The Immunological Similarity of Rat Glandular Kallikreins

By DAVID PROUD and GRAHAM S. BAILEY

Department of Chemistry, University of Essex, Wivenhoe Park, Colchester CO4 3SQ, Essex, U.K.

and KJELL NUSTAD and KÅRE M. GAUTVIK

Department of Clinical Chemistry, The Norwegian Radium Hospital, Montebello, Oslo 3, Norway

(Received 25 August 1977)

The immunological properties of the submandibular kallikrein, urinary kallikrein, pancreatic kallikrein and pancreatic prekallikrein of the rat were studied by immunodiffusion, immunoelectrophoresis and radioimmunoassay. Although they behaved differently electrophoretically, all the antigens showed identical immunological behaviour. The implications of this are discussed.

Glandular kallikreins (EC 3.4.21.8) are serine proteinases that produce small peptide hormones called kinins. The enzymes are glycoproteins that are found in tissue extracts and secretions of salivary glands, kidneys and pancreas (Webster *et al.*, 1970). In the last-named organ the enzyme exists as the inactive zymogen prekallikrein.

In a previous study (Brandtzaeg *et al.*, 1976) rat urinary and rat submandibular kallikreins were shown to behave similarly on immunodiffusion.

The object of this investigation was to extend the comparison to include the prekallikrein and kallikrein of the rat pancreas. In this study, however, immunodiffusion, immunoelectrophoresis and a radioimmunoassay for rat submandibular kallikrein (Nustad *et al.*, 1977) were utilized to produce a more comprehensive survey of the immunological behaviour of these enzymes.

Experimental

Materials

Pure rat submandibular kallikrein (Brandtzaeg *et al.*, 1976) and pure rat urinary kallikrein (Nustad & Pierce, 1974) were used in the immunological studies. Crude rat pancreatic homogenates were prepared as described elsewhere (Proud *et al.*, 1977). The partially purified preparations of rat trypsinogen and rat pancreatic prekallikrein were prepared by ion-exchange chromatography (Fig. 1 of Proud *et al.*, 1977) and are represented by the second pre-gradient peak and the fourth gradient peak respectively. Pure pig pancreatic kallikrein (Kallikrein) was kindly supplied by Bayer A.G., Wuppertal-Elberfeld, W. Germany.

Antibody to rat urinary kallikrein was raised in a sheep (Nustad & Pierce, 1974) and antibody to rat submandibular kallikrein was raised in two rabbits by injecting 150 µg of the pure enzyme in complete

Freund's adjuvant at multiple intracutaneous sites at 2-week intervals until bleedings with sufficiently high titre and avidity were obtained. Bz-Arg-OEt,* bovine trypsin, Amido Black 10B and agarose (type 1) were obtained from Sigma Chemical (London) Co., Kingston-upon-Thames, Surrey, U.K. Soya-bean trypsin inhibitor was purchased from BDH Chemicals, Poole, Dorset, U.K. ¹²⁵I was purchased from The Radiochemical Centre, Amersham, Bucks., U.K., and DASP sheep anti-(rabbit γ-globulin) immunoprecipitant was from Organon, Oss, The Netherlands. All other materials used were of analytical grade.

Methods

(a) *Protein determinations.* These were carried out on crude homogenates and partially pure preparations by the method of Warburg & Christian (1942).

(b) *Activation procedures.* Crude homogenates and partially purified rat trypsinogen were allowed to autoactivate at 25°C for 24 h.

Partially purified pancreatic prekallikrein was activated with bovine trypsin as follows. Trypsin solution (1 mg/ml) was prepared in 0.05 M-Tris/HCl, pH 7.75. Equal volumes of prekallikrein (about 2 mg of protein/ml) and trypsin solution were then incubated for 30 min at 25°C, after which time 10 mg of soya-bean trypsin inhibitor/ml was added to inhibit the trypsin.

To maintain identical conditions soya-bean trypsin inhibitor was added to all impure preparations. This addition also served to prevent activation of pre-enzyme preparations and, in activated samples, prevented any possible attack of antibody or enzymes by trypsin present in these preparations.

All preparations were tested for esterase activity to determine that they were active or inactive as required.

* Abbreviation: Bz-Arg-OEt, α-N-benzoyl-L-arginine ethyl ester hydrochloride.

(c) *Esterase assays.* Preparations were tested for esterase activity against Bz-Arg-OEt by a titrimetric assay with a Radiometer pH-stat assembly (Radiometer, Copenhagen, Denmark). The pH was maintained at 8.0 with 0.01 M-NaOH. Substrate was used at a concentration of 20 mM in 0.15 M-NaCl. Temperature was maintained at 25°C and a continuous N₂ flow was used to exclude atmospheric CO₂.

(d) *Immunodiffusion.* This was carried out in 1% agarose gels prepared in 0.07 M-barbitone buffer, pH 8.6. The pattern and dimensions of the wells were described by Beutner *et al.* (1968). Pure enzymes were used at a concentration of 1 mg/ml, and active and inactive partially purified preparations were concentrated to approx. 50 mg of protein/ml. Active and inactive crude homogenates were concentrated to approx. 500 mg of protein/ml. Best results were obtained when diffusion was carried out for 40 h at 4°C.

(e) *Immunoelectrophoresis.* Immunoelectrophoresis of 1 µl samples was carried out by using a Millipore immunoelectrophoresis system AR311 with accompanying pre-prepared 1% agarose slides. The electrophoresis was carried out in 0.07 M-barbitone buffer, pH 8.6. Two plates were electrophoresed simultaneously for 25 min at 100 V (current 25–30 mA). Antiserum (50 µl) was then added to each channel and diffusion allowed to occur over a period of 16 h at room temperature (20°C). The plates were press-dried and soluble protein was removed. The plates were then stained with Amido Black 10B (1% in 5%, v/v, acetic acid) for 5 min, and background stain was removed with 5% acetic acid. Sample concentrations were the same as those used for immunodiffusion.

(f) *Labelling.* Pure rat submandibular kallikrein was labelled with ¹²⁵I by the method of Hunter & Greenwood (1962) by using chloramine-T as oxidant. The labelled enzyme was separated from free iodide

by two successive gel filtrations on a column (1 cm × 20 cm) of Sephadex G-75.

(g) *Radioimmunoassay.* The radioimmunoassay procedure of Nustad *et al.* (1977) was used.

Samples were assayed as follows: 200 µl of standard or unknown, at a suitable dilution, was incubated with 100 µl of labelled enzyme (10000–20000 c.p.m.) and 100 µl of antibody to rat submandibular kallikrein (1:20000 dilution) for 20 h at 2°C. The antigen-antibody complex was separated from free antigen by immunoprecipitation. DASP sheep anti-(rabbit γ-globulin) immunosorbent (0.5 µl) was added to each tube and incubated in a shaker bath at 25°C for 2 h. Barbitone buffer, pH 8.6 (2 ml, 0.07 M), containing 0.1% bovine serum albumin and 0.01% merthiolate was added to each mixture. The samples were then centrifuged on an MSE Mistral 4L centrifuge for 10 min at 2500 g (*r*_{av}, 22.1 cm). The supernatant was removed to a fixed mark by aspiration. The buffer addition, centrifugation and aspiration procedure were repeated, and the radioactivity in the precipitated antigen-antibody complex was counted on a Tracerlab coru/matic 200 scintillation counter.

Results

As a preliminary measure bovine trypsin was shown to produce no cross-reaction with either antibody.

Fig. 1 shows the immunodiffusion patterns obtained against the antibody to rat submandibular kallikrein. The submandibular, urinary and pancreatic kallikreins all cross-react, as does the pancreatic prekallikrein (Fig. 1a). A single continuous precipitin ring with no spurs is produced, showing identical behaviour of the glandular kallikreins and prekallikrein from the rat on immunodiffusion. No cross-reaction occurred with either pig pancreatic kalli-

Fig. 1. *Immunodiffusion of antigens against antibody to rat submandibular kallikrein*

Antigens may be identified as follows. (a) 1, Pure rat submandibular kallikrein; 2, crude pancreatic homogenate; 3, activated crude pancreatic homogenate; 4, pure rat urinary kallikrein; 5, partially purified rat pancreatic kallikrein; 6, partially purified rat pancreatic prekallikrein. (b) 1, Pure rat submandibular kallikrein; 2, partially pure rat pancreatic kallikrein; 3, crude pancreatic homogenate; 4, pure rat urinary kallikrein; 5, pure pig pancreatic kallikrein; 6, partially pure rat trypsin. See the text for experimental details.

Fig. 2. Immunoelectrophoresis of antigens against antibody to rat submandibular kallikrein

Antigens may be identified as follows. 1, Pure rat submandibular kallikrein; 2, partially purified rat pancreatic prekallikrein; 3, partially purified rat pancreatic kallikrein; 4, pure rat urinary kallikrein; 5, partially pure rat trypsin; 6, crude pancreatic homogenate; 7, activated crude pancreatic homogenate; 8, pure pig pancreatic kallikrein. See the text for experimental details.

krein or rat trypsin (Fig. 1b), suggesting an immunological species specificity among the kallikreins. Identical results were obtained with the antibody to rat urinary kallikrein. Owing to the high protein concentration in the inactive and activated crude homogenates, precipitation tended to occur around the sample wells. This phenomenon also occurred on immunoelectrophoresis, but in neither case did it seem to have any effect on the results obtained.

The immunoelectrophoresis patterns obtained against the antibody to rat submandibular kallikrein are shown in Fig. 2. Again no cross-reaction was found with either pig pancreatic kallikrein or rat trypsin, whereas the submandibular and urinary enzymes both produced single precipitin arcs. The crude pancreatic homogenate also produced only a single arc, which was in the same position as that of the partially pure pancreatic prekallikrein, suggesting that this is the only cross-reacting species in the pancreas. Activation of either the crude or partially purified prekallikrein preparations results in an increase in electrophoretic mobility. Both the pan-

Fig. 3. Comparison studies using a radioimmunoassay for rat submandibular kallikrein

See the text for experimental details. Potential antigens are as shown. $\circ$, Standard curve for rat submandibular kallikrein; the curve for rat urinary kallikrein was identical with this; $\square$, partially pure rat pancreatic prekallikrein; $\triangle$, partially pure rat pancreatic kallikrein; $\bullet$, pig pancreatic kallikrein; $\blacksquare$, partially pure rat trypsin.

creatic kallikrein and its precursor have significantly lower electrophoretic mobilities than either the submandibular or urinary kallikreins. Once again identical results were obtained with the antibody to rat urinary kallikrein.

Owing to the very high sensitivity of the radioimmunoassay technique (0.1 ng detection limit), it was necessary to use a range of dilutions of impure preparations to obtain a range of antigen concentrations comparable with those of the pure enzymes (0–10 ng/0.2 ml). Cross-reactions are demonstrated by some degree of displacement of the antibody-bound radioactivity. Identical immunological behaviour would result in an identical displacement pattern and identical curves would be produced. For impure preparations, where exact antigen concentrations are not known, parallel curves would indicate identity.

The results of the experiments with the radioimmunoassay are shown in Fig. 3. Pig pancreatic kallikrein and rat trypsin caused no displacement of antibody-bound radioactivity, as indicated by the straight lines parallel to the axis. This again shows that these two enzymes do not cross-react. Rat submandibular kallikrein produced a typical standard curve on to which the values for rat urinary kallikrein could be directly superimposed, suggesting immunological identity between those two enzymes. The curves obtained with partially purified prekallikrein

and kallikrein from the pancreas are both parallel to the standard curve. Crude homogenates also produced parallel curves, but were omitted from Fig. 3 for clarity.

Discussion

The immunological properties of the submandibular kallikrein, urinary kallikrein, pancreatic kallikrein and pancreatic prekallikrein of the rat have been studied by immunodiffusion, immunoelectrophoresis and radioimmunoassay. Pig pancreatic kallikrein and rat trypsin were included in the study in an attempt to show the limits of specificity of the antigenic reactions involved.

Although pig pancreatic prekallikrein has been isolated (Fiedler *et al.*, 1970), this is the first time that the immunological properties of a pancreatic prekallikrein have been studied in comparison with the other glandular kallikreins from a given species. This is also the first time a radioimmunoassay has been utilized in a detailed immunological comparison of glandular kallikreins.

The fact that neither pig pancreatic kallikrein nor rat trypsin cross-react on any assay against either antibody suggests that, not only is the immunological reaction species specific, but also it is highly specific in terms of structure, since the antibodies do not cross-react with the trypsin of the same species.

All three rat glandular kallikreins and the pancreatic prekallikrein show identical immunological behaviour, but show differences in their electrophoretic mobilities. It is particularly noteworthy that, although activation of the prekallikrein results in a different electrophoretic mobility, as shown on immunoelectrophoresis, it does not affect its behaviour on immunodiffusion or radioimmunoassay. This suggests that whatever proteolysis occurs during activation has no effect on the highly specific antigenic site of the molecule. The fact that the crude pancreatic homogenate shows only a single arc on immunoelectrophoresis in the same position as that for the partially purified prekallikrein is further evidence that this is the only cross-reacting species in the pancreas and that rat trypsin does not cross-react. Immunoelectrophoresis also shows that the pancreatic kallikrein and its precursor have significantly lower electrophoretic mobilities than the other glandular kallikreins at the pH used. If it is assumed that the glandular kallikreins all have comparable molecular weights, then this lower electrophoretic mobility may indicate that the pancreatic enzyme may have a higher isoelectric point than either the

submandibular or urinary enzymes. The increase in mobility after activation of the prekallikrein may be due to a decrease in size after release of an activation peptide and/or a decrease in the isoelectric point of the enzyme as a result of the activation.

The radioimmunoassay provides a particularly sensitive and discerning method for the immunological comparison of antigens. Any degree of immunological similarity is shown up as a displacement of antibody-bound radioactivity. The closer the similarity of an antigen to the submandibular enzyme the closer its displacement curve is to the standard.

The identity shown by all the rat glandular kallikreins and the prekallikrein is indicated by the production of curves that are all parallel to the standard. For the urinary enzyme, where the antigen concentrations could be accurately determined, the experimental points chosen were the same as for the submandibular standard, and the curves produced were identical.

This immunological identity of the glandular kallikreins of the rat is a property that obviously has several useful ramifications. It allows, for example, the use of antibodies to the submandibular or urinary kallikreins in the cellular localization of the pancreatic zymogen. Also the production of parallel curves on the radioimmunoassay means that the assay for the submandibular kallikrein can be utilized for quantitative determinations of any of the other antigens. If the immunological identity of the glandular kallikreins within a species should be shown to be a common phenomenon, widespread applications could be envisaged.

References

- Beutner, E. H., Sepulveda, M. R. & Barnett, E. V. (1968) *Bull. W.H.O.* **39**, 587-606
- Brandtzaeg, P., Gautvik, K. M., Nustad, K. & Pierce, J. V. (1976) *Br. J. Pharmacol.* **56**, 155-167
- Fiedler, F., Hirschauer, C. & Werle, E. (1970) *Hoppe-Seyler's Z. Physiol. Chem.* **351**, 225-238
- Hunter, W. M. & Greenwood, F. C. (1962) *Nature (London)* **194**, 495-496
- Nustad, K. & Pierce, J. V. (1974) *Biochemistry* **13**, 2312-2319
- Nustad, K., Ørstavik, T. B. & Gautvik, K. M. (1977) *Microvasc. Res.* in the press
- Proud, D., Bailey, G. S., Ørstavik, T. B. & Nustad, K. (1977) *Biochem. Soc. Trans.* **5**, 1402-1404
- Warburg, O. & Christian, W. (1942) *Biochem. Z.* **310**, 384-421
- Webster, M. E., Mares-Guia, M. & Diniz, C. R. (1970) *Handb. Exp. Pharmacol.* **25**, 131-155