ENZYMES OF *ASPERGILLUS ORYZAE*

V. ETHANOL FRACTIONATION AT LOW IONIC STRENGTHS*

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Summary

Moving boundary and paper electrophoresis were applied to crude and deionized enzyme preparations from cultures of *Aspergillus oryzae*. Five main protein components were observed by both methods and four additional minor components were demonstrated with the paper method.

After paper electrophoresis the main protein component was found to correspond with a protease and an esterase with isoelectric points at pH 5.1. A second protease less active than the other protease in digesting gelatin, with an isoelectric point of 4.2, and a second esterase of isoelectric point 3.9, were also characterized. Sucrase appeared as a single peak whereas amylase and cellulase showed at least six and seven peaks respectively.

Solubility curves of dialysed crude mould enzyme in ethanol-water mixtures at low temperatures were atypical in that variation of pH and ionic strength had little effect on the solubility owing to the presence of 70 per cent. ionic material. Removal of this material with ion exchange resins also removed about 50 per cent. of the enzyme protein, whereas electrodialysis removed it almost completely without loss in enzyme activity. Material treated in this way was found to provide typical pH-solubility curves having well-defined solubility minima near the isoelectric point of the major protease (pH 5.1). Using this data two proteases were separated from each other and, after reprecipitation at the isoelectric point, the major protease was obtained electrophoretically pure.

A crystalline preparation of mould protease was shown to contain a single component representing approximately 90 per cent. of the protein present and having mobility corresponding to that of the major protease.

I. INTRODUCTION

Cohn *et al.* (1946, 1950) have developed the basic procedures for separating plasma proteins by ethanol fractionation at low temperatures and low ionic strengths and their methods have been used for enzyme fractionation by Ellis and Fruton (1951) and Askonas (1951) and in the investigations described in the present paper.

By ethanol fractionation followed by salting-out Crewther and Lennox (1950) prepared crystals containing protease from culture filtrates of a strain of *Aspergillus oryzae*. In subsequent papers Maxwell (1952*a*, 1952*b*) described the growth conditions for maximum enzyme production by this strain, and

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Crewther and Lennox (1953*a*, 1953*b*) published further information concerning the nature of the enzymes formed and the methods employed in the early fractionation experiments. The techniques of filter paper electrophoresis and chromatography (Gillespie, Jermyn, and Woods 1952; Jermyn 1953) have shown the extreme complexity of the culture filtrates. They contain not only many different types of enzymes, but also several components specific for a single substrate; for example, there are at least six amylase components. Fractionation was further complicated by the presence of salts in the mould culture filtrate even after dialysis. Further purification was therefore a necessary preliminary to successful fractionation.

The present paper reports the results of further fractionation studies of the enzymes produced by *A. oryzae*. As in earlier studies, ethanol was used as the precipitant but additional means were used to remove ionic material, thereby enabling solutions of known ionic strength to be fractionated. The homogeneity of the fractions has been examined by electrophoresis.

II. Methods

(*a*) *Enzyme Estimations*

Most of the protease estimations were made by the azoprotein technique of Charney and Tomarelli (1947) using sulphanilamide azocasein as substrate at pH 8. To cover a wider pH range the sulphanilic acid azo-albumin substrate of Tomarelli, Charney, and Harding (1949) was also employed. The method of Anson (1938), using haemoglobin and casein as substrates, the gravimetric gelatin technique of Crewther (1952), and the viscometric gelatin method of Lennox and Ellis (1943) were used also to provide comparative data with other substrates. The estimation of esterase, amylase, and sucrase is described by Crewther and Lennox (1953) and cellulase (C_x) by Jermyn (1952).

(*b*) *Protein Estimation*

The modified biuret procedure of Mehl (1945) was used initially in which each series of readings was standardized against crystalline bovine plasma albumin. For most of the estimations, however, a procedure based on measurement of the optical density at 278 mμ was employed. Electrodialysed mould enzyme preparations had an $E\,{}^{1\%}_{1\,cm}$ at 278 mμ of 8.6, whereas crude mould enzyme had an extinction coefficient of nearly 14. There was remarkably little variation in extinction coefficient between mould enzyme fractions.

(*c*) *Electrophoresis*

(i) *Moving Boundary Electrophoresis.*—The crude enzyme and electrodialysed preparations in 2 per cent. solution were dialysed for 48 hr at 1° C against 0.1 $\Gamma/2$ veronal buffer at pH 8.6. Electrophoresis was carried out at this pH in a Tiselius apparatus* at 1°C for 3 hr with a current of 15 mA.

* LKB Type 3021 from LKB-Produkter, Stockholm 12, Sweden.

(ii) *Filter Paper Electrophoresis.*—A modification was used of the method of Cremer and Tiselius (1950) as described by Woods and Gillespie (1953). The distribution of protein along the paper was determined by the dyeing procedure of Durrum (1950) and also by the less sensitive spectrophotometric measurement of water eluates at 278 mμ. Owing to differences in affinity of different proteins for dye and differences in the absorption of radiation at 278 mμ, neither method provides an absolute measure of protein distribution.

The distribution of enzymes was followed by cutting the paper transversely into 1-cm strips, eluting with water, and estimating the activity of the eluates. Many enzymes were extraordinarily labile in the dried state on the paper after electrophoresis, the viscometric protease being inactivated during electrophoresis (Crewther and Lennox 1953*a*), inactivation of catalase and alkaline phosphatase occurring within 1 hr at room temperature and many of the other enzymes being inactivated within 18 hr after drying. The strips were therefore stored at −20°C until required, to retain full activity of the enzymes studied.

(*d*) *Dialysis and Electrodialysis*

Dialysis in cellophane tubing against distilled water was ineffective in removing all the salts from crude mould enzyme. Electrodialysis, on the other hand, was successfully used for this purpose. A 10 per cent. solution was electrodialysed between concentric cellophane bags against running distilled water for 8-12 hr at 400 V with an initial current of 5 mA and a final current of less than 0.5 mA. Least enzyme damage occurred at temperatures approaching 0°C. After electrodialysis a small quantity of globulin-like non-enzymic protein was centrifuged off and the supernatant freeze-dried. The yield corresponded to 30 per cent. of the material in solution before electrodialysis.

(*e*) *Ion Exchange*

A column of Amberlite MB1 monobed ion exchange resin* was used. This is composed of equal parts by volume of Amberlite IR-120 and IRA-400 separately regenerated and then mixed. The progress of deionization was followed by measuring the conductivity of the issuing protein solution at 1000 c/s. The monobed resin was regenerated by floating apart the two resins with an inorganic salt solution of suitable specific gravity and then washing and treating with acid or alkali.

(*f*) *Freeze-drying*

All precipitates and solutions containing protein were dried as quickly as possible to remove the solvents, minimize digestion by the protease components, and convert the protein to a stable form suitable for storage. The drying apparatus used was a modification of that designed by Flosdorf and Mudd (1935). The dry protein was stored at −5°C.

(*g*) *Solubility Studies*

Solubility studies were made by adjusting aliquots of an aqueous solution to the desired pH, temperature, ionic strength, ethanol concentration, and pro-

* Röhm and Haas Co., Washington Square, Philadelphia 5, Pa., U.S.A.

tein concentration. The resulting suspensions were allowed to equilibrate for 24 hr at the desired temperature, after which the solid phase was separated by centrifugation at the same temperature and the concentration of protein in solution estimated. The solid phase was in the amorphous state. The ethanol concentrations are recorded as percentages of the commercial (95 per cent.) solvent in the system.

(*h*) *pH Measurement*

The pH was controlled wherever possible by the use of buffers to yield the desired ionic strength. The recorded pH values do not refer to the ethanol-water mixtures at low temperatures but to solutions obtained by dilution of these systems to ethanol concentrations of 5 per cent. or less at room temperature; 0.02M NaCl was used as the diluent in systems of low ionic strength.

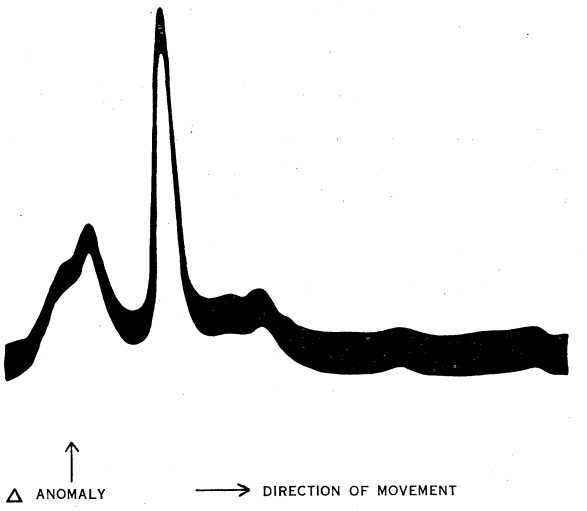

Fig. 1.—Moving boundary electrophoresis of electrodialysed mould enzyme in veronal buffer Γ/2 0.1, pH 8.6, 2 per cent. protein, 180 min, 15 mA, ascending boundary.

(*i*) *Preparation of Mould Culture Filtrate*

The mould culture filtrate was prepared, as described by Maxwell (1952*a*, 1952*b*), by growing a selected strain of *A. oryzae* for 10 days at 22°C. It was a clear amber fluid having a pH of approximately 7, containing about 0.1 per cent. protein, and being approximately 0.5M with respect to salts.

(*j*) *Preparation of Dry Crude Mould Enzyme*

Pooled culture filtrate (20 l) was dialysed against running tap water for 2 days at 10°C, whereby the salt concentration was reduced to a constant value, then cooled to 0°C, and maintained at that temperature until it was converted to an ice slurry. A coil through which ethanol passed at −25°C was immersed in the solution to facilitate cooling. Ethanol at −20°C was then run in beneath the surface from two jets at a rate of 200 ml per min with vigorous stirring to

give a final concentration of 60 per cent. As the ethanol was added the temperature gradually fell to $-20°C$, at which it was maintained for 8 hr. The precipitate was collected in a refrigerated Sharples super-centrifuge at $-20°C$, suspended in an ice-water slurry and freeze-dried to yield 20-30 g of light powder which was sealed in bottles and stored at $-5°C$. There was no loss in enzyme activity during a period of 12 months.

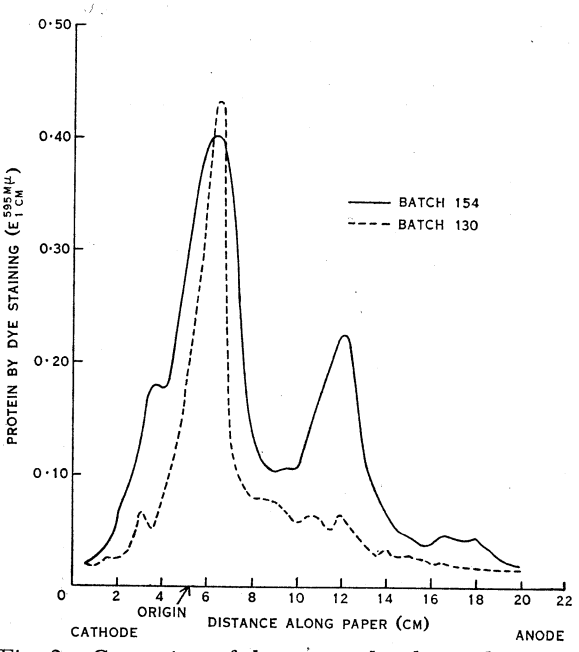

Fig. 2.—Comparison of the protein distribution by paper electrophoresis of two batches of electrodialysed mould enzyme in veronal buffer, Γ/2 0.025, pH 8.6 for 6 hr at 7.15 V/cm.

III. Results

(*a*) *Moving Boundary Electrophoresis*

Figure 1 shows the electrophoretic pattern of electrodialysed mould enzyme in veronal buffer Γ/2 0.1, pH 8.6, 2.0 per cent. protein. Five components are visible and a similar though less distinct pattern was obtained before electrodialysis. The electrophoretic mobility of the major component was found to correspond with that of the crystalline material of Crewther and Lennox (1950). The electrophoresis pattern gives no indication of the great complexity of the mould culture filtrates in terms of the enzyme components reported by Gillespie, Jermyn, and Woods (1952) and in the previous papers of this series.

(*b*) *Paper Electrophoresis*

Nine components can be seen in typical paper electrophoresis experiments but these are not always obvious in graphs plotted from absorption measurements on eluates.

(i) *Variability Amongst Batches.*—Mould enzyme was produced in weekly batches and although growth conditions were apparently identical, considerable variation occurred from one experiment to another. Such variations could be detected by enzyme estimation and also by paper electrophoresis. Electrophoretograms from two batches of enzyme, both run in veronal buffer pH 8.6 Γ/2 0.025, are shown in Figure 2. It can be seen that one of the main protein components is absent from batch 130. Some of the minor components are also missing or occur at a lower concentration. Similar differences were also reflected in the enzymic composition, one batch, for example, being rich in protease and poor in amylase whilst the reverse was true for another batch.

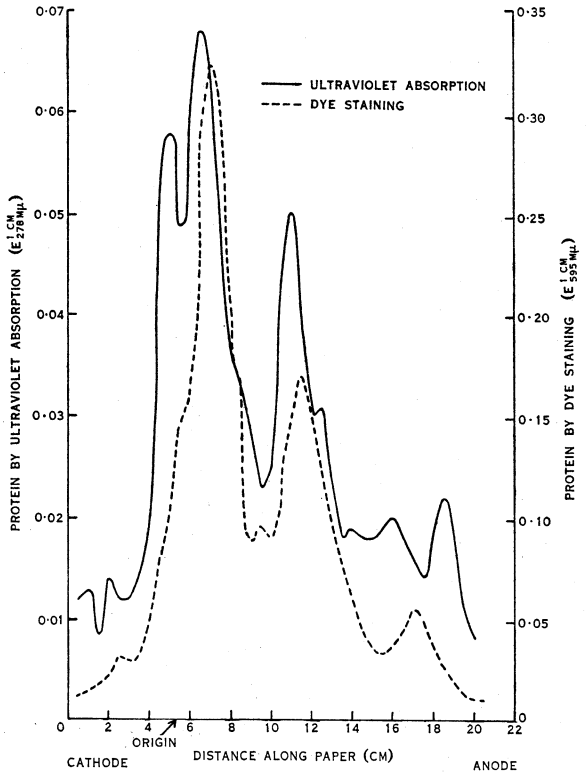

Fig. 3.—Protein distribution in electrophoretograms of electrodialysed mould enzyme, determined by two methods. Run in borate buffer Γ/2 0.1, pH 8.6 for 6 hr at 7.15 V/cm.

(ii) *Protein Distribution Along the Paper After Electrodialysis.*—Electrodialysed enzyme was electrophoresed on duplicate strips of paper at pH 8.6 in Γ/2 0.1 borate buffer at 7.15 V/cm for 360 min and the protein distribution along the paper was measured by the two methods. It can be seen from Figure 3 that the main protein components, as measured by each method, have migrated to almost identical positions on the paper but the relative heights of the peaks

vary. A more distorted pattern was obtained with the mould enzyme before electrodialysis.

(iii) *Distribution of Enzymes Along the Paper.*—As reported earlier, the mould culture filtrate contains multi-component enzymes (Gillespie, Jermyn, and Woods 1952). Before proceeding with further fractionation experiments, paper electrophoresis was employed to determine the complexity of the protease, esterase, sucrase, amylase, and cellulase and to ascertain the electrophoretic mobility of each component. Typical results of experiments on solutions of the dried electrodialysed mould enzyme are shown in Figure 4.

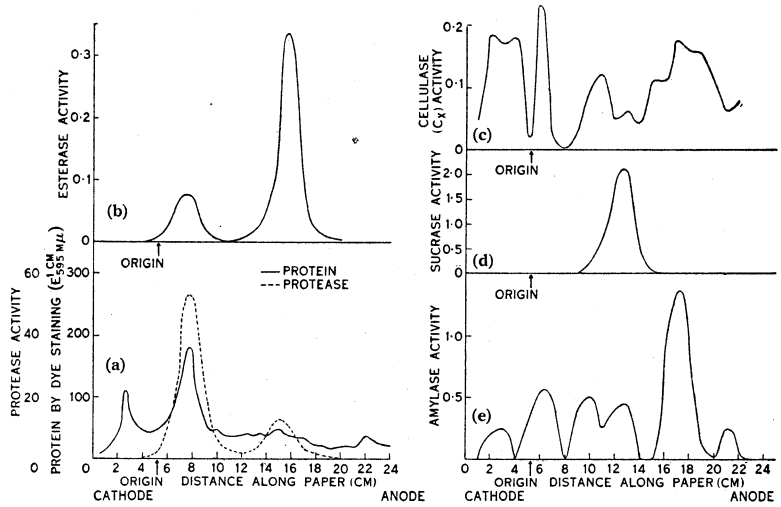

Fig. 4.—Enzyme distribution on paper electrophoretograms of electrodialysed mould enzyme. Run in veronal buffer Γ/2 0.025, pH 8.6 for 6 hr at 7.4 V/cm. Enzyme activities in arbitrary units.

Protease.—Using azocasein as a substrate the protease system was resolved into a large anodic component 2.5 cm from the origin and a smaller anodic component 8 cm from the origin. This experiment was repeated at pH values between 3 and 10 but no further resolution could be achieved. The results obtained at pH 8.6 and pH 5.0, using azocasein as substrate at pH 8.5 and 37°C, resemble those obtained using Anson's method with casein as substrate. Both methods gave similar twin peaks. The minor protease could be well distinguished from the major component for it gave barely measurable hydrolysis of gelatin, haemoglobin, and serum and egg albumins, whereas the latter readily attacked these substrates. The best separation of proteases was obtained at pH 5 in acetate buffer, one protease migrating to the anode, the other to the cathode. Their approximate isoelectric points are 4.2 and 5.1 (Woods and Gillespie 1953). Crewther and Lennox (1953*a*, 1953*b*) have shown that a third protease in the crude enzyme, which is capable of rapidly reducing the viscosity of gelatin, is inactivated during paper electrophoresis.

Esterase.—The mould enzyme esterase, which hydrolyses simple aliphatic esters, has been resolved into two components. The first, of isoelectric point pH 5.1, is closely associated with the major protease at a point 2.5-3.0 cm on the anodic side of the origin, the second is about 10.5 cm on the anodic side of the origin, has an isoelectric point of pH 3.9, and is not associated with protease activity. Between pH 4 and 10 the slow esterase component and the major protease component appear to travel together; they have not been separated by any method of fractionation yet employed.

Carbohydrases.—Whereas sucrase appeared as a single component, amylase was resolved into six well-defined components on paper electrophoresis. In the course of fractionation experiments evidence was obtained for the existence of nine amylases, all having different mobilities. From comparison of these results with those in Figure 4 it appears that some amylases with different solubilities in ethanol-water mixtures have approximately the same electrophoretic mobilities. Some of the amylase components were absent from some batches of mould enzyme having low total amylase activity. The multi-component nature of the cellulase is also evident from Figure 4.

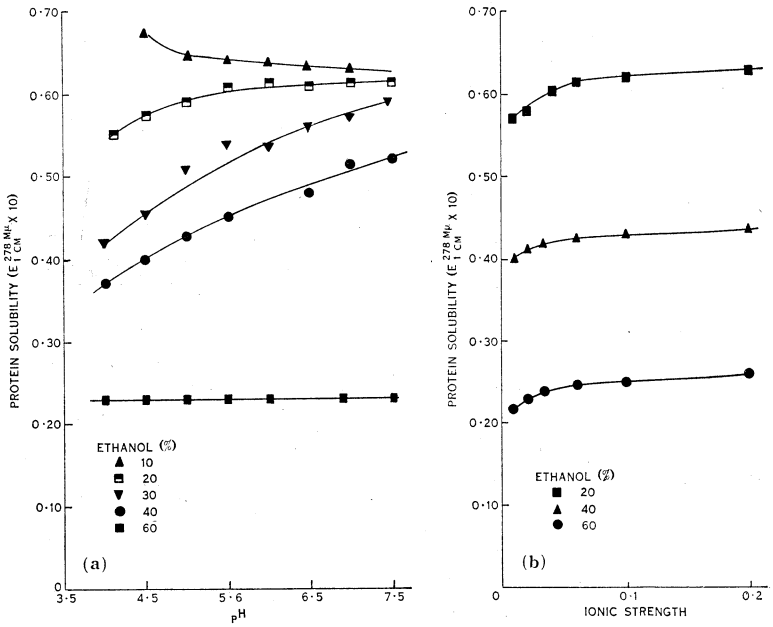

Fig. 5.—Solubility of crude mould enzyme at $-5°C$.

(*a*) In 0.01 Γ/2 salt solutions as a function of ethanol concentration and pH.

(*b*) At pH 4.5 as a function of ionic strength (sodium acetate) and ethanol concentration.

(*c*) *Fractionation Experiments*

(i) *Solubility Studies on the Mould Enzyme.*—The solubility of the mould enzyme preparation before treatment with ion-exchange resin or electrodialysis has been studied, using ethanol-water mixtures at $-5°C$ under controlled con-

ditions of pH and ionic strength with the solid phase in the form of amorphous protein. The results are presented in Figure 5. Variation in pH and ionic strength had very little effect on the solubility and no points of minimum solubility were observed. Subsequently it was found that the preparation contained only about 30 per cent. by weight of protein. The remainder was shown by Dr. M. A. Jermyn, of this laboratory, to comprise mainly salts of tartaric and valeric acids, bound more or less firmly to the protein. The ionic strength of the solution was therefore sufficient to mask solubility changes due to added electrolyte.

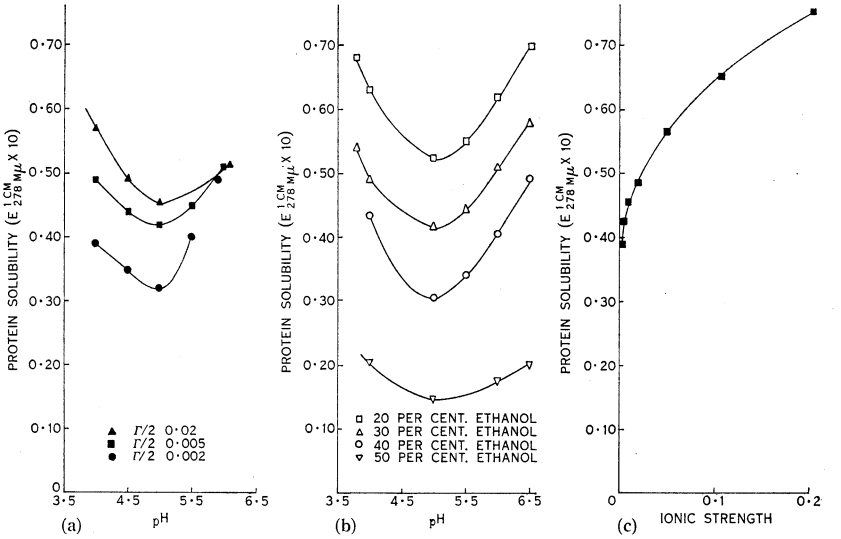

Fig. 6.—Solubility of electrodialysed mould enzyme at − 5°C.
(*a*) In 30 per cent. ethanol as a function of pH and ionic strength.
(*b*) In 0.005 Γ/2 salt solutions as a function of pH and ethanol concentration.
(*c*) In 30 per cent. ethanol at pH 5 as a function of ionic strength of sodium acetate.

Following the recommendation of Edsall and Surgenor (personal communication 1950) a 10 per cent. aqueous solution of the mould enzyme was run through a column of mixed ion-exchange resin. Aliquots of the effluent were collected and the conductivity, protein concentration, and the protease, esterase, amylase, and sucrase activities were measured. The conductivity fell to approximately 1 per cent. of its initial value. When the enzyme activities of each aliquot of effluent were plotted graphically against the fraction number the peaks in the curves for some of the enzymes suggested that chromatographic separation may have occurred. Approximately 50 per cent. of the protein was lost during the operation, presumably by uptake on the resins, and this included certain enzymes, notably the viscometric protease, catalase, alkaline phosphatase, and some components of the amylase and β-glucosidase complexes. Furthermore, removal of non-protein material was not complete for the pooled effluent after freeze-drying still contained only 75 per cent. protein.

The solubility of some samples obtained in this way was studied in ethanol-water mixtures at $-5°C$ under controlled conditions of pH and ionic strength. Though the protein became more soluble with increase in ionic strength, pH had little effect on the solubility. Because of the imperfect removal of small molecules and the loss of some of the enzymes this method was discontinued.

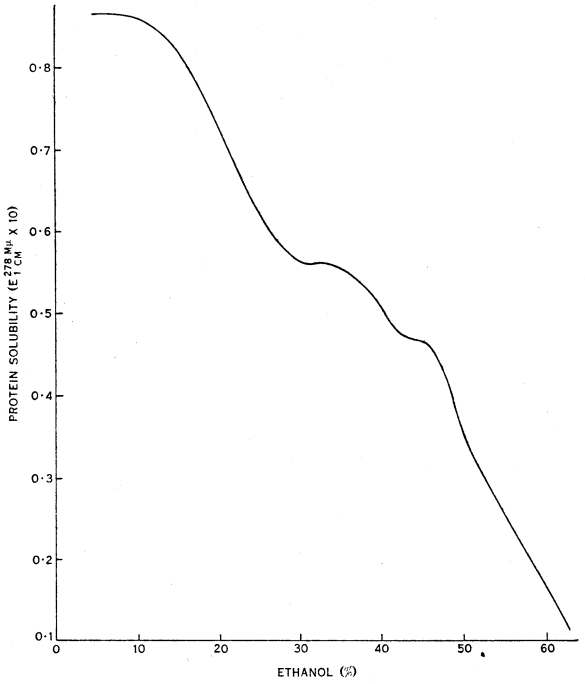

Fig. 7.—Solubility of electrodialysed mould enzyme at $-5°C$ in acetate buffer pH 4.19, $\Gamma/2$ 0.005 as a function of ethanol concentration.

The most successful method of deionizing the mould enzyme proteins was by electrodialysis. A 10 per cent. aqueous solution of the crude enzyme was electrodialysed, centrifuged to remove globulins, and freeze-dried. The yield of dry material corresponded to 30 per cent. of the starting product and it contained over 90 per cent. protein, according to the biuret method of protein estimation using albumin as a standard. It was a white powder soluble in water at room temperature to the extent of about 40 per cent. Most of the original enzyme activity was retained, the activity per unit weight in general increasing in proportion to the removal of inactive material during dialysis. This material apparently includes a compound having a high absorption coefficient at 278 mμ since the absorption fell from 14 to 8 during the treatment. On storage at 0°C for several weeks, certain enzymes, including catalase and alkaline phosphatase, commenced to lose activity and eventually became completely inactive. Previous storage experiments with the mixed enzymes in solution revealed similar loss in activity for these two components (Crewther and Lennox 1953*a*).

The solubility of electrodialysed enzyme in ethanol-water mixtures at $-5°C$ under controlled conditions of pH and ionic strength is shown in Figure 6. The minimum solubility in acetate buffers is seen to occur near to pH 5.1, which is the isoelectric point of the major protease component and protein. The solubility in acetate buffers was markedly affected by changes in pH and ionic strength. Increase in ionic strength from 0.002 to 0.02 produced approximately 45 per cent. increase in solubility at pH 5.1 in 30 per cent. ethanol, whereas at pH 6.2 the salting-in effect was negligible. It was further noted that in the high ethanol concentration range, 50-60 per cent., pH and ionic strength had very little effect on solubility. The solubility curves are of the type often found with proteins (Koechlin 1952). Sodium chloride had virtually the same salting-in effect as sodium acetate but both effects were much less than those reported from similar experiments with several blood plasma proteins.

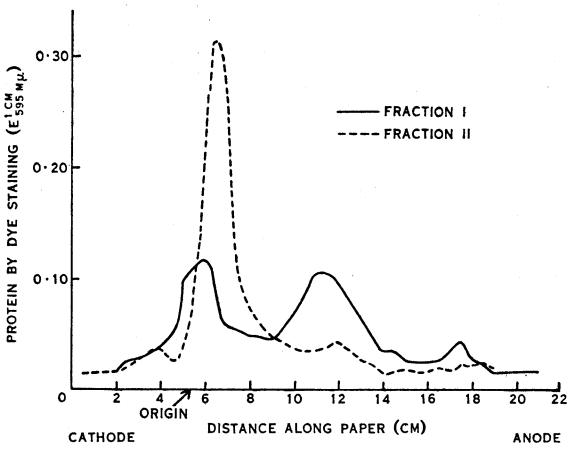

Fig. 8.—Paper electrophoresis of fractions I and II from electrodialysed mould enzyme in veronal buffer $\Gamma/2$ 0.025, pH 8.6, for 6 hr at 7.4 V/cm.

(ii) *Fractionation of Electrodialysed Enzyme.*—It is generally accepted that for satisfactory separation between proteins using ethanol fractionation a pH outside the range of isoelectric points is desirable. This was difficult to achieve in the present study, for above pH 7.0, the isoelectric point of one of the amylases, the proteins were too soluble, and below pH 3.9, the isoelectric point of the acid esterase, the enzymes were rapidly inactivated. A pH of 4.2 was therefore chosen as a compromise.

The solubility of the electrodialysed mould enzyme at this pH was measured in acetate buffer of ionic strength 0.005 at $-5°C$ with 1 per cent. protein present over the range 0-60 per cent. ethanol (Fig. 7). Practically no decrease in solubility occurred until a level of 11 per cent. ethanol was reached, thereafter small increments in ethanol concentration caused a sharp decrease in solubility. Marked inflexions in the curve were obtained at ethanol concentrations of 35 per cent. and 44 per cent. These points of inflexion were taken to mark the completion of precipitation of different sets of components. Select-

ing the first point of inflexion the electrodialysed enzyme was divided into two fractions, fraction I being insoluble in the presence of 35 per cent. ethanol, Γ/2 0.005 acetate, pH 4.2, and fraction II being soluble under these conditions. The two fractions were freeze-dried and paper electrophoretic studies were made, with the results shown in Figure 8. In fraction I the main protein peak is much reduced but the fraction is enriched in other components—a second peak of equal height to the first, and third and fourth peaks of smaller height can be seen. In fraction II the first peak makes up the bulk of the protein. These differences were also reflected in the physical nature of the fractions, fraction I being light brown in colour, sticky, and rich in carbohydrate, whilst fraction II was white and granular. There are marked differences also in the enzyme contents of the two fractions. Paper electrophoresis showed that fraction I contained all the protease having an isoelectric point of pH 4.2 but also some of the second protease, several amylases corresponding to the major portion of the amylase activity of this system, the sucrase, and a trace of both esterases. Fraction II contained most of the protease having an isoelectric point of pH 5.1, the viscometric protease, the two esterases, and several of the amylase components, although only a minor part of the total amylase activity.

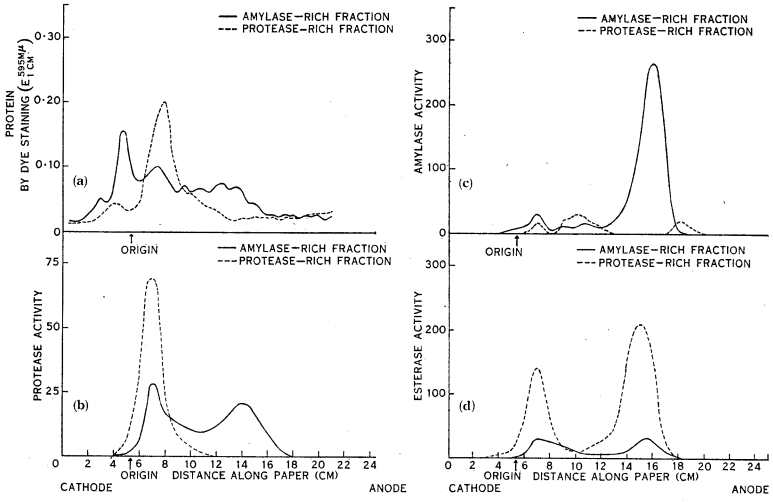

Fig. 9.—Distribution of protein and enzymes in amylase-rich and protease-rich fractions. Electrophoresis carried out in veronal buffer pH 8.6, Γ/2 0.025 for 6 hr at 7.4 V/cm. Enzyme activities in arbitrary units.

These two fractions were next subfractionated. Fraction I was split into I*A* and I*B* under the conditions of 1 per cent. protein, Γ/2 0.005 acetate, pH 4.2, − 5°C; I*A* was material insoluble in 25 per cent. ethanol, and I*B* that which was soluble. Similarly, II*A* was that part of fraction II insoluble in 44 per cent. ethanol and II*B* the part which was soluble. It can be seen that the fractionation of II takes place at the second point of inflexion on the solubility curve. The four fractions were freeze-dried and examined by paper electrophoresis. It was found that fraction I*A* contained a greatly enriched protease of iso-

electric point 4.2, whilst II*B* contained the protease of isoelectric point 5.1 and both esterases. ' The other two fractions contained protease and amylase components distributed in varying amounts. Fraction II*B* seemed to warrant further study, for it corresponded approximately to the protease-containing fraction of Crewther and Lennox (1953). In the next experiment II*B* will be referred to as the protease-rich fraction and I*A* + I*B* + II*A* as the amylase-rich fraction.

(iii) *Separation of Electrodialysed Enzyme into Protease-rich and Amylase-rich Fractions.*—From the fractionation experiments just described it can be seen that fraction II*B* consisted mainly of the major protease. To obtain a larger quantity of this material the electrodialysed enzyme was divided into two fractions by using the following conditions; protein 1 per cent., ethanol 47 per cent., $-5°C$, $\Gamma/2$ 0.005 acetate, pH 4.5 These fractions were examined by paper electrophoresis in veronal buffer $\Gamma/2$ 0.025 at pH 8.6 at 7.4 V/cm for 6 hr. As can be seen from Figure 9, the protease-rich fraction contains not only the major protease, having an isoelectric point of pH 5.1, but also three amylase components and both esterases. The amylase-rich fraction contains three amylase components, corresponding to most of the amylase activity, also all of the minor protease, portion of the major protease, the catalase, and the alkaline phosphatase.

(iv) *Re-precipitation of the Protease-rich Fraction near the Isoelectric Point.*—In an attempt to purify, re-precipitation was effected at its isoelectric point, pH 5.1, under the following conditions: 1 per cent. protein, pH 5.1 acetate buffer $\Gamma/2$ 0.005, and 50 per cent. ethanol at $-5°C$. The product was freeze-dried. Paper electrophoresis showed that it contained one protein component corresponding with the protease and esterase of isoelectric point pH 5.1.

Attempts were made to crystallize it from a solution containing 10 per cent. protein, acetate buffer $\Gamma/2$ 0.01, pH 5.1, and 30 per cent. ethanol, at $-5°C$. On several occasions evidence was obtained of the formation of microcrystals but insufficient material was available to carry out more systematic attempts at crystallization.

(*d*) *Paper Electrophoresis Examination of Protease Crystals*

The protease crystals of Crewther and Lennox (1950), which were formed at higher ionic strength and at a higher temperature than the protease-rich fraction prepared in the present investigation, were examined electrophoretically.

The crystals (5 g) were dissolved in water, filtered to remove approximately 1 per cent. of insoluble material, and electrodialysed for 24 hr at 5°C against distilled water. After some hours a further 1 per cent. of insoluble matter formed which was filtered off and was found to have no enzyme activity on the standard substrates. The filtrate was freeze-dried to yield 1 g of white powder.

A paper electrophoretogram run on solutions of the electrodialysed crystals provided the results in Figure 10, which shows that they contained approximately 90 per cent. of a single component. This agrees with the results obtained by electrophoresis in the Tiselius apparatus and ultracentrifugation (Crewther and Lennox 1950). However, as Figure 10 shows, both proteases

are present, but that with isoelectric point pH 5.1 was the more abundant. The esterase with isoelectric point pH 5.15 was also present. Similar results were obtained over a wide range of pH. As shown earlier, a sharp separation of these two proteases can be obtained by fractionation with ethanol at low ionic strength.

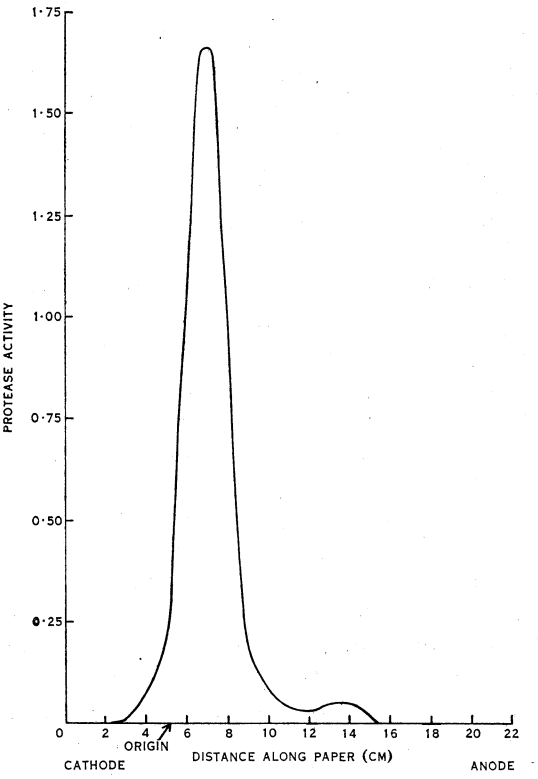

Fig. 10.—Distribution of protease in an electrophoretogram of electrodialysed enzyme crystals run in veronal buffer Γ/2 0.025, pH 8.6 for 6 hr at 7.4 V/cm. Protease activity in arbitrary units.

IV. Discussion

Salting out with neutral salts has been widely used for the fractionation and preparation of crystalline enzymes (Northrop, Kunitz, and Herriott 1948), but although this method does not damage proteins it is relatively insensitive to the specific characteristics of the various proteins in a mixture since it depends largely on the size and shape of the molecules and on the volume of water displaced. The salting-out of protein by sulphates or phosphates varies by a factor of 2 or 3 to 1 from one protein to another, and the limitations of the method were illustrated by the failure to fractionate the mould enzymes with ammonium sulphate (Crewther and Lennox 1953*b*).

In contrast with the salting-out method, the salting-in of proteins at low ionic strengths has revealed solubility ratios of the order of 10 to 1 or even 100 to 1 between different proteins. As in plasma fractionation (Cohn *et al.* 1946, 1950), ethanol has been used successfully at low ionic strengths, and at low temperatures to reduce the solubilities of the enzyme components of the *A. oryzae* culture filtrates sufficiently for their fractionation. The improved response in protein solubility to changes in ionic strength, pH, and ethanol concentration, reported for other protein mixtures to accompany a reduction in the salt concentration (e.g. Edsall 1947), is also evidenced by the solubility curves reported in the present paper. Difficulty has been experienced, however, in removing sufficient of the ionic material from the mould preparations to bring the ionic strength into the sensitive low range. Even after prolonged dialysis, the crude mould enzyme contained approximately 70 per cent. of material other than non-dialysable protein and, although partial removal was obtained by passage through a mixed-bed column of ion exchange resins, it was necessary to resort to electrodialysis for complete removal of the salts.

The change in the solubility curves of crude mould enzyme, after repeated fractionation and re-precipitation with ethanol, from a form showing minimum solubility over a wide range of pH to one having a well-defined and relatively sharp minimum (Crewther and Lennox 1953*b*) is probably attributable also to removal of ionic material.

The inflexions in the curve relating protein solubility and ethanol concentration indicated conditions for obtaining satisfactory separation of certain groups of enzymes. One of these inflexions corresponded closely with the conditions selected by Crewther and Lennox (1953*b*) for the fractionation of the mould enzyme preparatory to the production of crystals containing protease. Comparison by paper electrophoresis of the crystalline material and a protease fraction obtained from electrodialysed enzyme showed that, whereas the crystals contained a certain amount of the protease having an isoelectric point at pH 4.2 and traces of amylase, the other preparation was free of these enzymes and was, in fact, electrophoretically homogeneous, thus demonstrating the value of fractionating at low ionic strength.

The results provide further evidence of the multiple nature of the enzyme systems acting on single substrates. Thus during fractionation it has been possible to separate the two esterases previously demonstrated by paper electrophoresis (Woods and Gillespie 1953), and the amylases have been separated into groups. It is interesting to note that the traces of amylase appearing in the crystalline material correspond with one of the peaks of amylase activity shown up by paper electrophoresis. It has further been shown that, in addition to the viscometric protease and gravimetric protease of Crewther and Lennox (1953*a*) the mould enzyme contains a third protease which can be separated from the major gravimetric protease by paper electrophoresis. This third protease, which has considerable activity as measured by the azocasein method, corresponds with that having relatively low, though readily measurable, gravimetric protease activity with gelatin as substrate. It is likely therefore that the main peak of protease activity corresponds with an enzyme which acts readily on most proteins, including those lacking the aromatic amino acids.

The minor protease, having an isoelectric point at pH 4.2, apparently requires certain of the aromatic amino acids in its substrate since it acts readily on azocasein but, like chymotrypsin, is relatively inactive with gelatin as substrate. The results serve to emphasize the utility of paper electrophoresis in the study of the fractionation of complex enzyme mixtures.

The great difficulty of removing the last traces of salts from the mould enzyme suggests that the binding forces between the mould proteins and the dicarboxylic acid salts are greater than have been encountered in research with plasma proteins. The loss of enzyme activity on removal of the salts by passage of the mould enzyme through an ion exchange column was very marked. Similar observations were reported by Gilbert and Swallow (1950) for urease, α- and β-amylase, and Q-enzyme. Separation of the salts from the mould enzymes during paper electrophoresis may also have been responsible for the extreme lability of the enzymes in these experiments.

V. References

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