Br. J. Pharmac. Chemother. (1967), **30**, 134–142.

PURIFICATION OF A KININ-INACTIVATING ENZYME FROM CULTURES OF *PSEUDOMONAS AERUGINOSA*

BY

H. E. RUGSTAD

From the Institute of Physiology, University of Oslo, Oslo, Norway

(*Received December* 12, 1966)

Cultures of the microbe *Pseudomonas aeruginosa* (*Ps. aer.*) can produce considerable amounts of an enzyme which inactivates plasma kinins. This kininase is found mainly in the culture medium (Amundsen & Rugstad, 1965; Rugstad, 1966), where it can be detected in concentrations as low as 10^{-10}–10^{-11} g/ml. (Rugstad, 1966). A kininase of microbial origin is of special interest in connexion with the possible function of kinins as mediators in the production of inflammation. It might also be useful in the study of extracellular enzymes, because it can be identified in such small quantities.

This paper describes how the enzyme may be purified and some of the properties of the purified material.

METHODS

Determination of enzyme activity. Full details of the estimation of kininase activity have already been given (Rugstad, 1966). The fluid to be tested was incubated with synthetic bradykinin (BRS 640, Sandoz, Basle, Switzerland) and aliquots were removed at intervals and the residual bradykinin assayed on the rat uterus preparation. One unit of kininase has been defined as the amount of enzyme which will destroy 75% or more of 500 ng bradykinin in 11 min (but not in 6 min) at 37° C.

Bacterial strain. The strain of *Pseudomonas aeruginosa* used was the same as that described in a previous paper and was grown in medium "B" (Rugstad, 1966), which contains: K_2HPO_4 11 g, KH_2PO_4 1 g, Na_3-citrate · $5H_2O$ 1 g, $MgSO_4$ · $7H_2O$ 0.1 g, $(NH_4)_2SO_4$ 2 g, Glucose 5 g/l.

The water used for the medium contained the diffusate of 10 g yeast extract per litre.

Culturing of microbes. One and a half litre of medium was autoclaved in a 3 l. glass container equipped with a stirring device and an inlet tube for aeration. The container was incubated in a water bath at 37° C and inoculated with 100 ml. of a 12 hr old culture of *Ps. aer.* from the same medium. The stirring device was adjusted so that a vortex was formed and sterile air was then blown through the medium.

From the 10th hr after inoculation and onwards the amount of enzyme in the medium was determined every hour. When it was desired to harvest the culture the medium was centrifuged in batches at 10,000 *g* for 15 min, and the supernatant fluid pooled.

The protein content at various stages in the purification process was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) using human serum albumin as standard.

The protein content of fractions obtained after gel filtration and ion-exchange chromatography was estimated by measuring the absorbency at 280 mμ in a Zeiss model PMQ-II spectrophotometer, using quartz cells with an optical path length of 10 mm.

Gel filtration was performed with Sephadex G-100, bead form (lot no. 7120), obtained from A. B. Pharmacia, Uppsala, Sweden. The gel was allowed to swell for 4 days in 0.02 M Na-phosphate

buffer of pH 7.5, and packed in a Sephadex laboratory column, measuring 2.5 cm×100 cm, by the method of Flodin (1962). The height of the gel bed was 68 cm. The flow rate was adjusted to 12–15 ml./hr. Gel filtration was carried out at 5° C and portions of 5 ml. were collected with an automatic fraction collector.

Ion exchange chromatography was performed with the anion exchanger DEAE-Sephadex A-50, bead form (lot no. TO-7372, A. B. Pharmacia, Uppsala, Sweden). This was allowed to swell in de-ionized water for 4 days. The water was decanted and the gel material suspended in 1 N H_3PO_4 for 30 min. This gel suspension was centrifuged for 5 min at 1,000 *g* and thereafter equilibrated with 0.02 M Na-phosphate buffer of pH 8.1. The gel was then packed in a column with a cross section of 0.9 cm^2 until the volume of packed gel was 9 cm^3. The flow rate was kept at 0.2 ml./min by adjusting the hydrostatic pressure. Fractions of 5 ml. each were collected. This ion-exchange chromatography was carried out at room temperature.

Evaporation of water was carried out at 37° C and reduced pressure, using a rotating evaporator connected to a high vacuum pump.

Electrophoresis on cellulose acetate strips was carried out with a Shandon electrophoresis outfit (Shandon Scientific Company Ltd., London, England) and with 2½×12 cm Oxoid cellulose acetate strips (Oxoid division of Oxo Ltd., London, England). The procedure was that described in the booklet issued by Oxoid (1965): *Electrophoresis with Oxoid Cellulose Acetate Strips.* Three different buffers were used (1) a 0.1 M barbitone-acetate buffer of pH 7.5 corresponding to that described by Owen (1956); (2) a 0.1 M Na-phosphate buffer of pH 7.5 and (3) a 0.1 M Na-phosphate buffer of pH 5.8.

At the beginning of electrophoresis a constant voltage of 150 V was used. If the resistance changed during the electrophoretic run the voltage was adjusted so that the current was always kept below 0.4 mA/cm width of strip. Electrophoresis was performed with each buffer for periods of 1, 2 and 4 hr. The strips were then cut transversally and the enzyme content of the pieces determined.

Disc gel electrophoresis on polyacrylamide gel was carried out with the Canalco Model 12 system (Canalco Corporation, Bethesda, Maryland, U.S.A.) with glass tubes of 7×0.5 cm as described by Davis (1964). The theory of this method is discussed by Ornstein (1964). Unless otherwise stated, preparation of stock solutions as well as preparation and staining of the gel columns was performed as described in *Canalco Chemical Formulation and Instructions for Disc Electrophoresis* (1965). In one set of experiments standard gel (7%), stacking at pH 8.9, running at 9.5, was used. The special procedure for very dilute protein solutions was employed.

The working solution for the preparation of sample gel was modified by dissolving riboflavin 4 mg/100 ml. directly in stock solution (B), instead of using a separate riboflavin solution. There was, therefore, less dilution of the protein in the sample gel. The electrophoresis was carried out with a constant current of only 2.5 mA per column to avoid unnecessary heating of the columns.

In another set of experiments low pH gel (7.5%), stacking at pH 5.0, running at pH 4.3, was used as described in the *Formulation.* Again the special procedure for very dilute protein solutions was employed.

In each experiment two or more gel columns were used for staining and two or more for enzyme assay. The latter columns were cut in 3 mm thick slices with a modification of the gel column cutter described by Heideman (1964). Each slice was transferred to a clean tube containing 2 ml. of a 0.02 M Na-phosphate buffer at pH 7.5 and then gently crushed. The tubes were shaken in a bath at 37° C for 2 hr, and the kininase activities in these fluid contents were then assayed.

RESULTS

Purification procedures

The starting material was 1,400 ml. supernatant from an 18 hr old culture of *Ps. aer.* The protein content of this supernatant was 1.25 mg/ml. and its kininase activity 200 u./ml. This is apparently the maximal amount of enzyme which can be produced under

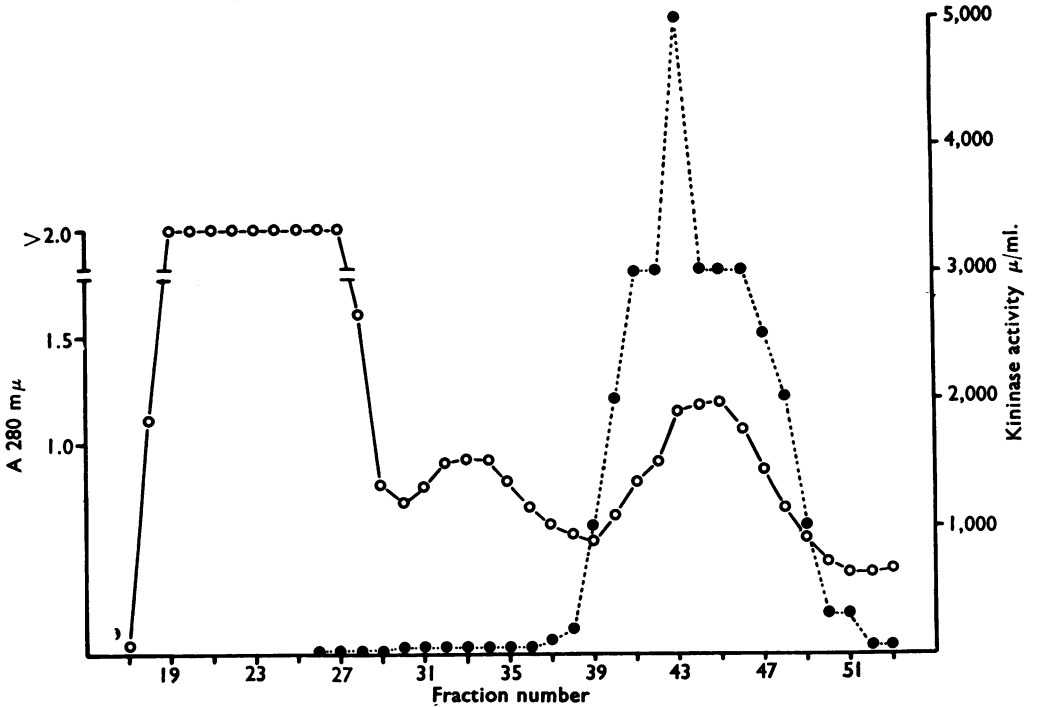

Fig. 1. Results from gel filtration on a Sephadex G-100 column (2.5×68 cm) of the concentrated, dialysed medium from a *Ps. aer.* culture (see Methods). ●---● = kininase activity in u./ml.; O——O = protein content, estimated as optical density (A) at 280 mμ.

these conditions, and the culture was therefore harvested when this amount of enzyme in the medium had been reached.

Evaporation of water and dialysis. The volume of the supernatant was reduced to 100 ml. with a rotating evaporator and the concentrated portion dialysed through a Visking dialysis tube, 18/32 (Visking Dept., Union Carbide International Co., New York, U.S.A.) against running tap water for 14 hr whereby the volume increased to 270 ml. This material was again concentrated by evaporation of water until the volume was 26 ml. After these procedures the kininase activity in the preparation was 10,000 u./ml. and the protein content 60 mg/ml.

Gel filtration. One half of this material, 13 ml., was submitted to gel filtration, the results of which are illustrated in Fig. 1. The kininase activity appeared relatively concentrated in about 10 fractions. Of these, the fractions from numbers 40 to 48 (inclusive) were pooled. This pooled material, which after testing measured 42 ml., had a kininase activity of 3,000 u./ml. and a protein content of 0.64 mg/ml.

Ion-exchange chromatography. One half of the pooled material from the gel filtration was dialysed for 2 hr against 0.02 M Na-phosphate buffer at pH 8.1 and then applied to the A-50 gel equilibrated with the same buffer (see Methods). Thereafter the column was washed with 0.02 M Na-phosphate buffer at pH 8.1 until the protein content of the eluate was zero as judged by absorbency at 280 mμ (Fig. 2).

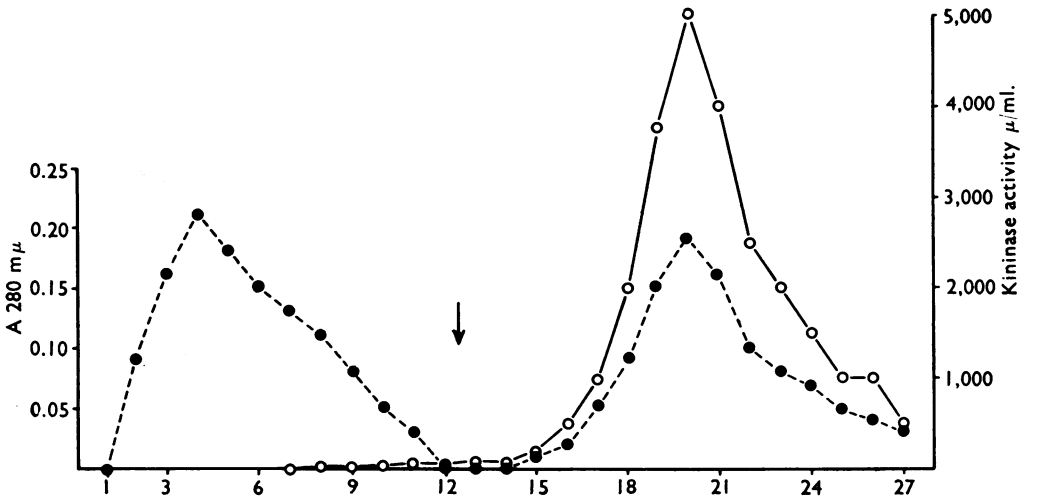

Fig. 2. Elution pattern from a DEAE-Sephadex A-50 column (1×10 cm) of the pooled material of fractions no. 40–48 from the gel filtration illustrated in Fig. 1. ●---●=kininase activity in u./ml.; O——O=protein content, estimated as optical density (A) at 280 mμ. The arrow indicates the point when elution with 0.05 M NaCl in the buffer was started.

The enzyme was then eluted with 0.05 M NaCl in the same buffer. The three fractions with the highest kininase content, nos. 17–19 (inclusive), were pooled, and this material constituted the final product. It had a kininase content of 4,000 u./ml. and a protein content of 0.16 mg/ml. Almost the same relationship between enzyme activity and protein content was found in all the fractions eluted with 0.05 M NaCl and with a high kininase content. The main data from one typical run of the various purification procedures are given in Table 1. A 166-fold purification had been obtained, and 90.1% of the kininase was recovered.

Properties of the enzyme

Ultraviolet absorption spectrum was investigated in a solution containing 0.17 mg/ml. of the enzyme in 0.02 M Na-phosphate buffer of pH 7.5. The examination was carried

TABLE 1

STAGES IN THE PURIFICATION OF THE KININASE FROM CULTURES OF *PSEUDOMONAS AERUGINOSA*

Stage in purification process	Volume	Activity u./ml.	Protein mg/ml.	Specific activity u./mg protein	Yield	Enrichment
Starting material (supernatant from cultures)	1,400	200	1·25	160		
After evaporation, dialysis and further evaporation	26*	10,000	60·0	166	92·9	1·04
Pooled fractions 40–48 from filtration on Sephadex-G ×100 column	42*	3,000	0·64	4,680	85·7	29·25
Final product	15	4,250	0·16	26,560	90·1	166

* One half of the material only subjected to further purification.

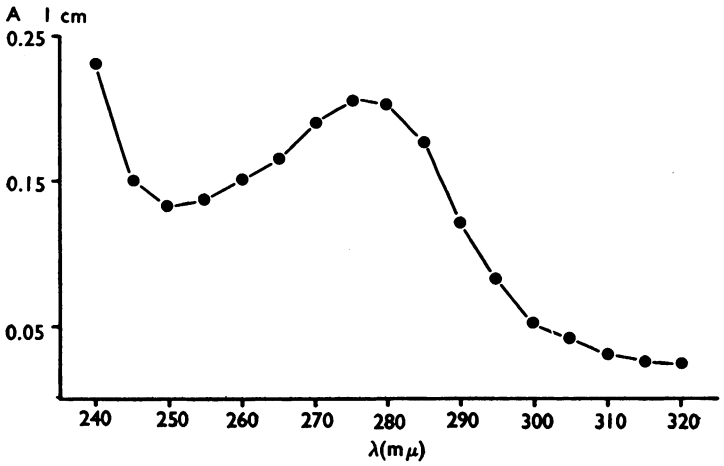

Fig. 3. Ultraviolet absorption spectrum of the purified enzyme dissolved in 0.02 M Na-phosphate buffer of pH 7.5. Protein concentration in the solution: 0.17 mg/ml. The light path was 1 cm.

out in the whole ultraviolet part of the spectrum and at 5 mμ intervals. The light path was 1 cm. The results are seen in Fig. 3. The enzyme solution has a maximum absorption at 275 mμ, and a minimum one at 250 mμ.

Absorbency of the enzyme solution was less than 0.02 at all wave lengths between 325 and 1,000 mμ.

Behaviour on cellulose acetate electrophoresis. The enzyme did not move on cellulose acetate strips under the conditions described in Methods, but was always found at the place of application.

Behaviour on disc electrophoresis on polyacrylamide gel. In one experiment with standard gel (7%) (stacking at pH 8.9, running at pH 9.5) the sample was submitted to electrophoresis for 70 min, by which time the tracking dye was near the bottom of the gel column. Only one single band of protein was seen after staining. This band had moved 6 mm into the separating gel (Fig. 4A). Enzyme assay of slices of another column which had been run simultaneously showed kininase activity corresponding to the protein band. No kininase activity was found in other parts of the gel. In another experiment with the same gel and buffer the electrophoresis was continued for 160 min. Again one single band of protein was found. This time it had moved 25 mm into the separating gel (Fig. 4B). On enzyme assay of an unstained column, kininase activity was again found corresponding to the protein band and in no other parts of the gel.

In another set of experiments, low pH gel (7.5%, stacking at pH 5, running at pH 4.3) was used. When samples were incubated for 1 hr at room temperature with the buffer used in this experiment, however, there was irreversible loss of enzyme activity. No enzyme activity could thus be identified after electrophoresis at this pH, although a single protein band was obtained. This band had moved 4 mm into the separating gel (Fig. 4C) after 55 min electrophoresis and 39 mm into the separating gel after 135 min.

The supernatant from a *Ps. aer.* culture, which had been dialysed and concentrated three times, gave at least six different protein bands after disc electrophoresis run in a standard gel column at high pH (Fig. 4D) for 75 min.

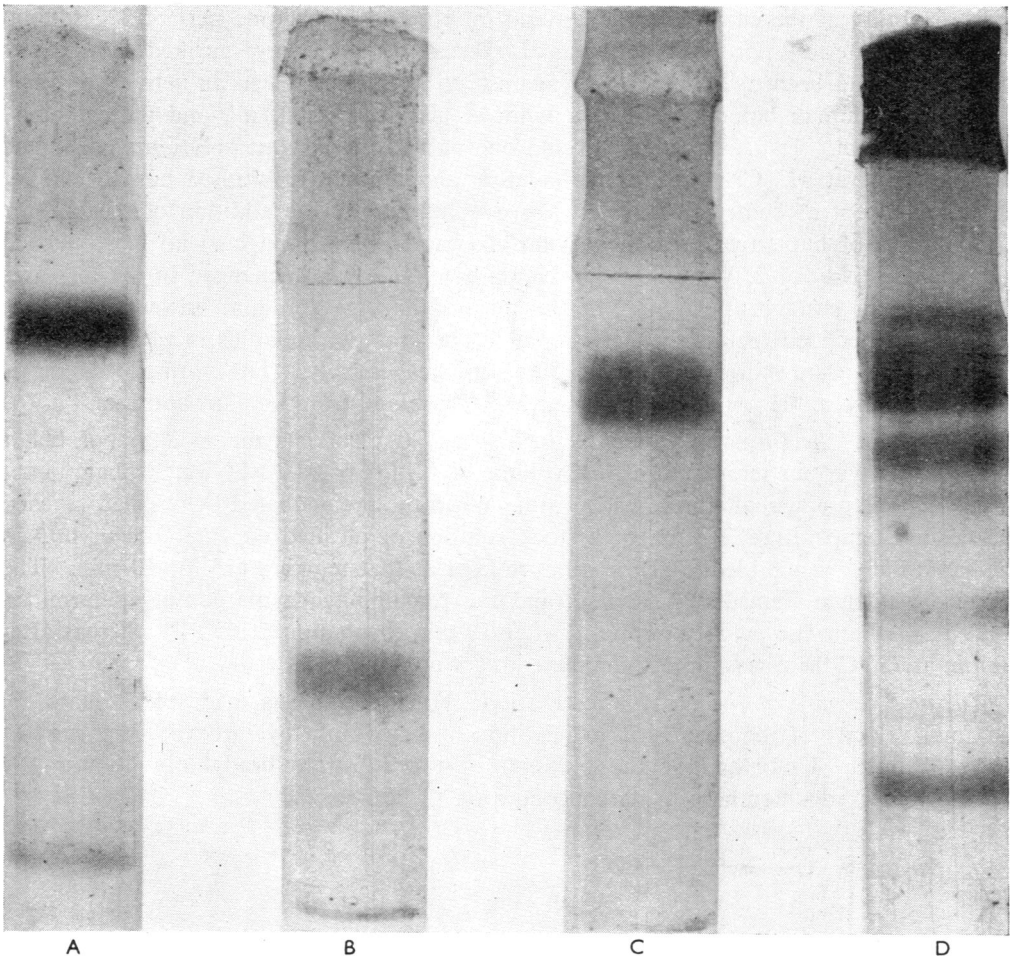

Fig. 4. Disc electrophoresis on polyacrylamide gel of a purified enzyme preparation and of starting material in purification procedure. (A) purified enzyme from *Ps. aer.*, standard gel (7%, pH 9.5) after electrophoresis for 70 min. (B) same as A, after electrophoresis for 160 min. (C) purified enzyme low pH gel (7.5%, pH 4.3) after electrophoresis for 55 min. (D) starting material—i.e., supernatant from the culture, dialysed and concentrated three times (see text). Standard gel, after electrophoresis for 75 min.

Stability in buffered solutions and in blood. The enzyme activity in the supernatant from *Ps. aer.* cultures remained constant for 25 hr at 37° C. The partly purified material obtained after gel filtration was also very stable. This material could be kept at 37° C for 24 hr with loss of activity less than 25%. When this material was kept at −20° C in the same phosphate buffer as used in the gel filtration, no loss of activity was detectable after 2 months. The final product was, however, less stable. The purified enzyme lost half of its activity in 6 weeks when kept in 0.02 M Na-phosphate buffer at pH 7.5 at −20° C. When kept at −90° C loss of activity was almost the same. Addition to the buffered solution of the purified enzyme of the salts present in the culturing medium (in the same concentration as used there) did not prevent loss of activity.

The stability of the enzyme in human and rat citrated blood (one part of 3% sodium citrate dihydrate to 9 parts of blood) was also tested. A solution of purified enzyme was used, which had been dialysed for 4 hr against 0.9% NaCl and had an activity of 1,000 u./ml. The human blood itself had a kininase activity of 5 u./ml. and the rat blood one of 30 u./ml. Two parts of blood and one part of the enzyme solution were mixed and incubated at 37° C. The enzyme solution and samples of citrated human and rat blood were kept as controls at 37° C. Five minutes after the addition of the enzyme the mixtures of human blood and enzyme and of rat blood and enzyme both showed a kininase activity of 250 u./ml. This kininase activity was unchanged in the mixtures after 24 hr of storage at 37° C. In the purified enzyme solution, however, kininase activity had declined to less than 750 u./ml. The kininase activities in the portions of citrated blood were still 5 u./ml. and 30 u./ml. respectively. The purified enzyme is thus apparently stable for at least 24 hr at 37° C in blood from humans and rats.

The effect of heating was tested by incubating 40 u. of enzyme in 2 ml. of buffer for 10 min at various temperatures. A volume of 1.8 ml. of 0.02 M Na-phosphate buffer at pH 7.5 was preincubated in a water bath. When the preincubated buffer had reached the desired temperature, 0.2 ml. of a stock solution of purified enzyme (200 u./ml.) in the same buffer was added, and the mixture kept at that temperature for 10 min. The mixture was then immediately cooled, and the remaining enzyme activity determined (Fig. 5). All enzyme activity remained after 10 min incubation at 65° C, whereas after 10 min at 75° C there was almost complete loss of activity.

Maximal velocity of the enzymatic reaction. An attempt was made to estimate the maximal velocity of the enzymatic inactivation of bradykinin by saturating the enzyme with substrate. Unfortunately the bioassay of the substrate bradykinin allows only relatively marked differences in its concentration to be detected.

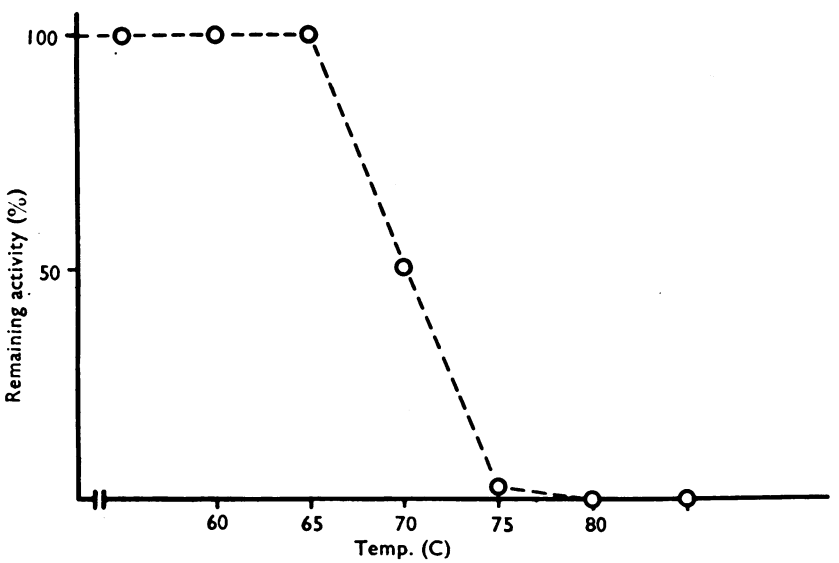

Fig. 5. Residual activity (in %) after heating of purified enzyme preparation for 10 min at various temperatures. Test samples contained 40 u. of enzyme dissolved in 2 ml. of 0.02 M Na-phosphate buffer of pH 7.5.

In one set of experiments 0.5 ml. of a solution containing 25,000 ng bradykinin was incubated with 4.5 ml. of an enzyme solution containing 1/10 of a u./ml. The purified enzyme was newly prepared and dissolved in 0.02 M Na-phosphate buffer at pH 7.5. Aliquots of 1 ml. of the mixture were taken out after 15, 30 and 45 min, and the reaction stopped by transferring each of them into 9 ml. of boiling saline. The mixture was kept boiling for 5 min, after which it was cooled, and the volume adjusted to 10 ml. This procedure destroyed the kininase activity completely, whereas control experiments showed that it caused no detectable loss of bradykinin. The concentration of bradykinin in such a 10 ml. sample would be 500 ng/ml. if no inactivation had taken place. The actual content of bradykinin in the various samples was estimated by a "bracketing" assay using 0.1 ml. doses of the samples added to the rat uterus. The samples taken after 15 min contained about 400 ng/ml. bradykinin. The 30 min and 45 min samples contained about 300 ng and 200 ng bradykinin/ml. This means that about 0.07 μg synthetic bradykinin is inactivated per min at 37° C and pH 7.5 by 0.9 ml. of an enzyme solution containing 1/10 of a u./ml. In another set of experiments twice the initial concentration of bradykinin was used but the enzyme concentration was kept the same. The amount of bradykinin inactivated per min by 0.9 ml. of the same enzyme solution was again about 0.07 μg. This indicates that 1 mg of enzyme could inactivate $(0.07 \times 26{,}560 \times 10 \times 10/9)\mu$g or 20.7 mg (0.02 m mole) bradykinin/min at 37° C.

DISCUSSION

Purification of extracellular enzymes from bacterial cultures may involve special problems. The enzyme concentration in the culture medium is usually low, and large quantities of salts and other extraneous material must be removed. Fortunately the growth medium used in the present experiments contains little protein and, further, the enzyme is very stable. There is little loss of total activity with the purification process described here.

The final enzyme product has apparently a high degree of purity. It is electrophoretically homogeneous on polyacrylamide gel disc electrophoresis at pH 9.5 and pH 4.3. Moreover, when the purified enzyme is finally eluted from the ion exchange column, an almost constant relationship exists between enzyme activity and protein content in the various fractions.

The enzyme can be dialysed without loss of activity and there is no indication that a co-factor is necessary. Although in its pure form the enzyme is relatively unstable, its activity is well maintained in blood. The reason for this stabilizing effect of blood is unknown, but the stability of the kininase in blood suggests that it may play a part in inflammatory reactions caused by *Pseudomonas aeruginosa.*

The specific activity of the enzyme with bradykinin as substrate is very high. Erdös, Renfrew, Sloane & Wohler (1963) used purified carboxypeptidase B (Worthington) from swine pancreas and found that "at a molar ratio of higher than 1:10,000 over half of the effect of bradykinin was abolished by the enzyme within a half hour." The amount of bradykinin used was 3.4 μg. If 1.7 μg bradykinin were inactivated and the molar ratio were 1:10,000, this would mean that 0.01 μg carboxypeptidase B had inactivated 0.057 μg bradykinin per min. The same amount of the kininase from *Ps. aer.* will inactivate more than three times as much. This comparison, however, is only a rough

estimate. Apart from the approximations in the above calculations, the comparison is not based on the initial rates of the reaction. These cannot be measured by this method because a large fraction of the substrate must be inactivated before the decrease in concentration can be detected. The only other kinin-inactivating enzyme hitherto purified, chymotrypsin, is even less active. Its specific activity with bradykinin as substrate is between 1-50th and 1-100th that of carboxypeptidase B (Erdös *et al.*, 1963).

SUMMARY

1. A kinin-inactivating enzyme has been purified from the culture medium of *Pseudomonas aeruginosa*. The medium used was composed of salts, glucose and the diffusate of yeast extract. The purification steps are: concentration by evaporation of water, dialysis, gel filtration on Sephadex G-100 and ion-exchange chromatography on DEAE-Sephadex A-50.

2. The purified enzyme is homogeneous on disc electrophoresis on polyacrylamide gel at pH 9.5 and 4.3.

3. The crude and partially purified enzyme is remarkably stable. It is also stable for 24 hr at 37° C in human or rat blood. The purified enzyme is less stable and lost half its activity in 6 weeks when stored at −20° C.

4. The reaction of the enzyme with bradykinin was found to proceed at a velocity corresponding to the inactivation of about 0.02 mM of bradykinin/mg enzyme/min at 37° C and pH 7.5. The difficulties in estimating the maximal velocity of the reaction are discussed.

Financial support from the Norwegian Research Council for Science and the Humanities, from the Nansen Foundation and from the Norwegian Council on Cardiovascular Diseases is gratefully acknowledged. I also wish to thank Miss M. Tschabold for skilful technical assistance.

REFERENCES

AMUNDSEN, E. & RUGSTAD, H. E. (1965). Influence of some pathogenic bacteria on kinin formation and destruction. *Br. J. Pharmac. Chemother.*, **25**, 67–73.

Canalco Chemical Formulation and Instructions for Disc Electrophoresis (1965). Canalco Industrial Corporation, Bethesda, Maryland.

DAVIS, B. J. (1964). Disc electrophoresis—II. Method and application to human serum proteins. *Ann. N.Y. Acad. Sci.*, **121**, 404–427.

Electrophoresis with Oxoid Cellulose Acetate Strips (1965). Oxoid Ltd., London.

ERDÖS, E. G., RENFREW, A. G., SLOANE, E. M. & WOHLER, J. R. (1963). Enzymatic studies on bradykinin and similar peptides. *Ann. N.Y. Acad. Sci.*, **104**, 222–235.

FLODIN, P. (1962). Experimental technique. In *Dextran Gels and their Application in Gel Filtration*, pp. 42–48. Pharmacia, Uppsala.

HEIDEMAN, M. L., JR. (1964). Disc electrophoresis of I^{131}-labeled protein hormone preparations and their reaction products with antibodies. *Ann. N.Y. Acad. Sci.*, **121**, 501–524.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275.

ORNSTEIN, L. (1964). Disc electrophoresis—I. Background and theory. *Ann. N.Y. Acad. Sci.*, **121**, 321–349.

OWEN, J. A. (1956). Determination of serum-protein fractions by zone electrophoresis on paper and direct reflection photometry. *Analyst, Lond.*, **81**, 26–37.

RUGSTAD, H. E. (1966). Kininase production by some microbes. *Br. J. Pharmac. Chemother.*, **28**, 315–323.