A Protease Inhibitor in Ox Lung Tissue

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Procedures are described for the isolation and partial purification of a protease inhibitor from ox lung tissue. The active compound has a characteristic thermostability. It is most stable at acid reaction, and is destroyed at neutral and alkaline reaction. The inhibitor differs from heparin and shows similarity with pancreatic trypsin inhibitors.

Ox lung tissue inhibits the proteolytic effect of a number of proteases\(^1\). The present paper describes the isolation of a powerful protease inhibitor from ox lung tissue. The inhibitor differs from heparin and appears similar to the trypsin inhibitor present in pancreatic tissue. The active compound has been termed *pulmin*\(^2\).

EXPERIMENTAL

1. Estimation of inhibitory effect. Trypsin (crystalline, Armour, containing less than 50 % MgSO\(_4\)) was used in estimating the inhibitory effect. The proteolytic activities of appropriately mixed enzyme solution (1 vol.) and inhibitor dilutions (1 vol.) were estimated by the fibrin plate method and recorded as the products in mm\(^2\) of two diameters of the lysed zones (average of 3 determinations)\(^3\). In the first part of this work the activity was estimated at pH 6.5 or 7.2 and at 32\(^\circ\). Later when the improved fibrin plate method (pH 7.8; 37\(^\circ\)) was available\(^4\), this was used.

Though the form of the inhibition curves does not allow a precise quantitative estimation of the inhibitory activity at low concentrations of inhibitory compounds, the fibrin plate method has important advantages in preparative work. The presence of salts at different concentrations, of dissolving or precipitating agents, of small amounts of alcohol, or even of solid matter in suspension, does not significantly interfere with the estimations. This makes the estimation of the inhibitory effects of the preparations obtained very simple. Thiocyanate (1 M) had no influence on the trypsin activity or on the inhibitory effect, and was therefore preferred as the dissolving medium in the estimation of the activity of the preparations. In order to increase the accuracy of the estimations during the final steps of the purification procedure, the inhibitory effect towards chymotrypsin (crystalline, Armour) was also measured, because the curves obtained here were more suitable for interpolation. The yields given below are approximate. They vary from batch to batch of crude material, and the amount of total substances in a given preparation is not always easily estimated (see later).

2. Crude material. The ox lung tissue was freed from water-soluble proteins by repeated treatment of the ground tissue with 0.9 % NaCl and water, followed by dehydration with acetone\(^1\). The aqueous phase was removed through gauze instead of by centrifug-
ing, and the final treatment with ether was omitted. One kg of ground lung tissue produced about 70 g of dried tissue containing about 13.3 % N. The inhibitory effects of these preparations were compared by shaking a trypsin solution with varying amounts of the dried tissue, followed by estimation of the protease activity retained in the solution 3. Larger amounts, prepared according to our description, were kindly placed at our disposal by the Løvens kemiiske Fabrik, Copenhagen.

3. Crude extract. Extraction of the inhibitory compound was accomplished with thiocyanate, as used by Snellman, Jensen and Sylven 4, in the isolation of heparin. Because of considerable swelling of the lung tissue an amount of 5 g dried tissue per 100 ml solution was found most convenient.

The dried tissue (100 g) was stirred for 2 hours at room temp. with 2 000 ml M potassium thiocyanate and then passed through a double layer of gauze, yielding about 1 650 ml extract. The nitrogen content was estimated to 0.2 mg/ml (Micro-Kjeldahl) after removal of thiocyanate by dialysis (discarding a small inactive sediment). This gave about 350 mg N in the extract from 100 g dried tissue.

Low yields were obtained in extraction experiments with secondary phosphate (M), NH₂OH, sodium salicylate (50 %), dextrose (50 %), thiosulphate (M) and pyridine (5 %). Urea (50 %) gave potent solutions but inconsistent results. Treatment with NaOH yielded very potent solutions, but produced also an extreme swelling of the lung tissue, resulting in poor yields. Potassium thiocyanate (M) produced potent solutions without too much swelling. Treatment for 2, 6 and 20 hours (at room temp.) gave the same potencies of the extracts, but the content of impurities increased with time. Best yields were obtained with 0.5 - 1.0 M potassium thiocyanate. Extraction at room temp. produced slightly higher activity than at 0°, but also a higher N content. The turbid thiocyanate extract could be clarified without loss of activity by passing through a Seitz clarification filter (asbestos filter pad). Dialysis produced a slight, inactive sediment and from the resulting solution the active compound could be precipitated with acetone or ethanol. Repeated treatment of the lung tissue with thiocyanate did not significantly increase the yield of soluble inhibitor, though a certain amount of inhibitor effect still remained in the lung tissue. Extraction of the washed lung tissue before dehydration yielded inferior results because of an immense swelling of the tissue, and because of the presence of large amounts of inert material in the resulting solution. The activity and N content of the crude extract varied considerably with the sample of lung tissue used.

4. Fractional precipitation. Large amounts of inactive proteins could be removed by means of sulphasalicylic acid whereafter the inhibitory fraction could be precipitated by means of tungstic acid. The procedure finally adopted was as follows:

Five ml of 0.1 M sodium sulphasalicylate was added to each 100 ml of undialyzed thiocyanate extract. The mixture was stirred mechanically, and N HCl was added until pH 2.8 was attained. An inactive precipitate (amounting to 150 mg N per 100 g dried tissue) was removed by centrifugation. 10 ml of 0.1 M sodium tungstate were added to each 100 ml of the supernatant followed by 10 ml N HCl (mechanical stirring). The pH was now about 1.3. After standing in the icebox for 2 hours for complete sedimentation, the mixture was centrifuged and the inactive supernatant discarded. The precipitate could be redissolved in about 100 ml H₂O per 100 g tissue by addition of solid sodium bicarbonate to neutral reaction. The solution had about 200 mg N per 100 g tissue, and contained most of the activity of the crude extract. The methods used in this fractional precipitation were derived from previous studies on the precipitation of proteins with anions 5, 6. If the precipitation was carried out on a dialyzed extract the precipitate with sulphasalicylic acid contained the active material.

5. Acetone precipitation. When the solution containing the dissolved tungstic acid precipitate was dialyzed against tap water (24 hours), a precipitate containing part of the inhibitory activity was formed. In order to retain this substance in solution it was found necessary to add an equal volume of 1 M potassium thiocyanate to the dialyzed mixture. Some insoluble matter (amounting to 50 mg N per 100 g tissue) was removed by centrifugation and discarded. The supernatant was still turbid and had to be clarified by passing through a thin layer of diatomaceous earth (Kieselguhr) in a Büchner funnel. After this treatment the solution contained about 125 mg N per 100 g tissue. After dialysis against tap water (24 hours) and addition of a few drops of saturated NaCl solution an active precipitate was obtained in the cold with 3 volumes of acetone. After treatment with acetone and ether it amounted to about 1 g (≈ 120 mg N) per 100 g tis-

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suie. This preparation was soluble in water after addition of alkali and contained almost the total activity of the crude extract.

Further purification was attempted by fractionation with ethanol. The thiocyanate solution of the tungstic acid precipitate was clarified and dialyzed as described. Then 0.6 vol. of 96% ethanol and a few drops of saturated NaCl solution were added. An active precipitate was isolated by centrifugation, and dried with ethanol and ether (pulmin I). It amounted to about 0.9 g per 100 g tissue. A re-precipitation was performed by dissolving pulmin I in 0.9% NaCl (16 mg per ml) and adding 0.5 vol. of 96% ethanol. The centrifugate was again inactive, and the active precipitate was dried with abs. ethanol and ether (pulmin II). It amounted to about 250 mg per 100 g tissue.

6. Purification of the thiocyanate solution. The good solubility of the precipitates in M thiocyanate suggested a fractional precipitation of such solutions.

100 mg of an ethanol precipitated product (pulmin II) was dissolved in 20 ml M potassium thiocyanate and precipitated with 50 ml of 96% ethanol. The precipitate was inactive and was discarded. The centrifugate was freed from ethanol by evaporation in vacuo, diluted to the original volume with water, and 6 g solid ammonium sulphate per 10 ml solution was added. The active precipitate obtained was extracted twice by stirring for half an hour with a mixture of 12.5 ml ethanol and 5 ml H₂O per 10 ml original volume. The united extracts were freed from ethanol in vacuo and the resulting solution dialyzed for a short time against 0.9% NaCl in a rapid dialysir. The solution was precipitated slowly by addition to 10 volumes of abs. ethanol, and the precipitate was washed and dried with ethanol and ether (pulmin III). This procedure was based on preliminary experiments about which the following could be stated: The solubility of the ethanol precipitated product (pulmin II) in thiocyanate solutions is of great advantage, because the presence of thiocyanate completely changes the order of precipitation. Without thiocyanate active components precipitate at low ethanol concentrations (see Section 5). In the presence of thiocyanate considerable amounts of inert matter (80% of the material) could be precipitated at low ethanol concentration. However, the subsequent separation of the active components was not possible even with excess of ethanol. The removal of thiocyanate was difficult. Dialysis of the solutions produced a considerable loss of activity. Slight losses had also been observed in the preparation of the previous products, but had been thought to be caused by losses during the procedures. With the more pure preparations the losses were so large that they could not be accounted for in this way. Dialysis against a number of different solutions was tried. Apparently the loss only depended upon time, and some activity could be recovered from the outer fluid. Best results were obtained by rapid dialysis in a dipping dialysir. These results suggest that the inhibitor has a fairly low molecular weight, and this possibility has to be taken into consideration when preparing larger quantities. Losses were not encountered during storage of the solutions.

Because of the difficulties involved in removing thiocyanate by dialysis without losing inhibitor activity, other ways were sought for the isolation of active compounds. It was found that the solution could be evaporated to dryness in vacuo (bath temp. <60°) without losing activity. Complete removal of thiocyanate by extraction of the residue with ethanol could not be accomplished without loss of activity. Removal of free thiocyanic acid was attempted by addition of glacial acetic acid and water during the evaporation. No loss in activity was encountered, but difficulties arose in the subsequent removal of potassium acetate. After evaporation of ethanol in vacuo the active components in the thiocyanate solution could be precipitated by addition of ammonium sulphate. Addition of 6 g solid ammonium sulphate per 10 ml solution produced a precipitate containing all activity in addition to inorganic components. Most of the activity could be extracted with a mixture of ethanol and water in the proportion 25:10. After removal of ethanol in vacuo and concentration to a smaller volume, a short dialysis could be performed and an active precipitate obtained by dropwise addition to 10 vol. abs. ethanol.

The yields during these last steps were not high. Losses were encountered during dialysis as already mentioned, and the extraction of the ammonium sulphate precipitate was far from quantitative. However, the precipitates as finally obtained (pulmin III) were extremely potent powders.

7. Properties. The experiments described above have revealed some interesting properties of the active component. It is extremely stable at acid reaction, tolerating treatment for several days at a pH between 1 and 2. The active substance appears to have.

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Fig. 1. Thermostability of ox lung inhibitor. (Acetone precipitated preparation). Appropriate solutions were made in a dilute maleic acid buffer. After heating in a boiling water bath for 1 hour the activity against trypsin was estimated on fibrin plates, and read on a dilution curve of the inhibitor. Abscissa: pH during heating. Ordinate: Remaining activity in per cent of untreated solution.

A fairly low molecular weight, disappearing slowly by diffusion during dialysis. This ability to diffuse appears to be especially pronounced in more pure preparations. Less pure preparations do not dialyze. In these solutions proteins probably combine with the active substance, preventing diffusion. When a dialysis is incorporated into the procedure, a certain loss is therefore encountered in all excepting the first few steps. The necessity of using thiocyanate solutions makes it impossible to follow the purification steps accurately by nitrogen estimations and the amount of dry matter isolated at different steps has to be used as an approximate measure of the total amount of substance present. In the first steps the amounts of nitrogen have been estimated after dialysis; the figures recorded represent a good estimate of the compounds present, because the active, dialysable component in these steps is only a small fraction of the total amount of solid matter. Because of the high solubility of the active components in alcohol-water mixtures, it was impossible in all intermediate steps to isolate the products quantitatively as a dry powder.

The preparation isolated as pulmin I contained about 11 % N and 6 % P. Pulmin II contained about 10 % N and 5 % P. The preparation isolated as pulmin III contained a large amount of inorganic salts so that the nitrogen content was reduced. It contained also thiocyanate if the dialysis had not been sufficient. The inactive product removed during the step leading to pulmin III (fractional precipitation of the thiocyanate solution with ethanol) contained about 12 % N and 7 % P, which by re-precipitation could be increased to about 8.9 % P. Measurements of ultraviolet absorption of solutions of this compound indicated that it consisted mainly of nucleic acids. Because of the high salt content in pulmin III, the solutions were analysed for N and P and the proportion P/N compared with that obtained from other products. For pulmin I and pulmin II P/N (mg/mg) was between 0.4 and 0.5. For pulmin III it was below 0.01 because of removal of most of the phosphorus. A rough idea of the degree of purification could be obtained from the amounts of nitrogen in the different preparations which produced complete inhibition of a chymotrypsin solution. One mg of chymotrypsin was inhibited by the following amounts: pulmin I, 0.9 mg N; pulmin II, 0.3 mg N; pulmin III, <0.1 mg N.

The lung inhibitor is a rather stable compound. Solutions of pulmin heated for 2 hours at 56° (pH between 1.6 and 9.7) did not lose activity. At acid reaction the com-

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pound tolerates heating for 1 hour at 100°, but at neutral and alkaline reactions it is destroyed (see the thermostability curve, Fig. 1).

A comparison was made between the inhibitory effects of heparin (obtained from Løvenga kemiske Fabrik, Copenhagen) and the acetone precipitated lung inhibitor on a solution of human globulin activated by streptokinase. This solution contains plasmin as well as an activator of plasminogen. The results are presented in Fig. 2. It can be seen that the lung inhibitor is a much more powerful inhibitor of fibrinolysis than heparin.

DISCUSSION

The method described here for the isolation and preparation of a protease inhibitor from ox lung tissue makes it possible to obtain this antiproteolytic agent as a stable, watersoluble dry powder of high potency. The losses encountered during dialysis indicate that the inhibitor is not a protein, but that it has a rather low molecular weight, possibly less than 10 000. The heat stability curve shows a characteristic optimum at rather acid reaction and in this respect the compound resembles trypsin inhibitors isolated from pancreas and colostrum (Laskowski and Laskowski, Jr.12). The yield of inhibitory compounds from the pancreas is low. Green and Work11 mention a figure of 1 mg pure inhibitor per kg of fresh pancreas, and they obtained only 10 g of a product about 50 % pure from 100 kg dried pancreas residues. The results with ox lung show that this organ is extremely rich in inhibitory substance. Apart from the pancreas it is the only organ in the animal organism, which has been found to contain large amounts of a protease inhibitor, though it may be mentioned that urine contains an inhibitor of apparently similar nature (Astrup and Sterndorff8). It is of interest that the inhibitor is absent from the lungs of most other mammalian species, including man. Lung tissue is usually a

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potent plasminogen activator. Even calf embryo lung is a potent plasminogen activator and does not contain the inhibitor.

Ox lung serves as a source for the production of heparin (Charles and Scott 10; Kuizenga and Spaulding 12). Initially we thought that the substance might contain heparin bound to protein. We have tried to prepare heparin from the lung inhibitor after methods devised by Charles and Scott 9 and Wilander 15 for the extraction of heparin from tissues. Only traces were obtained. A search for sulphuric acid residues after hydrolysis with HCl revealed only insignificant amounts. The comparison in Fig. 2 of heparin with a purified lung inhibitor shows definitely that the inhibitor is different from heparin, and we have therefore suggested the term pulmin for the ox lung inhibitor. Larger amounts of this compound are being prepared in order to study its properties and physiological effects.

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