indicating immunological identity. However, the precipitin line produced between antiserum and asialo-kallikreins seemed to be closer to the center hole containing antiserum than in the case of sialo-kallikreins.

Immunoelectrophoresis

The immunoelectrophoresis patterns of various glandular kallikreins against the antisera to hog pancreatic kallikreins A and B and HUK are shown in Fig. 6. Single precipitin lines were obtained with hog pancreatic kallikreins A and B, and hog submaxillary kallikrein against antisera to hog pancreatic kallikreins A and B. The hog pancreatic homogenate also produced a continuous precipitin line, which was in the same position as those of pure hog pancreatic kallikreins A and B, suggesting that kallikrein is the only cross-reacting protein in the pancreas. However, different electrophoretic mobilities were observed for the three hog glandular kallikreins (pancreatic kallikreins A and B, and submaxillary kallikrein). The electrophoretic mobilities of the three kallikreins decreased in the order hog pancreatic kallikrein A, kallikrein B and submaxillary kallikrein. Again HUK did not give any precipitin line against anti-hog pancreatic kallikrein A serum. HUK did produce a precipitin line against anti-HUK serum, at a position intermediate between those of hog pancreatic kallikreins A and B. Furthermore, our anti-HUK serum produced a single precipitin line against Bartter's HUK and human salivary kallikrein. Bartter's HUK migrated slightly more slowly than normal HUK, and human salivary kallikrein produced a very broad precipitin line against anti-HUK serum (Fig. 6).

Immunoelectrophoresis patterns obtained between anti-hog pancreatic kallikrein and various forms of hog pancreatic kallikrein are shown in Fig. 7.

The hog pancreatic asialo-kallikreins A and B also produced only a single arc, but each asialo-enzyme showed a lower electrophoretic mobility than the corresponding sialo-enzyme. However, hog pancreatic asialo-kallikreins A and B did not appear at the same position.

Discussion

The immunological properties of pancreatic, submaxillary, renal and intestinal kallikreins from hog, and urinary, pancreatic and salivary kallikreins from humans have been studied by means of immunodiffusion and immunoelectrophoresis. Pancreatic kallikreins from rat, dog and monkey, trypsin and elastase from hog pancreas, and bovine pancreatic α-chymotrypsin were included in the study in order to define the limits of antigenic specificity of pancreatic kallikrein and other serine proteases.

The highly purified hog pancreatic kallikreins A and B injected into rabbit induced the production of specific anti-hog pancreatic kallikrein A serum and anti-B serum, respectively, as assessed by Ouchterlony double immunodiffusion and immunoelectrophoretic methods. A single precipitin line showing complete identity of hog pancreatic extract (*i.e.* crude hog pancreatic kallikrein fraction), purified submaxillary kallikrein and partially purified renal and intestinal kallikreins from hog as well as highly purified hog pancreatic kallikreins A and B was observed. Hog pancreatic kallikreins A and B also showed identical behavior on immunodiffusion. We interpret these results as indicating complete immunological identity of the four glandular kallikreins (pancreatic, submaxillary, renal and intestinal kallikreins) as well as the various forms of pancreatic kallikreins from hog. The antisera to hog pancreatic kallikreins A and B were each prepared in a sensitized rabbit, and hence the possibility remains that these anti-sera might be polyclonal antibodies. Further experiments with monoclonal antibody thus seem to be necessary to establish definitively the immunological identity of hog pancreatic kallikreins A and B. There is no cross-reaction with either HUK or pancreatic kallikreins of other species (human, dog and rat), and even though there is a close relationship between hog pancreatic kallikrein and other mammalian serine proteases (trypsin, chymotrypsin, elastase, *etc.*), which have 29—44% identical amino acid residues,[19,31] anti-hog pancreatic kallikrein serum did not cross-react with hog pancreatic trypsin, bovine pancreatic α-chymotrypsin or hog pancreatic elastase. The only dissimilarity among the various hog glandular kallikreins was their different electrophoretic mobilities. Similar differences have been observed using human glandular kallikreins including urinary, salivary and pancreatic kallikreins.[21] As regards hog glandular kallikreins, it is possible that the different electrophoretic mobilities are due to differences in the carbohydrate moieties, including sialic acid residues[13,19,32] or hog pancreatic kallikrein B might be formed by partial deamination, which may occur *in vivo* or during isolation.[13,19,21,32,33]

Anti-HUK serum produced a single precipitin line of complete identity with crude HUK as well as with pure HUK. Further, there was immunological cross-reaction among normal urinary, Bartter's urinary, pancreatic and salivary kallikreins from human, and monkey pancreatic kallikrein, though there was no cross-reaction with hog pancreatic kallikreins A and B against anti-HUK serum. The only observed dissimilarity of the human glandular kallikreins was their different electrophoretic mobilities, as recently concluded by Fink *et al.*[21b] These results strongly suggest that there is an immunological species-specificity among the glandular kallikreins, as recently suggested by other observations.[6,13,19-21,32,34-36]

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