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The Proteolytic Enzymes of *Aspergillus oryzae*

I. Methods for the Estimation and Isolation of the Proteolytic Enzymes

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Methods are presented for the separation and estimation of proteolytic enzymes of moulds. Three proteolytic enzymes have been isolated and purified from *Aspergillus oryzae* both with laboratory techniques and with a method suitable to be carried out on a larger scale.

It has been known for many years that the great majority of moulds produce appreciable amounts of proteolytic enzymes. Much attention has been focused on this field during the last ten years and many enlightening results have been presented but, with few exceptions, these enzymes have not been purified and studied extensively.

Mould protease is seldom one single enzyme but consists of a system of proteolytic enzymes. Thus, such simple expressions as *Aspergillus oryzae* protease and *Rhizopus* protease have caused much confusion, and some reasonable classification of mould proteases has been required. Our knowledge of the complicated mixture of proteolytic enzymes produced by different moulds is still very limited; therefore, the proteases have been classified into groups according to the analysis of the apparent pH-activity curves of various mould cultures with regard to casein digestion [1,2].

A wide range of pH optima has been reported for the mould proteases depending on the substrate and the organism used. Most workers have accepted the evidence for three types of protein hydrolyzing enzymes having optimum activity at acidic, neutral, and alkaline pH's, respectively. An acid protease is a major component in various black *Aspergilli* such as *Asp. niger*, *Asp. awamori*, and *Asp. saitoi* [3]. *Aspergillus oryzae* grown in a carbohydrate rich medium, which leads to a lowered pH, is said to produce mainly this type of enzyme but only little of it in a protein rich medium [1,4,5]. A neutral protease has been separated from Taka-diastase [1], a commercial enzyme preparation from *Asp. oryzae*, and a similar enzyme has been isolated from *Asp. sojae*. *Rhizopus* and *Penicillium* have been reported to produce an acid protease abundantly along with other types of proteases. An alkaline protease is said to be the main protease in *Asp. oryzae* culture [6], and many other *Aspergilli* and some *Penicillia* also produce this type of enzyme [7].

A classification and characterization of the different proteolytic enzymes based on the region of pH in which the optimal enzyme activity is obtained is not sufficient.

Enzymes of different origins may produce the same action on a simple substrate but may be essentially different in nature. Therefore some workers have tried to classify the proteolytic enzymes by studying their behaviour towards a number of proteolytic inhibitors [8]. In this way it was thought that it might be possible to differentiate between proteolytic enzymes with identical action on simple substrates. This interaction gives a more detailed classification of the proteolytic enzymes, and by this method it has been possible to show the complexity of enzyme preparations which were formerly thought to be homogeneous. Since our knowledge of the specificity of the proteases is far from complete other means of classifying these enzymes have also been attempted but these are, however, subject to some criticism. Although the proteolytic enzymes have been the subject of many investigations since 1932 most of the available data on this enzyme system have been obtained with crude, unfractionated, and partly inactivated preparations [9]. Relatively few attempts have been made to determine absolute and relative amounts of the individual enzymes.

The proteolytic enzymes of moulds appear to be exceedingly numerous, yet our information concerning them is very sparse as compared to our present knowledge of the animal enzymes. It has, however, become evident that the enzyme content varies as much between members of the same genus as between moulds of different genera. The medium in which the mould is grown and the time of cultivation have an important effect upon the amount of enzyme formed.

The relatively unexplored study of mould proteases appears to be closely concerned with the great difficulties in isolation and fractionation of the different enzymes. A number of methods have been presented for the separation of proteases, but most of these appear to have certain failings. Purification of the enzymes involves very delicate and intricate problems. Some of the proteolytic enzymes are unstable and easily loose their activity; consequently their purification must be carried out with great care under special and very limited conditions. In addition to this, there is another important problem due to the fact that each enzyme is stable only under limited conditions which differ from enzyme to enzyme. Therefore each enzyme has its own requirement for a satisfactory purification with a good yield, and few routine procedures have been adopted. These conventional purifying methods always cause some impurities to accumulate together with the protease. In order to obtain a satisfactory result, purification must be carried out under strict conditions remarkably reducing the yield of the different proteases.

Our studies of the proteolytic enzymes produced by *Aspergillus oryzae* were started in the beginning of 1959 after the stimulating report by Stefanini *et al.*[10] on a fibrinolytic material produced by this fungus. Stefanini considered the active substance to be non-protein in nature. From preliminary studies it seemed more likely to us that the substance searched for was a proteolytic enzyme. The present paper describes investigations undertaken with the object of devising methods free from the above limitations for the isolation and purification of microbial proteases without any appreciable loss of any of them or their activity. Our methods have been applied to the separation of the proteolytic enzymes of several different moulds, and in this paper a study of the enzymes produced by *Aspergillus oryzae* is presented.

The crude protease solution, obtained by submerged cultivation contains, in addition to the proteases and other enzymes, various impurities, such as polysaccharides, glucoproteins, nucleoproteins, coloring matter, *etc.*, which are metabolic and autolytic products of the microorganism used, and low molecular substances from the culture medium. Although the activities of the protein hydrolyzing enzymes were high enough to be accurately measured, the total protein concentration was too low for efficient purification with routine procedures, such as salting out with ammonium sulphate or precipitation with organic solvents.

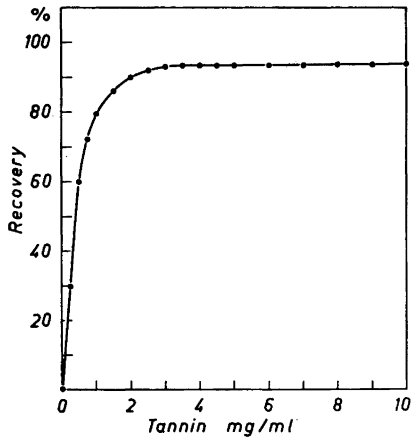


Fig. 1. Recovery of proteases in culture fluid from *Aspergillus oryzae* after precipitation at pH 5.5 with varying amounts of tannin and removal of tannin by washing with acetone. The protease-tannin complex was allowed to precipitate for 2 hours at 20°C.

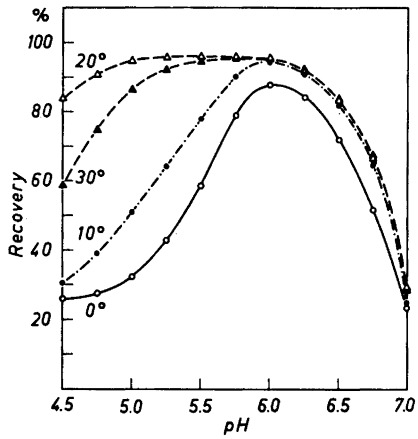


Fig. 2. The recovery of proteases after tannin precipitation at different pH's and at different temperatures. The proteases were precipitated by the addition of tannin to the final concentration of 3.0 mg per ml, and after 2 hours the precipitate was freed from tannin by washing with acetone.

Several methods have been tried and the best purification was initiated by selective precipitation of the proteins including the proteolytic enzymes with tannin, and then removing of the tannin by washing with acetone. The chief factors which determined the effectiveness of the precipitation of the proteases were tannin concentration, pH, and temperature. The relationship between tannin concentration and the amount of proteases precipitated is shown in Fig. 1. Another important variable was pH, and the range for maximum precipitation at different temperatures can be seen from Fig. 2.

On the basis of these results, the following procedure was adopted for the concentration of the proteins. The culture filtrate, obtained from the harvested culture was adjusted to pH 5.5. Almost quantitative precipitation of the proteases was obtained by the addition of tannin to a final concentration of 3.0 g of tannin per 1000 ml of the solution (filtrate). After standing for not less than 2 h at room temperature the precipitate was isolated by centrifugation and freed from tannin by repeated dispersal in acetone, centrifuging, and finally drying in a desiccator. The crude protease mixture thus obtained was much more susceptible to further purification. The dry powder retained its proteolytic activity for more than a year. It was easily soluble in water and salt solutions and in this way it was possible to prepare protease solutions more than 2000 times as active as the original solution.

The first information about the complexity of the protease mixture produced by *Aspergillus oryzae* was obtained by testing solutions of the crude enzyme against various protein substrates but it is evident that no decision can be made in this way as to the number of enzymes in the preparation.

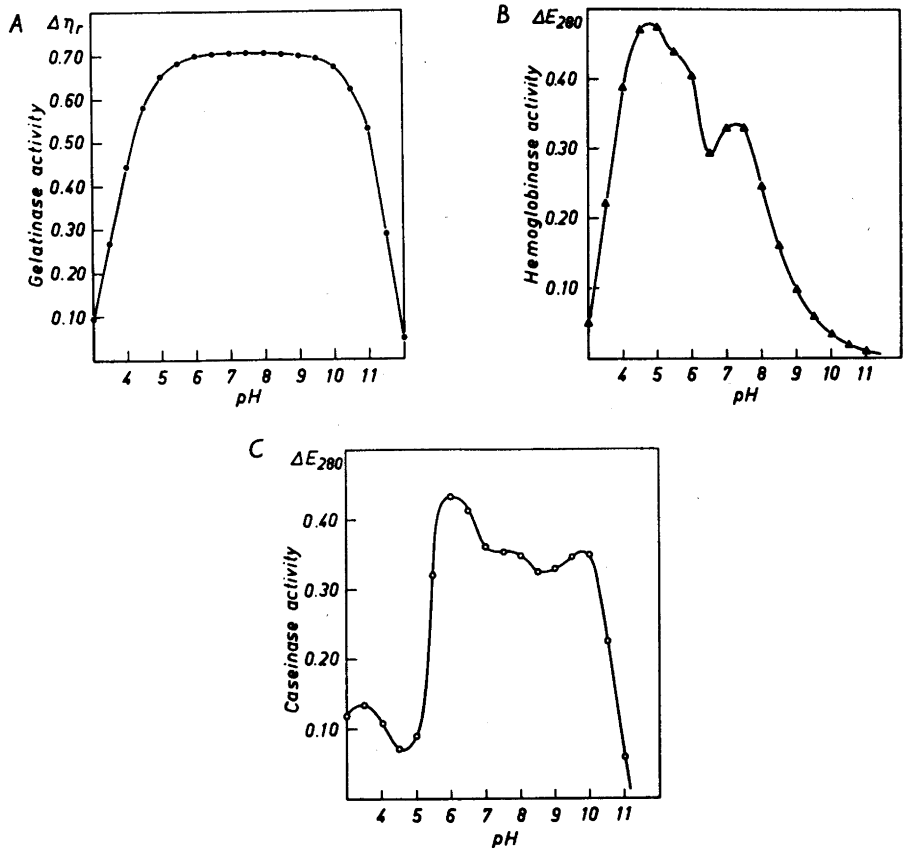


Fig. 3. pH-activity of the proteases produced by *Aspergillus oryzae* on different protein substrates.

A. Gelatinase activity was estimated as the reduction in relative viscosity of a 5 % gelatin solution in Britton-Robinson buffer of the appropriate pH after a digestion period of 5 minutes at 37°C.

B. Hemoglobinase activity was estimated as the hydrolysis of about 2 % urea denatured hemoglobin after incubation for 10 minutes at 37°C. Increase in optical density of trichloroacetic acid filtrate was used as a measure of protease activity.

C. Caseinase activity was determined by the rate of hydrolysis of 1.5 % casein in Britton-Robinson buffer after incubation for 30 minutes at 37°C. Increase in optical density of trichloroacetic acid filtrate was used as a measure of protease activity.

The rates of hydrolysis of three different proteins can be seen from Fig. 3. The broad pH maximum for the digestion of gelatin indicated the possibility that more than one enzyme may be present. The effect of pH on the rate of hydrolysis of casein suggested that at least three proteases were present. However, casein, which is insoluble at pH 4 to 5 was not a perfect substrate, and the existence of a protease with optimum at about pH 3 could be misleading. When denatured hemoglobin was used as the substrate the pH-activity curve

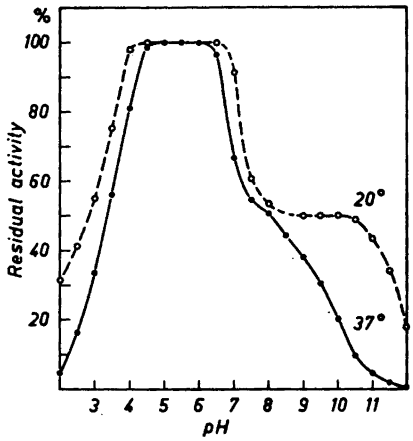


Fig. 4. pH-stability of the protease mixture. The enzyme mixture was incubated for 30 minutes at 20°C or at 37°C in Britton-Robinson buffer adjusted to the desired pH. At the end of incubation samples were mixed with 0.2 M phosphate buffer of pH 6.0 and the remaining activity was estimated by casein digestion.

differed greatly from those with casein and gelatin. Several enzymes were also involved in its digestion. The results presented underline the obvious limitations in utilizing the pH optima for the identification and classification of proteases from different moulds. The substrate specificity is ignored as well as the fact that different enzymes show different pH optima depending on the nature of the substrate. The differences of activity against different substrates are also due to the fact that the presence of the substrate frequently has a specific stabilizing effect on the enzyme.

The proteolytic enzymes are relatively fragile substances with a tendency to undergo denaturation and inactivation under unsuitable conditions. Therefore it is advisable to map out the stability limits of the mixture of proteolytic enzymes, especially with respect to pH, so that one knows the pH-range within which the operations must be carried out. As shown graphically in Fig. 4, the proteolytic enzymes produced by *Aspergillus oryzae* were stable at room temperature over the range pH 4.0 to 6.5, and might become irreversibly destroyed on one or both sides of this pH range. The occurrence of an irreversible destruction was tested by exposing the enzymes to a range of pH values for a definite period before measuring the activity at a pH at which they are stable.

Further information about the number of different proteases in the crude preparation can be obtained by studying the effect of pH on the inactivation. The residual activity after preincubation for different time periods at pH 3.0 and 9.0, respectively, showed clearly the presence of three different proteolytic enzymes. The inactivation exhibited a stepwise course when plotted against time as in Fig. 5. The pH values were chosen so that the proteases were inactivated with very different time courses. Therefore the graph which was obtained consisted of three straightline portions, each corresponding to a region where the inactivation of only one of the enzymes was dominating. The slope is a measure of the enzyme lability at the actual pH value. The occurrence of an irreversible destruction of the proteases at certain acid and alkaline pH values made it possible to get both a qualitative and a quanti-

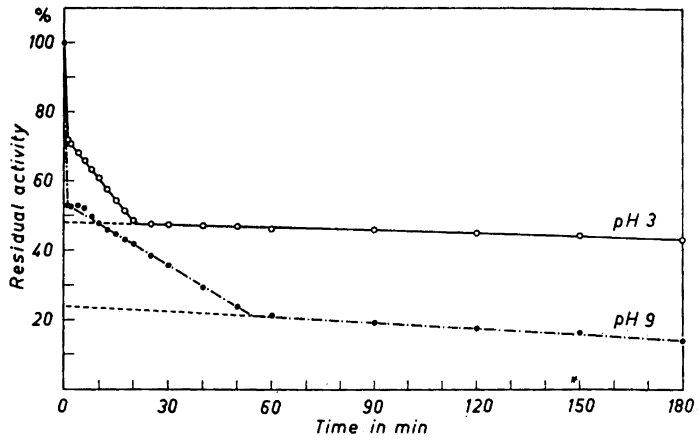


Fig. 5. The rate of inactivation of the proteases at pH 3.0 and 9.0 after incubation for different time periods at 37°C. The residual activity was estimated at pH 6.0 using casein as substrate.

tative estimation of the different components of the crude protease mixture without any further fractionation.

The data in Table 1 show the contribution of the individual proteases to the total proteolytic activity at pH 6.0. Correlation of the values obtained at the different pH conditions could, however, be made first after further studies of the enzymes and their properties. The complexity of the protease preparation was apparent and was also revealed by electrophoresis. The speed of migration of zones depended greatly on the properties of the buffer used in the analysis. The pH and ionic strength influenced the distance of migration and often the extent of separation. Therefore orientating experiments were first performed by filter paper electrophoresis for choosing suitable buffers and pH. Acetate, phosphate, veronal and phosphate-borate buffers, all with an ionic strength of 0.05, were tested, and the pH range covered was from 4.0 to 9.0.

Electrophoresis was carried out on duplicate strips of paper, one of which was used for staining of the protein zones with bromophenol blue and the other

Table 1. Estimation of the amounts of the different proteases by studying the effect of pH on their inactivation. The contribution in per cent of the individual enzymes to the total proteolytic activity at pH 6.0 was estimated with casein as substrate. The correlation of the values obtained at pH 3.0 and 9.0 was made first after studying the properties of the purified components.

Component	Protease I	Protease II	Protease III
pH 3.0	27.0	25.0	48.0
pH 9.0	29.0	24.0	47.0

for detection of proteolytic activity. The proteins could be seen by visual inspection and a measurement of the components was made roughly by direct scanning of the stained strips. The proteolytic activity could be followed by cutting the paper transversely into 5 mm strips, eluting with water or buffer, and estimating the activity by digestion of casein. The proteases could also be detected directly on the paper by studying the fibrin decomposing activity. Therefore the small paper strips were placed on the surface of a fibrin plate and this was incubated at 37°C for 3 h. The lysed zones were a semiquantatitive measure of the proteolytic activity on the strips.

The electrophoretic separation of the protease mixture into three components was clearly demonstrated with buffers of pH 4.5—6.0. The best resolution of the protein mixture was obtained by a potential of 10 V/cm for 6 h in phosphate-borate buffer at pH 8.5. One of the proteases was, however, denatured at this pH and therefore only two of the proteases could be detected together with at least six other protein components. The small zones, which often might be seen by visual inspection, could not be clearly detected on the results graphed by scanning. This can be seen from Fig. 6, which shows the schematic diagram and pattern of the separation at pH 8.5. The method presented may not be used for quantitative evaluation of the proteins in a crude preparation but affords a qualitative demonstration of particular enzymes, *e.g.* proteases.

When sufficient material is available a continuous electrophoretic separation is preferable because of its much greater capacity. The separation of the proteases by this method has opened up a simple way for a quantitative determination of these enzymes. Amounts of the different substances sufficient for tests of homogeneity and preliminary studies of their properties can be separated.

One fundamental advantage of the continuous method is that the mixture to be separated is continuously fed into the apparatus and the separated components can be continuously removed. The crude protease mixture to be separated was dissolved in the buffer used for the separation, and the feed rate for putting it on the paper was 10—20 mg/h. The concentration of the proteins in the different fractions was determined by measuring the optical density at 280 mμ or by the modified biuret-Folin reaction described by Lowry *et al.*[11] The proteolytic activity was determined by the rate of hydrolysis of casein at pH 6.0 or 7.4.

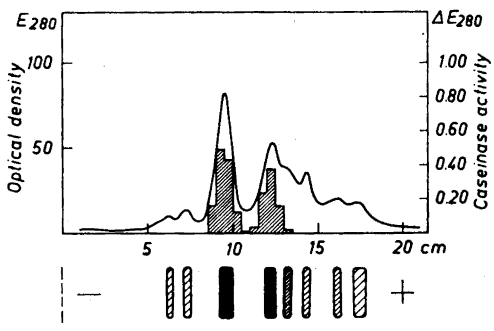


Fig. 6. Schematic diagram of an electrophoretic pattern of the proteins in the crude protease mixture in pH 8.5 and 0.05 ionic strength phosphate-borate buffer. The resolution shown was obtained after a run for 6 hours at 10 V/cm.
The lower part of the figure shows a diagram of the directly visible protein bands after dyeing. The upper part shows the distribution of the bromophenol blue color after photometric scanning (solid line). The proteolytic activity was also estimated by the caseinase method (crossed area).

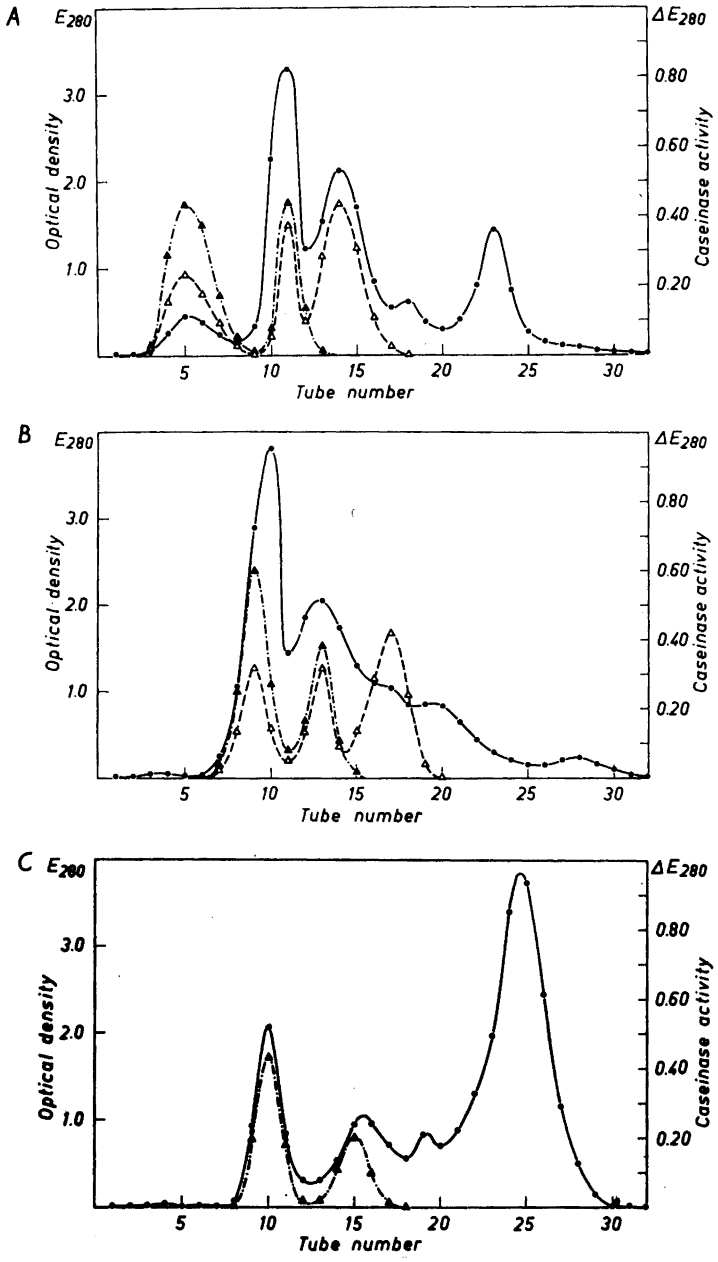


Fig. 7. Distribution patterns for separations of proteins in the crude protease mixture by continuous electrophoresis.

Solide line represents absorption at 280 mμ. Caseinase activity was estimated at pH 6.0, —△—△—; and at pH 7.4, —▲—▲—.

The separations were carried out under the following conditions:

A. Acetate buffer, pH 4.5, $\mu = 0.025$ at 80 mA.

B. Phosphate buffer, pH 6.0, $\mu = 0{,}05$ at 75 mA.

C. Phosphate-borate buffer, pH 8.5, $\mu = 0.05$ at 100 mA.

Distribution patterns for typical experiments are shown in Fig. 7. In accordance with the results from one-dimensional electrophoresis, the separations at pH 4.5 and 6.0 resulted in the complete separation of three different proteolytic enzymes. The denaturation of one of them on exposure to pH values above 6.5 was confirmed by the detection of only two proteases by the separation at pH 8.5.

Quantitative values of the different components of the crude protease preparation varied appreciably with the techniques of separation and activity measurements. Despite this variation among methods, the results were reproducible for a selected and standardized procedure using the same specimen. The estimation of all the proteases could be accomplished at pH 6.0. When one of the proteases was irreversibly inactivated at a separation above pH 6.5 it was preferred to estimate the activity of the remaining proteases at pH 7.4 because of their higher activity under these conditions. A summary of the results obtained by continuous electrophoresis can be seen from Table 2. The values obtained agreed closely with those found by enzyme inactivation at extreme pH's presented above (Table 1), in spite of the fact that the methods used were inherently different.

Table 2. Separation of the proteases by continuous electrophoresis at pH 4.5, 6.0 and 8.5.The fractions were analyzed by the caseinase method at pH 6.0 and 7.4. The amounts are expressed in per cent of the total activity.

Component	Protease I		Protease II		Protease III	
Caseinase pH	6.0	7.4	6.0	7.4	6.0	7.4
Electrophoresis at pH 4.5	29.0	66.0	23.5	34.0	47.5	—
at pH 6.0	28.5	65.0	24.0	35.0	47.5	—
at pH 8.5	—	65.0	—	35.0	—	—

The results presented showed that continuous electrophoresis may be used as a quantitative analytical or preparative method, depending on the amount of substance applied. Gram quantities of the crude preparation were fractionated and amounts of the different proteases isolated were sufficient for primary studies of their properties. It was sometimes necessary to concentrate the fractions for further studies. This was done by ultrafiltration through a collodion membrane or by precipitation with tannin.

The electrophoretic studies had clearly disclosed the presence of three proteases in the crude preparation obtained from *Aspergillus oryzae*. The protein mixture was, however, much more complex than that revealed by the electrophoretic studies. Examination of the shape of the graphs presented in Fig. 7 showed what might be a large number of protein species, some displaced from one another significantly, but often overlapping. Repeated electrophoresis at other pH values revealed that the different fractions obtained

did not represent single proteins but a complex mixture. The proteases could, however, be separated and quantitatively estimated. Their properties were studied and other methods for their further isolation were worked out.

The application of ion exchange chromatography to the fractionation of enzymes is a very promising method but is complicated by several factors which are of only minor importance in the chromatography of simpler substances. The instability of the enzymes themselves severely limits the choice of eluting agents and imposes special conditions of operation, such as low temperature and restricted pH range. The most important limitation of the ion exchange resins is their irreversible binding of proteins. This can be the result of the formation of too many bonds between the protein and the adsorbent, so many that simultaneous dissociation of all of them does not occur except under conditions destructive to the native configuration of the molecule. Two types of adsorbents have been developed during the last years: calcium phosphate gel and cellulosic ion exchangers.

Calcium phosphate gels have been used for the fractionation of small amounts of the crude mould preparation, but, in column work, the extremely high resistance imposed by this material to the flow of buffers was a serious handicap. Therefore the final purification of the proteases was achieved by the use of ion exchange cellulose.

The cellulose adsorbents are available as either anion or cation exchangers, and both types have been used in the chromatography of the proteases. The adsorbed protein is tightly bound, and the formation of multiple electrostatic bonds between the protein and the adsorbent is undoubtedly involved. The elution can be accomplished by changing the pH to reduce the number of charges on the protein or adsorbent, or by raising the salt concentration to compete for the existing charges.

The crude protease mixture was examined chromatographically on DEAE-cellulose, an anion exchanger containing diethylaminoethyl groups attached to cellulose. The eluting buffer was selected for its effectiveness in controlling pH in the region in which the proteases were stable and in which chromatography was to be conducted. The resolution of the components by gradient elution was not as good as by stepwise elution, therefore, the majority of the separations were carried out with the stepwise procedure and this method is also recommended for large scale operations.

In Fig. 8 is shown the elution diagram of the proteases, where a series of stepwise changes in salt concentration at constant pH, has resulted in a resolution of the crude product in at least 8 distinct peaks designated A to H in the order of their elution. The course of the elution of the proteins was followed by measuring the optical density of the fractions at 280 mμ. The effluent fractions were also examined for proteolytic activity and were characterized by electrophoretic and chemical techniques. For the described elution schedule, in both analytical and preparative columns, the reproducibility of the position of the chromatographic peaks was very good. On rechromatography each of the protease fractions were eluted as a single peak at the same salt concentration as in the first experiment. The behaviour upon rechromatography indicated that a true fractionation took place.

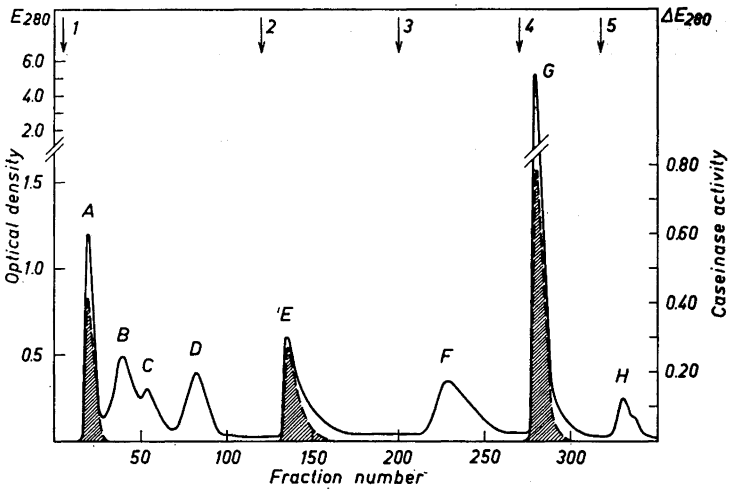


Fig. 8. Chromatography of the crude protease mixture on a DEAE-cellulose column (3.0 × 40 cm) buffered with 0.01 M phosphate buffer at pH 6.0. 2.0 g of dialyzed material dissolved in 15 ml of buffer were applied and the fraction volume was 5.0 ml; temperature, 4°C.
The solide line represents absorption at 280 mμ. The crossed sections show the relative caseinase activity at pH 6.0.
Stepwise changes in salt concentration of eluting agents at pH 6.0:

1) 0.01 M phosphate buffer
2) 0.10 M » »
3) 0.25 M » »
4) 0.50 M » »
5) 0.50 M » » + 0.50 M sodium chloride.

Previous studies had disclosed the presence of three different proteases and this was also revealed by ion exchange chromatography. The protein mixture was, however, more complex than that revealed by the electrophoretic studies. The three proteolytic enzymes earlier referred to as protease I, II, and III were found in peaks A, E, and G, respectively. Proteins were also found in the other peaks but they have not been characterized. About 85—95 % of the proteolytic activity applied to the column was recovered when the separation was carried out at low temperature (4°C). The activity of the different proteases calculated from the chromatography data can be seen from Table 3. The methods for the identification of the proteases and a more extensive chemical and enzymatic characterization will be given in a later paper.

The ion exchange chromatography described could be used for the separation of fairly large amounts of material, but the method was time consuming and not suitable for large scale separations. This can, however, be accomplished with a multistage batch procedure using a cation exchanger, CM-cellulose, containing carboxymethyl groups attached to cellulose. In this

Table 3. Amounts and identity of the protease fractions obtained by ion exchange chromatography on DEAE-cellulose. The fractions were analyzed by the caseinase method at pH 6.0.

Peak	Identity	Proteolytic activity in % of total
A	Protease I	28.0
E	Protease II	24.0
G	Protease III	48.0

way the crude preparation can be fractionated without an appreciable loss of the proteases and their activity. Further resolution of the proteases and the other proteins can then be accomplished on separate columns of the same type under different conditions or with other cellulose ion exchangers.

Preliminary batch experiments showed that the adsorption of the different proteases on CM-cellulose was very sensitive both to ionic strength and pH. The adsorption capacity of the cation exchanger at different pH was evaluated with solutions of the purified proteases. The results presented in Fig. 9 show that the proteases I, II, and III were quantitatively adsorbed on CM-cellulose at pH below 5.5, 4.5, and 3.0, respectively. Therefore, the purification procedure developed consisted of a selective adsorption of the proteases at these pH values. The outline of the procedure is shown in Fig. 10.

Preliminary studies had indicated that the less buffer and salts in the protein solution, the more sites of the ion exchanger were available for protein adsorption. Therefore the concentration of inorganic salts was kept as low as possible. Tannin precipitated protease preparations were chosen as starting material, and this was dissolved in water at pH below 6.5 to a final concentration of 2 g/l. The solution was adjusted to pH 5.5 and then stirred with

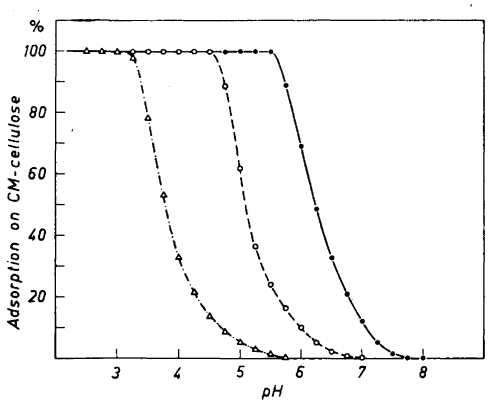
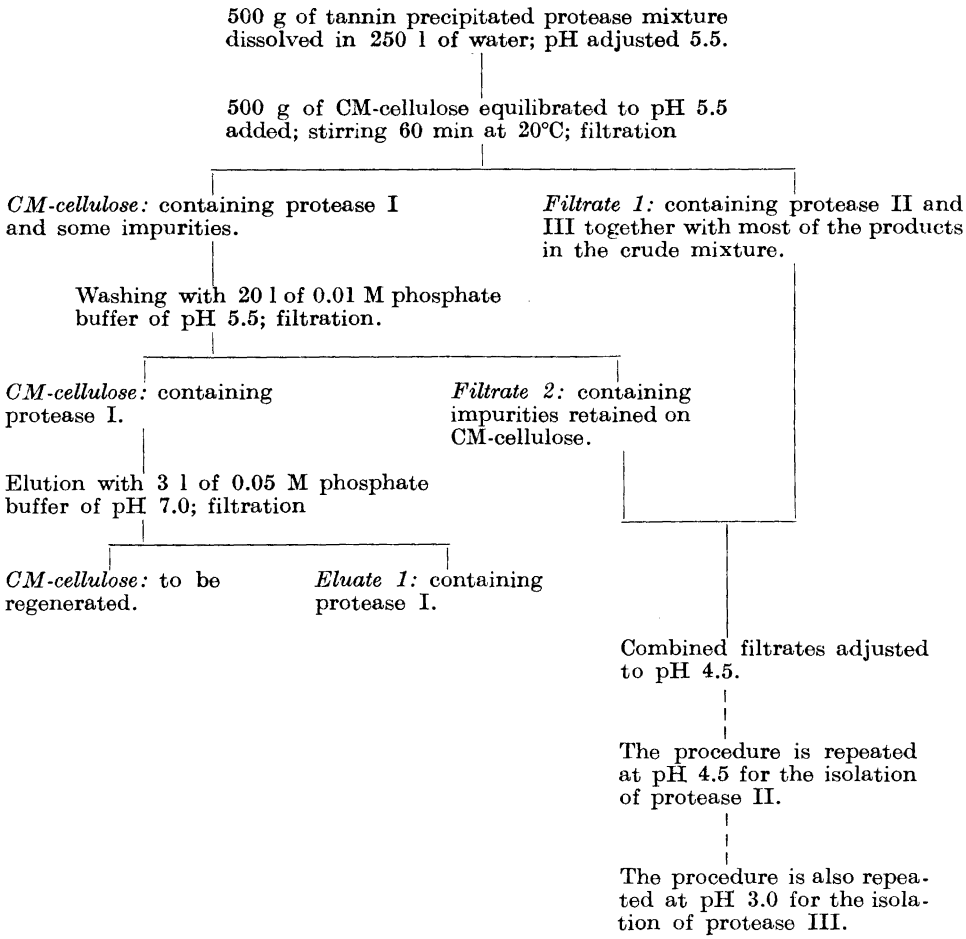


Fig. 9. Effect of pH on the adsorption of the purified proteases of *Aspergillus oryzae* on CM-cellulose. The adsorption of the enzymes was estimates by the caseinase method at pH 6.0. —●—●—, protease I; —○—○—, protease II; —△—△—, protease III.

Fig. 10. Separation of the proteases by selective adsorption on CM-cellulose.



the cellulose ion exchanger at room temperature. After equilibrating with the first batch of exchanger, the solution was removed by centrifugation. Although the protein-exchanger combination was separated from the bulk of the reaction solution on centrifugation, it nevertheless retained large amounts of solution, which of course contained unreacted proteins. Washing with 0.01 M phosphate buffer of pH 5.5 removed the nonadsorbed material without eluting the adsorbed proteins. The combined solutions were adjusted to pH 4.5 and the procedure was repeated at this pH for the isolation of protease II and then at pH 3.0 for protease III.

Table 4. Amounts of the different proteases calculated from the decrease in activity after adsorption on CM-cellulose at different pH. The activity was estimated at pH 6.0 and 7.4 using casein as substrate. The amounts are expressed in per cent of the total activity.

Component	Protease I		Protease II		Protease III	
Caseinase pH	6.0	7.4	6.0	7.4	6.0	7.4
Proteolytic activity in % of total	28.0	65.0	23.5	35.0	48.5	–

It was very important that the conditions of pH and ionic strength of the exchanger were kept substantially constant, the same as those of the protease solution to be treated during the operation. For this purpose, the CM-cellulose was carefully buffered. If the exchanger was not so conditioned the selective adsorptivity of the protease would be reduced. The amount of ion exchange material to be used was determined by the volume of the protease solution and its protease concentration, but it should be sufficient to be able to adsorb substantially all the actual protease in the solution. Therefore a large excess of CM-cellulose was used.

Elution, like adsorption, as one might expect, was a function of pH. The elution of the proteases was carried out by stirring with buffers of pH higher than that of the adsorption stage. In order to avoid inactivation of the proteases due to strong change in pH it was found most satisfactory to use 0.05 M phosphate buffer of pH 7.0 for the elution of protease I and II, and a buffer of pH 6.0 for protease III. Repeated elution resulted in a recovery of more than 90 % of the proteolytic activity. The over all recovery was high from preparations with either small or large amounts of proteases. The fact that the proteases could be eluted under conditions favorable to the enzymes was responsible for this high yield. For example protease III, which was unstable at pH's above 6.5 was eluted at pH 6.0. Another factor which favored the usefulness of the method in enzyme work was the rapidity at which adsorption and elution took place. The procedure can also be used on a small scale as an analytical method, and the results of these analyses are summarized in Table 4.

It was in most cases necessary to concentrate the eluates for storage or for further purification by another procedure. Thus, the proteases could be precipitated with tannin as described earlier or dialyzed and then lyophilized.

The effectiveness of the fractionation can be checked and the products further purified by chromatography on DEAE-cellulose. The conditions chosen were the same as described above and the resulting diagrams are shown in Fig. 11. The elution patterns obtained showed that a highly purified protease I was obtained by the selective adsorption on CM-cellulose. The product contained only small amounts of two inactive proteins. Protease II and III were also highly purified, but they contained still other protein components. A satisfactory purification can, however, be accomplished by the DEAE-chromatography.

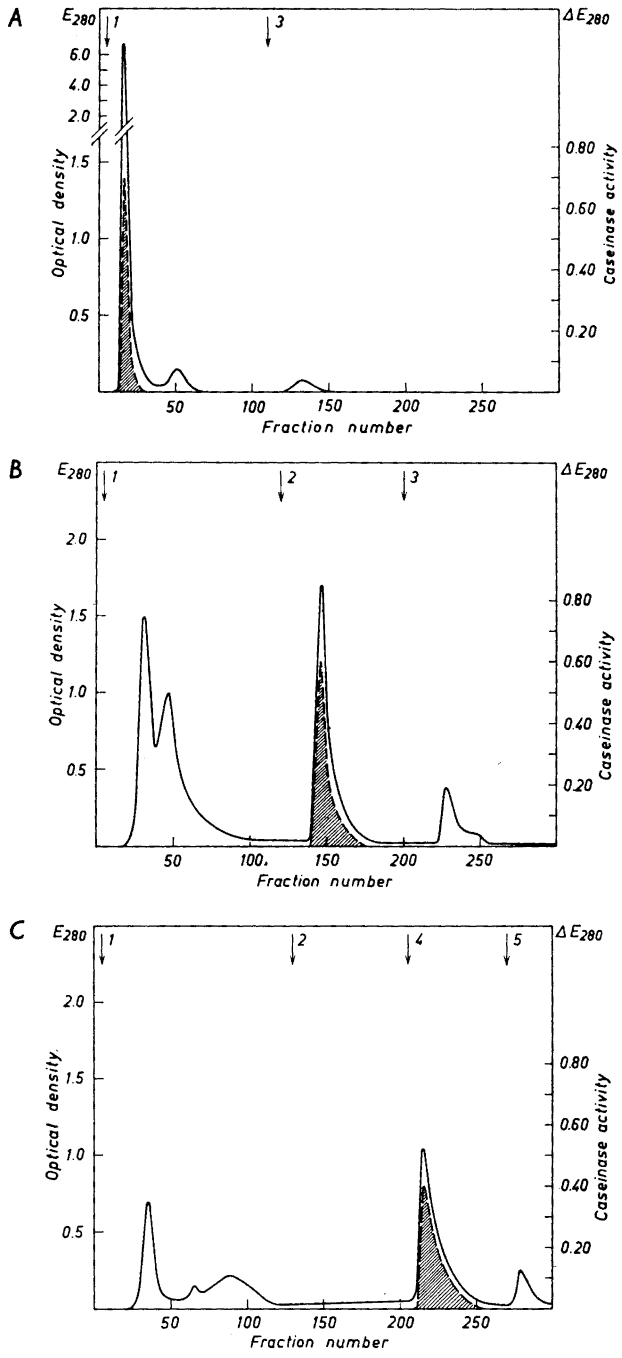


Fig. 11. Chromatographic behaviour of protease fractions obtained by the method outlines in Fig. 10, on DEAE-cellulose columns, 3.0 × 30 cm.

A. 300 mg of protease I material. B. 500 mg of protease II material. C. 500 mg of protease III material. The same experimental conditions were used as described in Fig. 8.

EXPERIMENTAL

Determination of proteolytic activity

The proteolytic activity was determined with casein, hemoglobin and gelatin as substrates. As a matter of convenience, the hydrolytic power of the enzymes on casein, hemoglobin and gelatin were called caseinase, hemoglobinase and gelatinase activity, respectively.

Caseinase determination. The method used was a modification of that described by Kunitz [12] for the caseinolytic assay of trypsin, which measures the digestion products by their ability to absorb ultraviolet light after precipitation of undigested protein with trichloroacetic acid.

The Hammarsten casein furnished by Merck was found to be appropriate material for the estimations. A stock solution of casein was made by suspending 15 g of casein in about 400 ml of water. Sodium hydroxide was added to convert the casein to the sodium salt and then the suspension was stirred vigorously to complete solution. Then the solution was adjusted to pH 7.4 and the volume was brought to exactly 500 ml by further addition of water.

The assay system for the estimation of the caseinase activity of the enzymes consisted of 3 ml casein solution and 3 ml of an appropriate buffer containing the enzyme. The enzyme solution was first incubated at 37°C for 15 min, the casein solution was added and the reaction was allowed to proceed at 37°C. 2 ml aliquots withdrawn at the start and also after 30 min were pipetted into each of two test tubes containing 3 ml of 10 % trichloroacetic acid. After standing for at least 30 min at room temperature the trichloroacetic acid mixture was centrifuged and the clear supernatant solutions decanted and filtered. The optical density of these solutions was measured in a Zeiss spectrophotometer. Digestion of casein solution for 30 min at pH 6.0 and 37°C was adopted and used as a standard method throughout our studies. All the enzymes were stable under these conditions. The amount of enzyme must be adjusted to give an optical density between 0.050 and 0.500 at 280 mμ with 1 cm quartz cuvettes, as at this level of activity the degree of hydrolysis was found to be directly proportional to the amount of enzyme present. Caseinase activity was alternatively estimated at pH 7.4 for some of the enzymes. The method was also used for estimation of the activity over a wider pH interval.

Hemoglobinase determination. The method used was a modification of that described by Anson [13]. Denatured hemoglobin was digested under standard conditions and the undigested hemoglobin was precipitated with trichloroacetic acid. The amount of unprecipitated hydrolytic products, corresponding to the proteolytic activity, was estimated spectrophotometrically in the ultraviolet region as described above.

The hemoglobin solution was prepared in the following way. A solution of 8 ml of 1 N sodium hydroxide, 72 ml of water, 36 g of urea, and 10 ml of 22 % hemoglobin was kept at 25°C for 30 – 60 min and then mixed with a solution containing 10 ml of 1 M KH_2PO_4 and 4 g of urea. The solution was stored at 5°C. Fresh solutions of hemoglobin at appropriate pH values were prepared each day. The estimation was carried out as follows: 2 ml of hemoglobin solution was added to each of a series of test tubes which were equilibrated at 37°C. 0.1 ml of a solution of a suitable enzyme concentration was added and the mixture incubated for 10 min at 37°C. Then a 1 ml aliquot withdrawn from the reaction mixture was pipetted into a centrifuge tube containing 4 ml of 5 % trichloroacetic acid. The increase in absorption at 280 mμ of material not precipitated by trichloroacetic acid was estimated as described for the caseinase determination. Alternatively the activity was estimated by measuring the blue color given by tyrosine and tryptophan derivatives with the phenol reagent of Folin and Ciocalteu. The use of the spectrophotometric measurement, however, almost completely superseded measurements by the phenol reagent.

Gelatinase determination. Estimation of the gelatinase activity was carried out by the gelatin viscosity reduction method. The gelatinase activity was defined as the ratio of the reduction in viscosity to the initial viscosity after incubation for 5 min at 37°C of a 5 % solution of gelatin containing the enzyme.

For the determination, one volume of 10 % gelatin solution and one volume of an appropriate buffer were mixed at 37°C, and 3 ml of this mixture was transferred to an Ostwald viscosimeter kept in a water bath at 37°C. The flow time of the mixture was

measured 5 min after mixing. The viscosimeter had a bulb capacity of about 2 ml and an average flow time of about 8 sec with water.

Production of enzymes

The enzymes of *Aspergillus oryzae* (B—1273 [10]) were obtained by submerged cultivation on a protein and carbohydrate rich medium. Maximum yield was obtained when the medium contained 1—1.5 % proteins from such sources as soybean meal our peanut meal and, in addition, 2—4 % carbohydrate, and salts of magnesium and phosphorus. The initial pH of the sterilized medium was 6.5 to 7.0. Optimal enzyme activity was usually reached in 3 to 4 days. As soon as abundant spore formation occurred all proteolytic enzymes were no longer intracellular but could be recovered quantitatively from the medium. Harvesting must then take place since a considerable decrease in enzyme potency was observed on further incubation. The culture filtrate obtained from the harvested culture was concentrated by tannin precipitation.

Precipitation of proteolytic enzymes by tannin

On the basis of the results presented above the concentration of the proteins was carried out as follows. The culture filtrate was adjusted to pH 5.5. The bulk of the protein was then precipitated by slowly adding a 10 % solution of tannin to the final concentration of 3.0 g of tannin per 1000 ml of the filtrate. A large precipitate was formed, which after settling for at least 2 h, was isolated by centrifugation and freed from tannin by repeated dispersal in acetone in the centrifugation vessels, recentrifuging and finally drying in vacuum. The proteolytic enzymes were found to retain their enzymatic activity after the precipitation and regeneration procedure. 80 to 100 % recovery of the proteolytic activity was obtained calculated on the basis of the assay of the original culture filtrate.

Electrophoresis

The enzyme preparation was subjected to electrophoresis under a variety of conditions. Paper electrophoresis was used for following the distribution of activity among the different protein fractions in each successive step during the course of fractionations. The amounts of material which could be purified by curtain electrophoresis were small; however, they were adequate to produce enough material for preliminary characterization of the proteases by analytical methods.

One-dimensional paper electrophoresis. All runs were carried out with an electrophoresis apparatus of the horizontal moist-chamber type. The best results were obtained after preceding zone-sharpening.[14] Whatman No. 1 filter papers gave the most satisfactory and reproducible separations. The use of buffers of different composition helped to reveal the components and therefore acetate, phosphate, veronal and phosphate-borate buffers, all with an ionic strength of 0.05 were tested, covering the pH range from 4.0 to 9.0. Visualization of protein components on the filter paper was accomplished through the development of a visible pattern with bromophenol blue. Measurement of components stained on the paper was made roughly by visual inspection or more precisely by direct scanning with an EEL scanner.

The paper strip was divided into two equivalent strips, one of which was used for staining of the protein zones and the other for detection of proteolytic activity. This was done by cutting the paper into segments each of which was subsequently eluted and tested for caseinase activity. When this was performed at pH 6.0 a white precipitate was formed and the time required to get a standard color was a rough measure of the proteolytic activity. The activity could also be measured by the conventional caseinase method.

The proteolytic activity was also directly estimated by studying the fibrin decomposing activity. Fibrin plates were prepared by the method of Astrup and Müllertz [15]. Nine ml of a 0.3 % fibrinogen solution (human or bovine fibrinogen) in an appropriate

phosphate or tris buffer, with ionic strength of 0.15, was clotted with 1 ml of thrombin solution (25 units per ml) in a 10 cm diameter Petri dish. The small paper strips were placed on the surface of the fibrin plate and this was incubated at 37°C for 3 h. The area of the dissolved zones obtained were taken as a measure of the proteolytic activity on the strips. The same results were obtained with fibrin plates which were heated to 85°C for 30 min.

Continuous electrophoresis. The continuous flow paper electrophoresis cell used was the Beckman/Spinco model C P.

The method is based on the principle of passing a background electrolyte down a filter paper sheet by gravity. The mixture to be separated is allowed to flow in from above in a fine stream and passes along with the electrolyte as a narrow band. An electrical potential is applied across the filter paper curtain at right angles to the direction of flow and the various substances are deflected towards the anode or the cathode in accordance with their mobility in the electrical field. The degree of separation is determined by the electrophoretic mobility of the different components, the composition of the buffer used, the electrical potential across the curtain and the length of time that the substances are subjected to the field.

With regard to selecting the optimum field intensities for a given separation, several points must be taken into consideration. In general, for a given flow rate down the paper curtain, the higher field intensities, the better will be the resolution attainable, for a given set of experimental conditions. When the current is too high, however, the water from the electrolyte may evaporate so rapidly that the downward flow is not sufficient to wash the paper, and in extreme cases solids may be deposited, thus disrupting the steady state. A cooling plate, through which tap water runs continuously, helps to dissipate heat produced. A high flow rate of buffer enables larger amounts of material to be separated, but the separation of the different components increases, the longer they remain on the paper. Thus the final choice in the selection of experimental conditions must be a compromise of the factors mentioned above.

The buffers used were the same as by one-dimensional electrophoresis except for lower ionic strength in some cases, as can be seen from Fig.7.

The filter paper curtain used was the Schleicher and Schüll No. 2668, cut to provide 32 drip points at the bottom of the curtain. The concentration of the proteins in the different fractions collected from the individual tongues was estimated by measuring the optical density at 280 mμ or by the modified biuret-Folin reaction described by Lowry *et al.* The proteolytic activity was determined by the rate of hydrolysis of casein at pH 6.0 or 7.4.

Separation of the proteolytic enzymes by column chromatography

Calcium phosphate chromatography. All chromatographic experiments were carried out on hydroxylapatite. The preparation of this modified calcium phosphate was described by Tiselius *et al.*[16] Stepwise elution, employing fractional increase in phosphate concentration was used to separate the various proteins in the mixture.

The method was only used for small scale separations of the proteolytic enzymes. For further information concerning protein chromatography on hydroxylapatite, the reader is referred to the paper mentioned above.

Ion exchange chromatography. Diethylaminoethylcellulose (DEAE) was either prepared from cellulose powder by the procedure of Peterson and Sober [16] or was obtained commercially. The anion exchange adsorbents used had a total exchange capacity of 0.4—0.6 mequiv./g dry weight.

Before packing a column, DEAE-cellulose was suspended in about 10 times its volume of 0.5 M phosphate buffer of the appropriate pH and stirred well at room temperature for 1 h. The cellulose was allowed to settle for half an hour, after which the supernatant was decanted in order to remove the smallest particles. Then the adsorbent was washed several times on a Büchner funnel with 0.01 M phosphate buffer, pH 6.0 the starting buffer, and then resuspended in the same buffer. After equilibration over night with this buffer, a slurry of the adsorbent was poured into glass chromatography columns of the

appropriate size. The adsorbent was allowed to settle by gravity, and then it was further compacted by air pressure until a constant column height was reached. Dimensions of the columns used are indicated in the figures. To insure thorough equilibration the columns were washed with 25—30 volumes of buffer or until the pH of effluent was that of the inflowing buffer.

Prior to being chromatographed, the products were dialyzed against the starting buffer. The majority of the separations described were carried out with stepwise elution as can be seen from the figures. Fractions were collected with a constant volume fraction collector.

The course of the elution of the proteins was followed by measuring the optical density at 280 mμ. The elution schedule could also be assessed more easily when the eluent was monitored by a UV absoption monitor and recording meter. In this manner a continuous graphic record of the absorption at 254 mμ of the emerging solution was obtained without necessity of collecting and analyzing individual fractions.

Those fractions which comprised discrete peaks were combined, dialyzed against cold water, and lyophilized or directly precipitated with tannin. The products were analyzed by paper electrophoresis and tested for their specific proteolytic activity. Smaller fractions were also concentrated by ultrafiltration through a collodion membrane according to Mies. The collodion sack was supplied by Membranengesellschaft, Göttingen. After use, DEAE-cellulose was regenerated by washing on a filter funnel with 0.5 N sodium-hydroxide and then with large volumes of water to neutral pH.

Selective adsorption procedure for separation of the proteases

We found that proteolytic enzymes could be isolated from a crude protease solution without an appreciable loss of the proteases or their activity by the use of an adsorption — elution process. The method, which is outlined in Fig. 10, was not only suitable for separating the proteases from each other but had also the advantage of removing most of the impurities accompanying the proteases. The method was rapid and also suitable to be carried out on an industrial scale.

The adsorbent used was the cation exchanger CM-cellulose prepared according to the method of Peterson and Sober [17] with Whatman cellulose powder and chloroacetic acid, or was obtained through commercial channels. Different batches, which contained 0.7—0.9 mequiv. of carboxyl groups per g of CM-cellulose, all behaved in the same manner during the separation process.

It was very important that the pH of the resin was the same as that of the protease solution to be treated, during the operation. Therefore the different batches to be used were buffered to the appropriate pH in the same way as described above for the DEAE-cellulose.

Any crude *Aspergillus oryzae* protease solution could be treated by the process, for example a culture fluid of the mould obtained by any known submerged cultivation. In most cases we used, however, crude protease mixtures obtained by precipitation with tannin.

500 g of tannin precipitated protease mixture was dissolved in 250 liters of water or 0.01 M phosphate buffer of pH 5.5. The pH was adjusted to 5.5 and then 500 g of CM-cellulose properly equilibrated to the same pH was added. The mixture was stirred at room temperature for 1 h and then the protein-exchanger combination was separated from the bulk of the solution by centrifugation in a basket centrifuge. The cellulose exchanger retained, however, large amounts of the solution which contained unreacted proteins and other products. Therefore the nonadsorbed material was removed by washing with 0.01 M phosphate buffer of pH 5.5. The combined solutions were adjusted to pH 4.5 with hydrochloric acid. The second protease was adsorbed on 500 g of CM-cellulose buffered to pH 4.5. The whole procedure was carried out as described above. Then the solution was adjusted to pH 3.0 and another 500 g of CM-cellulose buffered to pH 3.0 was added to adsorb the last protease component of the mixture.

Recovery of the proteases from the ion exchanger could be effected by use of any buffer of pH higher than that used in the adsorption step. In most cases both of the first

adsorbed proteases were eluted with 0.05 M phosphate buffer of pH 7.0 and the last one with a buffer of pH 6.0. Repeated elution resulted in a recovery of more than 90 % of the proteases and their activity. After use the CM-cellulose was recovered by washing with a solution of 0.5 M sodium chloride and 0.5 M sodium hydroxide, and then with water to neutrality.

The above mentioned process alone was sufficient when the only requirement was to separate the proteases from each other and from most of the impurities. Further purification of the proteases could, however, be achieved by ion exchange chromatography on DEAE-cellulose. These separations were carried out exactly as described earlier for the separation of the crude and unfractionated protease mixture.

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