The Major Alkaline Proteinase of *Aspergillus oryzae*, Aspergillopeptidase B.* I. Isolation in Homogeneous Form†

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A procedure has been devised to isolate the major alkaline proteinase of *Aspergillus oryzae* in gram quantities, as an apparently homogeneous preparation. The purification, using 500-g batches of a commercial concentrate, is accomplished in five steps: (1) dialysis, (2) batchwise treatment with ECTEOLA-cellulose, (3) fractional precipitation with ammonium sulfate (75–85% saturation), (4) chromatography on Amberlite CG-50 at *p*H 6.5, and (5) chromatography on Amberlite CG-50 at *p*H 7.5. The eluents used are 0.01 M sodium phosphate buffer, *p*H 6.5, for step 4 and the same buffer at *p*H 7.5 for the final chromatography. The enzyme is purified 55- to 60-fold during the isolation. A 500-g portion of the commercial concentrate yields about 0.9 g of the purified enzyme, amounting to about 22% recovery of total proteolytic activity. In digesting casein, the purified enzyme shows about the same specific activity as chymotrypsin, and three to four times the activity of trypsin. The purified enzyme travels as a single peak in the ultracentrifuge at *p*H 5.0 and at *p*H 8.0. On paper electrophoresis in buffers of *p*H 5.0, 6.0, 7.0, 8.0, and 10.0 and staining with amido black, only a single spot could be detected in each case. The enzyme also travels as a single peak in moving-boundary electrophoresis carried out in two buffers of different *p*H and ionic strengths (*p*H 4.9, μ 0.1, and *p*H 8.0, μ 0.2).

Species of the common mold in the genus *Aspergillus* are known to secrete large amounts of proteolytic and diastatic enzymes into their culture media (Thom and Rasper, 1945). Oshima (1922) first reported the proteolytic activity of *Aspergillus oryzae*. Since then several investigators have found, in the culture media of many species of Aspergilli, evidence for the presence of at least three groups of proteolytic enzymes designated from the *p*H of their optimum activities as acid, neutral, and alkaline proteinases. The reported *p*H optima of these three groups are, respectively, 3, 6–7, and 8–10 (Hagihara, 1960). An enzyme having an acid *p*H optimum (acid proteinase) is found to be the major proteinase component in various black aspergilli (e.g., *Aspergillus niger*, *Aspergillus saitoi*, *Aspergillus awamori*), while the major component in the normally grown cultures of *Aspergillus oryzae* is an alkaline proteinase (Hagihara, 1960).

Crystals of an alkaline proteinase of *Aspergillus oryzae* were first obtained by Crewther and Lennox (1950) by fractional precipitation of the proteins of the culture medium with cold alcohol, followed by crystallization from ammonium sulfate solution. Interestingly, the specific activities of the crystals were found to depend on the conditions of preparation (Crewther and Lennox 1953). Examination of the crystalline preparation by paper electrophoresis and by ultracentrifugation revealed it to be inhomogeneous, although the major component accounted for 80–90% of the total protein. Three other groups of workers have since crystallized the alkaline proteinase, but the homogeneity of these preparations has not been examined (Akabori *et al.*, 1953; Miura and Motonaga, 1954; Bergkvist, 1963). Besides these preparations, another aspergillus alkaline proteinase has been crystallized from *Aspergillus sojae* (Mizunuma and Iguchi, 1957). Few studies have been made, however, on the physical and chemical characterization and specificity of these enzymes, and also little is known about the genetic relationship between the proteinases from different species of this organism.

The studies described in this paper were undertaken with the aim of isolating the major alkaline proteinase of *Aspergillus oryzae* in homogeneous form, in order to be able to determine the physical constants, amino acid composition, specificity, and other enzymic properties of this enzyme. A purification procedure has been devised yielding gram quantities of the purified enzyme which appear to be homogeneous when checked by several methods.

Materials and Methods

Aspergillus oryzae Proteinase Concentrate.—The starting material for isolating the enzyme is a concentrate of the proteolytic enzymes of *Aspergillus oryzae*, called "fungal protease" (batch F. 8954; 80,400 hemoglobin units/g) purchased from Miles Chemical Co., Inc., Clifton, N. J. This commercial concentrate, obtained from the culture grown on wheat bran by extraction with water, alcohol precipitation, and vacuum drying (Hoogerheide, 1954; Underkofler *et al.*, 1958), is a gray-brown powder, the major portion of which dissolves in water to give a dark solution containing all the proteolytic activity.

Adsorbents for Chromatography and Gel Filtration.—ECTEOLA-CELLULOSE (capacity 0.48 meq/g), purchased from Bio-Rad Laboratories, Richmond, Calif., was used without any pretreatment and was regenerated after each use according to Peterson and Sober (1956). The alkali treatment sometimes had to be repeated, to displace the adsorbed pigments.

CELLULOSE PHOSPHATE (Whatman) was equilibrated,

* In the Report of the Commission on Enzymes of the International Union of Biochemistry, 1961, enzyme number 3.4.4.17 is listed as aspergillopeptidase A, an acid protease from *Aspergillus saitoi* (Yoshida, 1956). In a situation where the nomenclature and classification of proteolytic enzymes obtained from molds is confusing, this classification is helpful, and we have accordingly followed this system of classification and named the enzyme described in this paper as aspergillopeptidase B.

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after removing finely divided particles and treating with 4% NaOH for 3–4 minutes to remove alkali-soluble impurities, by suspension in 0.01 M sodium phosphate buffer, *p*H 6.5, and mechanical stirring as 20% (v/v)HCl was added dropwise to lower the *p*H to 6.5. The stirring and *p*H adjustment were continued until the *p*H remained steady at 6.5 ± 0.03 for a period of about 1 hour. The material was then filtered in a Büchner funnel and washed five to six times with buffer, and the filter cake was resuspended in the buffer for packing into columns.

AMBERLITE CG-50, 200–400 mesh (Mallinckrodt Chemical Co.), was equilibrated, after removing fine particles, with 0.01 M phosphate buffer, *p*H 6.5, as described for cellulose phosphate, using 40% NaOH to adjust the *p*H. Equilibration with 0.01 M phosphate buffer, *p*H 7.5, was accomplished in the same fashion.

CARBOXYMETHYL-CELLULOSE (CM-cellulose, capacity 0.67 meq/g; Bio-Rad Laboratories) was treated in the same manner as cellulose phosphate. SEPHADEX G-75 (Pharmacia, Sweden) used was the medium grade, 100–270 mesh.

Preparation of Columns for Chromatography.—These columns were prepared from a slurry of the equilibrated adsorbents in the desired buffer. All chromatographic runs were made in a cold room at 4–6°, unless otherwise noted, since extensive loss in activity (30–45% on Amberlite and CM-cellulose columns) was found to occur at room temperature. Columns used in exploratory experiments were 30 × 0.8 cm; those used for preparative purposes were 3.1, 4.5, and 6.5 cm in diameter.

Buffer Solutions.—Sodium phosphate buffer (0.01 M) was prepared in 10- to 20-liter quantities by 100-fold dilution from a 1.00 M stock solution $NaH_2PO_4 \cdot H_2O$. (Malinckrodt, analytical reagent); the *p*H was adjusted to 6.5 ± 0.03 or 7.5 ± 0.03 with 10% NaOH using a Beckman Zeromatic *p*H meter. Gel filtration was carried out in 0.1 M sodium phosphate buffer, *p*H 6.8, obtained by mixing equal volumes of 0.1 M solutions of mono- and disodium phosphate.

The buffers used for paper electrophoresis were 0.1 M acetate, *p*H 5.0; 0.05 M maleate, *p*H 6.0; 0.05 M and 0.1 M phosphate, *p*H 7.0; 0.1 M Tris, *p*H 8.0; and 0.05 M carbonate-bicarbonate, *p*H 10.0. Each buffer was prepared as described by Gomori (1955).

For moving-boundary electrophoresis 0.02 M buffers, containing enough sodium chloride to make the ionic strength either 0.1 or 0.2, were employed. The relative amounts of free acid and salt in these buffers were calculated from the data of Gomori (1955). Buffers used were (1) acetate buffer, *p*H 5.0, μ 0.1 (sodium acetate, 0.02 M; HCl, 0.0059 M; NaCl, 0.08 M), actual *p*H = 4.89, and (2) barbital buffer, *p*H 8.0, μ 0.2 (sodium barbiturate, 0.02 M; HCl, 0.007 M; NaCl, 0.18 M), actual *p*H = 8.04. The *p*H was measured with a Radiometer Model 25 *p*H meter using Beckman *p*H 4 and *p*H 7 buffers as standards.

The ultracentrifuge experiments were carried out in (1) 0.02 M sodium barbital buffer, *p*H 8.04, μ 0.2, prepared as described, and (2) 0.1 M sodium acetate buffer, *p*H 5.0, prepared as described by Gomori (1955).

Dialysis.—Dialyses were carried out in seamless cellulose dialysis tubing (Fisher Scientific Co.) at 4–6° against distilled water for 24–36 hours with changes of water at 5- to 6-hour intervals. Sulfate and phosphate were determined with $BaCl_2$ and by a spot test (Feigl, 1954) to check their removal by dialysis.

Assay Procedures.—Protein concentration was estimated from the ultraviolet absorption at 280 mμ in a Beckman Model DU spectrophotometer. For dilute solutions the Folin-Ciocalteau method, as modified by Lowry and co-workers, was used (Lowry *et al.*, 1951).

Proteolytic activity was measured by a modification of the method of Kunitz (Kunitz, 1947; Laskowsky, 1955). The enzyme was allowed to digest buffered casein at 35° for 10 minutes; then the reaction was stopped and the undigested proteins were precipitated by the addition of trichloroacetic acid. After the precipitate was centrifuged off the absorbance of the supernatant was measured at 280 mμ.

The substrate, a 0.5% solution of casein in 0.1 M sodium phosphate buffer, *p*H 7.6, was prepared by adding 1.00 g of casein (Hammersten quality, Nutritional Biochemicals Corp.) to a mixture of 13 ml of 0.2 M NaH_2PO_4 and 87 ml of 0.2 M Na_2HPO_4 (Gomori, 1955) diluted to 150 ml. The mixture was stirred with a magnetic stirrer and heated (but not allowed to boil) until a clear solution was obtained. It was then cooled and quantitatively transferred to a 200-ml volumetric flask and diluted up to the mark with distilled water. The solution was refrigerated when not in use.

The enzyme solution (100 μl) was transferred with a Lang-Levy pipet into test tubes (12.8 × 1.3 cm) which were placed in a water bath at 35°. After 1 minute, 1.9 ml of substrate was added at 0.1-minute intervals to each tube by means of a B-D Cornwall continuous-pipetting device (Becton, Dickinson and Co., Rutherford, N.J.). The substrate was prewarmed to 35° by immersion in the water bath for at least 30 minutes. After 11 minutes (i.e., 10 minutes after the reaction began) we stopped the reaction by pipetting 3 ml of 5% (w/v) trichloroacetic acid into each tube at 0.1-minute intervals. The contents of each tube was mixed well with a Vortex mixer, spun for 5 minutes in a refrigerated centrifuge, and filtered through small plastic funnels plugged with Pyrex wool, and the absorbance of the filtrate was measured at 280 mμ. The blanks were run exactly as above except for addition of the enzyme after the casein was precipitated with trichloroacetic acid. With partially purified preparations the enzyme may be omitted from the blanks, since the optical density was the same with and without enzyme addition. The absorbance of the blank was in the range 0.05–0.08; when it exceeded 0.1, the substrate was discarded

Unit of Enzyme, Purity, and Purification.—One unit of enzyme was defined as that amount which would give, in the foregoing assay, an optical density of 0.1. The efficacy of the different purification procedures and the progress of purification were followed by calculating at each step the purity and purification factor (Dixon and Webb, 1958). Purity is defined as the number of units of enzyme per ml of solution divided by the absorbance of the solution at 280 mμ. Purification is expressed as the purity at any step divided by the purity of the starting material.

Chromatographic eluents were monitored with an ultraviolet-detector unit (Gilson Medical Electronics) connected with a recorder (Rectiriter, Texas Instruments). The fractions were counted with either a drop counter or a timer unit.

Ultracentrifugation.—Sedimentation analyses were carried out at 20° in a Spinco Model E ultracentrifuge equipped with a phase plate using a synthetic-boundary cell. The movement of the boundary was observed with Schlieren optics and was photographed on metallographic plates at suitable intervals.

Paper Electrophoresis.—Whatman No. 1 filter-paper strips, 21 × 57 cm or 9 × 57 cm, spotted with 2.5–10 μl of a 1–10% protein solution, were held tightly sandwiched between two 6.4-mm-thick glass plates (23 × 50 or 11.5 × 50 cm) which had been evenly

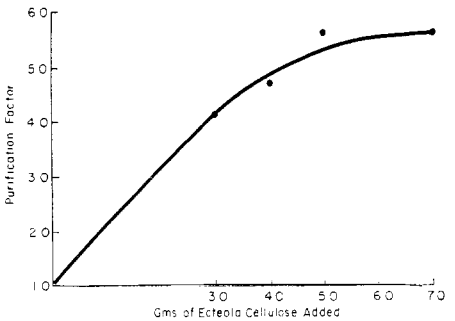

FIG. 1.—Purification of aspergillopeptidase B by batchwise treatment with ECTEOLA-cellulose. One g of the dialyzed and lyophilized "fungal protease" (purity = 11 units/OD_{280}; purification [assumed] = 1) was dissolved in 50 ml of distilled water and increasing amounts of ECTEOLA-cellulose were added. The solution was kept stirred for 10–15 minutes at room temperature and the supernatant was analyzed for protein and activity. Purification factor: purity attained divided by the purity before treatment.

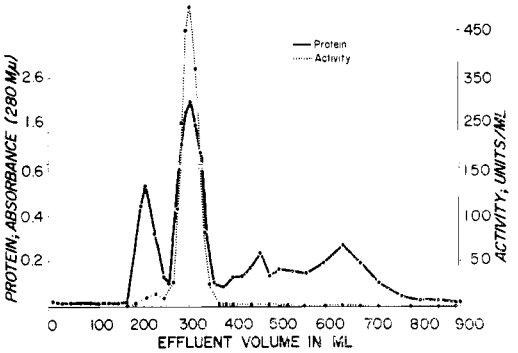

FIG. 2.—Gel filtration of aspergillopeptidase B on Sephadex G-75. A 51 × 4.4-cm column of Sephadex G-75 (medium) was prepared in 0.1 M sodium phosphate buffer, *p*H 6.8; 200 mg of proteinase preparation (purified with ECTEOLA-cellulose and $(NH_4)_2SO_4$ fractionation) was dissolved in 10 ml of the buffer, applied to the top of the column, and eluted with the same buffer at the rate of 48 ml/hr. The experiment was carried out at room temperature; protein recovered, 103%; activity recovered, 78%.

coated with a very thin film of silicone grease. The glass plates were laid on the buffer chamber so that the overhanging ends of the paper were in the buffer solution. A potential of 400–600 v (5–15 ma) for 4–6 hours was generally used. After electrophoresis the paper was allowed to dry at room temperature and dyed with amido black 10B (0.7% solution; 3-minute staining time). The solvent for making the dye solution and for washing off adsorbed dye from the paper was a mixture of methanol, acetic acid, and water, in the ratio 5:1:4 (v/v).

Moving-Boundary Electrophoresis.—The procedure was carried out in a Perkin-Elmer electrophoresis apparatus (Model 38) at 0° using 2-ml Tiselius cells. The protein solution was prepared by dissolving salt-free lyophilized material in buffer and dialyzing it for 16–24 hours at 4–6° against 1 liter of the same buffer. The power was kept at approximately 2 w to minimize heating of the solution.

Experimental and Results

Dissolution and Dialysis.—For preparative work, 500 g of the material was dissolved in 2 liters of ice-cold water using a gentle mechanical stirring to hasten the process. The solution was then centrifuged in large plastic cups in an International portable refrigerated centrifuge (2300 rpm for 30 minutes), and the sediment was washed twice with 200-ml portions of cold water. The combined solution and washings were then dialyzed for 24 hours against running cold (6–8°) tap water. About 70% by weight of the material was removed at this step with virtually no loss in activity.

Batchwise Treatment with ECTEOLA-cellulose.—ECTEOLA-cellulose removes little of the proteolytic activity from solution, while absorbing some of the proteins and most of the pigments. The results of an experiment to find the optimum amount of ECTEOLA-cellulose are shown in Figure 1. The purification levels off at approximately six. Most of the pigments and little of the activity were removed by 3 g of ECTEOLA-cellulose per g of dialyzed preparation (Fig. 1). Dialysis was essential for the ECTEOLA-cellulose step to be effective.

For preparative work, the dialyzed solution (approximately 3 liters) was stirred mechanically and 300 g of ECTEOLA-cellulose was added. Stirring was continued for 20–30 minutes and the solution was then filtered through a large Büchner funnel, keeping the suction low, so as to prevent foaming. The filter cake was pressed down by hand and was washed twice with 200-ml portions of distilled water. The treatment was repeated with 200 g of ECTEOLA-cellulose and the clear solution, yellow or pale-brown, was then used for ammonium sulfate fractionation.

Regenerated ECTEOLA-cellulose has a lower capacity for adsorbing the pigment and protein than does the fresh material. This was compensated for by using more ECTEOLA-cellulose. Purification was followed by diluting 0.2 ml of the supernatant to 10 ml and measuring its absorbance at 280 mμ. Treatment with ECTEOLA-cellulose was repeated until the absorbance was about one-fourth of that obtained with the untreated solution. Some latitude in the purity attained at this step is admissible, since it has no critical bearing on the purity of the final product. The recovery of activity was in the range of 80–90%.

Ammonium Sulfate Precipitation.—In preliminary experiments protein concentrations between 0.5 and 2% at different *p*H values (3.5–8.0) were tried. Adjustment of the *p*H of the protein solution before ammonium sulfate fractionation produced no improvement in purity. The results of fractional precipitation at different saturations are presented in Table I.

TABLE I
AMMONIUM SULFATE FRACTIONATION OF ASPERGILLOPEPTIDASE B[a]

$(NH_4)_2SO_4$ (% saturation)	Activity Precipitated (% of total)	Purity (units/OD_{280})
0		50
0–75	18	54
75–80	28	149
80–85	34	145
85–100	7	28
100	3[b]	10

[a] Material, "fungal protease" after dialysis and ECTEOLA-cellulose purification; protein, 1.3%; activity, 245 units/ml. [b] Activity in the supernatant.

Little precipitation occurred until the solution reached about 60% saturation. The highest purity was found in the two fractions precipitated between 75 and 85% saturation. In further work these two were collected as a single fraction.

In preparative work the volume of the solution was measured after ECTEOLA-cellulose adsorption and

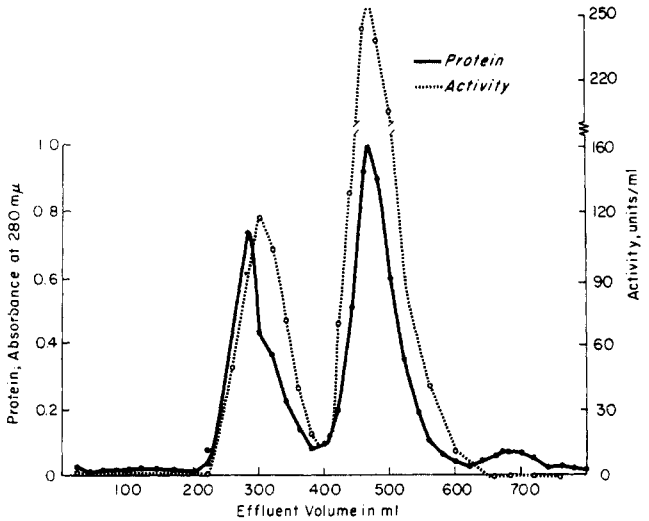

FIG. 3.—Chromatography of aspergillopeptidase B on Amberlite CG-50, *p*H 6.5. A 40 × 4.4-cm column was prepared in 0.01 M sodium phosphate buffer, *p*H 6.5. A 200-mg portion of the partially purified proteinase preparation obtained by gel filtration (the major peak in Fig. 2) was dissolved in 20 ml of the buffer, applied on top of the column, and eluted with the same buffer at the rate of 100 ml/hr. Temperature, 4–6°. Activity recovered, 95%; protein recovered, 95%.

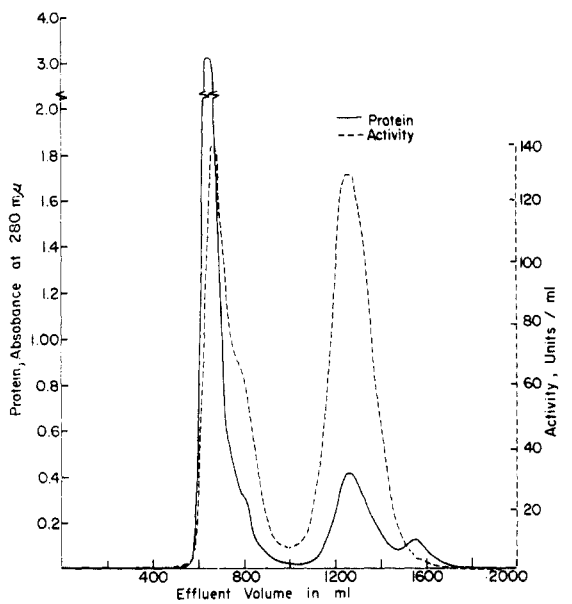

FIG. 4.—Chromatography of aspergillopeptidase B on Amberlite CG-50, *p*H 6.5 (omitting the gel filtration step). A 40 × 6.5-cm preparative column was prepared in 0.01 M sodium phosphate buffer, *p*H 6.5. A 1.5-g portion of partially purified preparation (purified with ECTEOLA-cellulose and ammonium sulfate fractionation) was dissolved in 25 ml of the buffer, applied to the top of the column, and eluted with the same buffer at the rate of 180 ml/hr in the cold room.

the ammonium sulfate required to give 75% saturation (Green and Hughes, 1955) was added slowly to the mechanically stirred solution. The solution was then kept at 0° for 6–10 hours to allow the precipitate to coagulate and settle, and the supernatant was collected by spinning in a Servall refrigerated centrifuge (6,000–10,000 rpm for 10–20 minutes). The precipitation procedure was repeated on the clear supernatant with enough ammonium sulfate to bring the saturation up to 85%. The solid which precipitated between 75 and

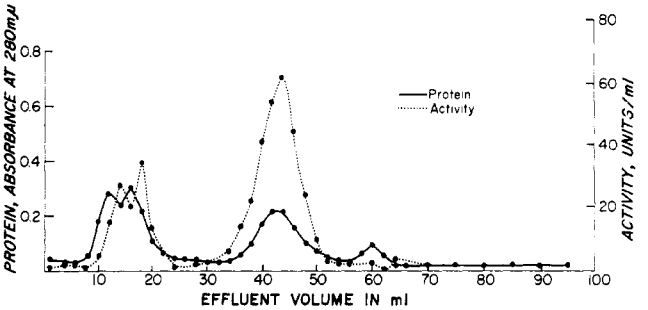

FIG. 5.—Elution pattern of aspergillopeptidase B on CM-cellulose. A 30 × 0.8-cm column, prepared in 0.01 M sodium phosphate buffer, *p*H 6.5, was used. A 5.1-mg portion of proteinase preparation purified by gel filtration was dissolved in 1 ml of the phosphate buffer, applied to the top of the column, and eluted with the same buffer at the rate of 5 ml/hr. Protein recovered, 100%; activity recovered, 56%; temperature 27°.

85% saturation was collected, dissolved in distilled water, dialyzed until free from sulfate, and then lyophilized.

Gel Filtration on Sephadex G-75.—The elution pattern of the ammonium sulfate–fractionated preparation is shown in Figure 2. The enzyme preparation was separated into several peaks, the second containing almost all the proteolytic activity. However, this active peak did not represent a single enzyme; chromatography on Amberlite CG-50 revealed it to be a mixture of at least two proteolytic enzymes and probably some inactive proteins.

In the purification procedure finally adopted, gel filtration has not been included. Chromatography on Amberlite CG-50 gave a material of the same purity whether the enzyme preparation had gone through the Sephadex purification step or not.

Chromatography on Amberlite CG-50, pH 6.5.—Preliminary experiments (carried out on 30 × 0.8-cm columns) demonstrated that no adsorption of the proteins occurs with sodium phosphate buffers of *p*H 6.5 and concentrations lying between 0.2 and 0.025 M. However, 0.01 M sodium phosphate buffer, *p*H 6.5, separated the active preparation (obtained after Sephadex G-75 gel filtration) into two major active peaks and a minor inactive one. A typical elution pattern is shown in Figure 3.

The major alkaline proteinase (hereafter called aspergillopeptidase B) appeared as the second peak. Its distribution coefficient between the adsorbent and the buffer, calculated from the position of emergence, was approximately 2 (Mayer and Tompkins, 1947). The position of emergence of aspergillopeptidase B, however, is very sensitive to changes in phosphate concentration. Increasing the latter above 0.01 M displaced this peak towards the origin, and above 0.025 M it merged with the first protein peak.

The contents of the tubes containing aspergillopeptidase B were pooled, dialyzed, and lyophilized. The purity of this product was the same, whether or not prior purification by gel filtration had been carried out. Figure 4 shows the elution pattern obtained when the column was loaded with ammonium sulfate–fractionated material directly. It appears that most of the proteins that accompanied aspergillopeptidase B through ammonium sulfate fractionation are not adsorbed.

The enzyme obtained from the pooled active peak after dialysis and lyophilization was rechromatographed on the same column. It emerged after the same volume in the same position as a single active peak.

TABLE II
PURIFICATION OF ASPERGILLOPEPTIDASE B[a]

Procedure	Weight (g)	Total Activity (units × 10^4)	Purity (units/OD_{280})	Yield (%)	Purification
None	500	203	5.6	100	1
Dialysis	150	199	8.0	98	1.5
ECTEOLA-cellulose	55	171	39	84	7.0
$(NH_4)_3SO_4$ precipitation (75–85%)	6.5	75	106	37	19
Chromatography on Amberlite CG-50; 0.01 M PO_4, pH 6.5	1.1	30	295	15	53
Chromatography on Amberlite CG-50; 0.01 M PO_4, pH 7.5	0.9	28	310	14	55

[a] The data were obtained from the purification of a 500-g batch of "fungal protease." The purification was carried out in one batch up to the ammonium sulfate fractionation; chromatography was done in a few batches. For definition of activity unit, purity, and purification, see Materials and Methods.

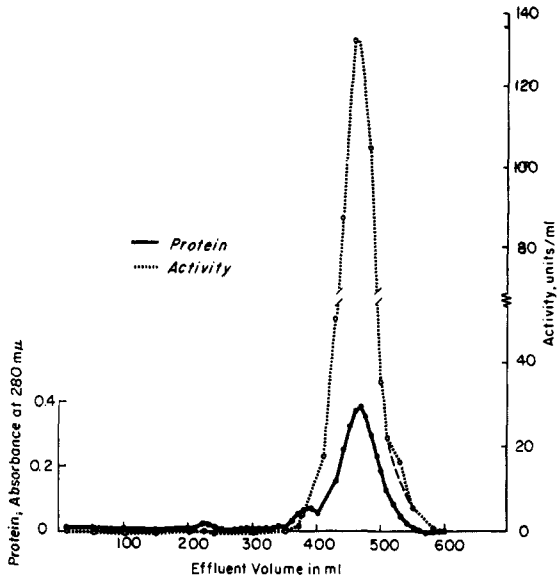

FIG. 6.—Elution pattern of aspergillopeptidase B on cellulose phosphate. A 47 × 3.1-cm column prepared in 0.01 M sodium phosphate buffer, pH 6.5, was used. 38 mg of Aspergillopeptidase B (purified with ECTEOLA-cellulose, $(NH_4)_2SO_4$ fractionation, and Amberlite CG-50, pH 6.5, chromatography), dissolved in 20 ml of the buffer, was applied to the top of the column and eluted with the same buffer at the rate of 60 ml/hr in the cold room at 4–6°.

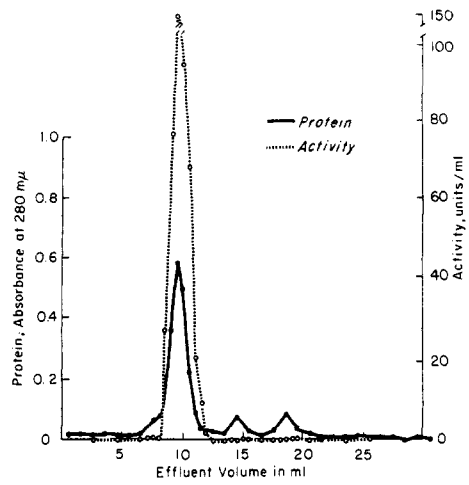

FIG. 7.—Chromatography of aspergillopeptidase B on Amberlite CG-50, pH 7.5. A 30 × 0.8-cm column prepared in 0.01 M sodium phosphate buffer, pH 7.5 was used. Proteinase (5 mg; purified with ECTEOLA-cellulose, $(NH_4)_2SO_4$ fractionation, and Amberlite CG-50, pH 6.5, chromatography) was dissolved in 1 ml of the buffer, applied to the top of the column, and eluted with the same buffer at the rate of 6.6 ml/hr. Activity recovered, 96%; protein recovered, 96%, temperature 4–6°.

Chromatography on CM-Cellulose.—The behavior of the Sephadex-purified preparation on another cation exchanger, CM-cellulose, was examined under conditions which afforded separation on Amberlite CG-50. Figure 5 shows the elution pattern, which appears to be similar to that obtained with Amberlite CG-50. The first peak, however, has begun to resolve into two proteolytic enzymes and further, aspergillopeptidase B emerges somewhat later.

Between the two adsorbents, Amberlite CG-50 was adopted for preparative work because it can stand strong acids and alkalies for regeneration, and it packs easily into columns that give high flow rates.

Chromatography on Cellulose Phosphate.—The purity of aspergillopeptidase B obtained on Amberlite CG-50 chromatography was next examined on another cation exchanger, cellulose phosphate. Figure 6 shows a typical elution pattern. All activity appeared in a single major peak, but two inactive proteins, present in small amounts, were seen to have separated; apparently, these two proteins accompanied aspergillopeptidase B through the last chromatographic step.

It was later found that similar separation of the two minor inactive proteins in the enzyme preparation could also be made on a column of Amberlite CG-50 itself, if the elution was performed at a different pH. Therefore cellulose phosphate was replaced by Amberlite CG-50, since the latter offers, as already mentioned, greater flow rate, ease of handling, and stability.

Chromatography on Amberlite CG-50, pH 7.5.—The elution pattern of the enzyme preparation (purified on Amberlite CG-50, pH 6.5) on Amberlite CG-50 at pH 7.5 is shown in Figure 7. The pattern strikingly resembles the one from cellulose phosphate: all the activity appears in a single major peak, and again two minor inactive proteins are separated. The protein impurities seen in this elution pattern are probably the same as those separated in the cellulose phosphate chromatography, for, when a sample of the cellulose phosphate–purified enzyme preparation was chromatographed on Amberlite CG-50 at pH 7.5, both the minor peaks were absent.

For preparative work, a 50 × 4.4-cm column was used, loaded with 500 mg of the proteinase preparation and eluted at a flow rate of about 125 ml/hr.

Recommended Scheme of Purification and the Purity of the Final Product.—A scheme of purification involving five steps has been devised from the results of the foregoing experiments. The steps are (1) dialysis, (2) batchwise treatment with ECTEOLA-cellulose, (3) fractionation with ammonium sulfate, collecting the fraction precipitated between 75 and 85% saturation, (4) chromatography on Amberlite CG-50, eluting with

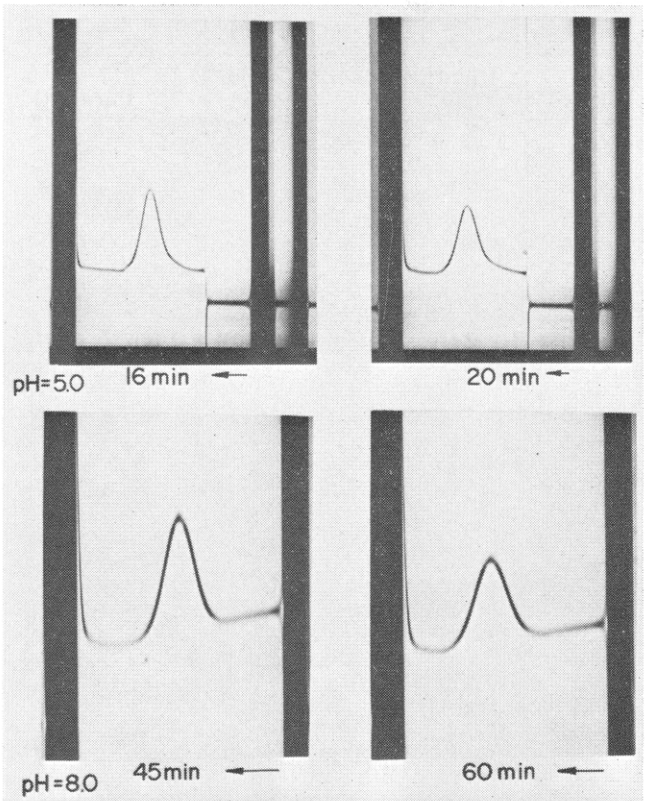

FIG. 8.—Sedimentation pattern of aspergillopeptidase B in the ultracentrifuge. The enzyme solution in a synthetic-boundary cell was spun at 59,780 rpm in a Spinco Model E analytical ultracentrifuge at 20°. The Schlieren patterns are shown at the indicated periods after attainment of full centrifuge speed. The arrows indicate the direction of movement of the boundary. Upper pair: 1% aspergillopeptidase B solution (purified on cellulose phosphate) in 0.1 M sodium acetate buffer (μ 0.07), *p*H 5.02. Lower pair: 1.2% aspergillopeptidase B solution (purified on Amberlite, *p*H 7.5) in 0.02 M sodium barbital–0.18 M NaCl buffer (μ 0.2), *p*H 8.04.

0.01 M sodium phosphate buffer, *p*H 6.5, and (5) chromatography on Amberlite CG-50, eluting with 0.01 M sodium phosphate, *p*H 7.5. The preparations were finally dialyzed in the cold against distilled water for 24–36 hours and then lyophilized.

It is convenient to carry out the purification in 500-g batches, and the details of each step are given for this amount. The amount of water used to dissolve the crude proteinase concentrate has been kept to a minimum to provide as concentrated a solution as possible, thus avoiding time-consuming concentrations between purification steps, and minimizing losses due to surface denaturation.

Table II presents a summary of the results obtained in the purification of a 500-g batch of the material. The final product is 0.9 g of a white fluffy powder with an activity of 310 enzyme units/unit absorbance at 280 mμ, representing a 55- to 60-fold purification over the starting material. (On a weight basis, the activity was 280 enzyme units/mg.) The yield of proteolytic activity was 14%; however, the actual yield is somewhat greater because the crude concentrate is a mixture of several proteinases. Aspergillopeptidase B is estimated to contribute 60–70% of the proteolytic activity of the crude concentrate. On this basis the actual yield rises to 20–23%.

Comparison of Activity with Chymotrypsin and Trypsin.—The activity of bovine chymotrypsin and trypsin in digesting casein was measured under the same assay conditions as used for aspergillopeptidase B in order to compare the relative peptide bond–splitting power of these three enzymes. Aspergillopeptidase B showed the same activity as 3 × crystallized α-chymotrypsin (Mann), and 3–4 times the activity of 2 × crystallized trypsin (Worthington).

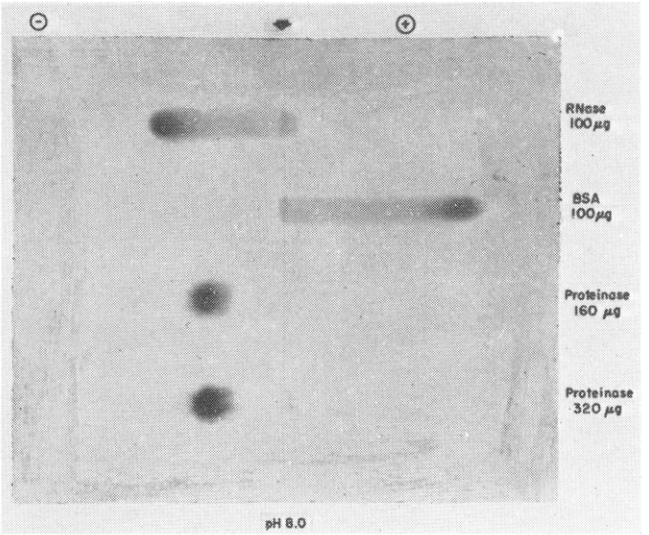

FIG. 9.—Paper-electrophoretic pattern of aspergillopeptidase B at *p*H 8.0. Electrophoresis was carried out on Whatman No. 1 paper at 600 v for 4 hours in 0.1 M Tris buffer and the protein was stained with amido black. The arrow shows the line of application.

Homogeneity of the Purified Enzyme Preparation.—ULTRACENTRIFUGATION.—The sedimentation patterns of solutions of enzyme preparation in two buffers of different *p*H and ionic strength are presented in Figure 8. Enzymes prepared by the two different final steps of purification (Amberlite, *p*H 7.5, and cellulose phospate, *p*H 6.5) were examined. Only one component was apparent at all times during these runs.

PAPER ELECTROPHORESIS.—The electrophoretic patterns in Tris buffer at *p*H 8.0 and in maleate buffer at *p*H 6.0 are presented, respectively, in Figures 9 and 10. For comparison, crystalline bovine serum albumin and crystalline bovine pancreatic ribonuclease were carried through the procedure with the aspergillopeptidase B preparation. The enzyme preparation gives one spot in all buffers examined, namely, at *p*H 5, 6, 7, 8, and 10. For the electrophoretic run shown in Figure 10 a rather large amount of the enzyme (500 μg) was used so that even a minor impurity would have been visible. None was detected.

MOVING-BOUNDARY ELECTROPHORESIS.—Figure 11 presents the results obtained with moving-boundary electrophoresis of the enzyme preparation. The concentrations of the enzyme solutions were reasonably high, resulting in the observed tall electrophoretic peaks which facilitate detection of impurities present as minor components. However, only a single peak was visible in these experiments.

DISCUSSION

Purification Procedure.—The major advantage from the use of ECTEOLA-cellulose is the removal of large amounts of the dark pigments. The color which still remains in solution after ECTEOLA-cellulose treatment is not removed on ammonium sulfate fractionation, but is completely separated from the enzyme during chromatography. The pigments emerge from the column together with the unadsorbed proteins.

Chromatography on Amberlite CG-50 separates

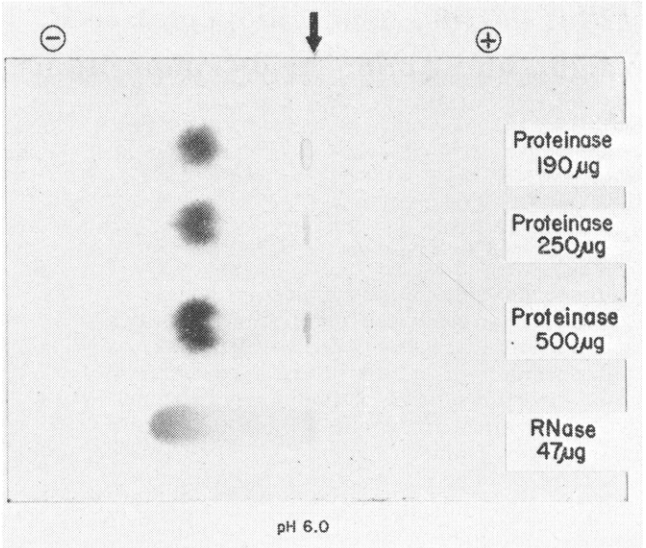

FIG. 10.—Paper-electrophoretic pattern of aspergillopeptidase B at *p*H 6.0. Electrophoresis was carried out on Whatman No. 1 paper at 600 v for 4 hours in 0.05 M maleate buffer and the protein was stained with amido black. The arrow shows the line of application.

aspergillopeptidase B from all other proteolytic activities and almost all the protein impurities. The two minor inactive protein impurities which accompany the enzyme are removed at the next chromatographic step with cellulose phosphate or Amberlite CG-50 at *p*H 7.5.

The columns of Amberlite CG-50 at *p*H 6.5 and 7.5 as well as the cellulose phosphate columns have been used repeatedly without repacking or regeneration. With each run, the elution was continued until no more protein was recorded by the ultraviolet detector-recorder unit. The column was then ready for use again.

The gel filtration experiments, although ineffective in separating the proteolytic activities, provide a tentative notion of the molecular weight of the enzyme. Figure 2 shows that the proteolytic enzymes were considerably retarded by the column; they emerged about 130 ml after the first protein peak appeared (calculated distribution coefficient, 0.3). This indicates a relatively small molecular weight for aspergillopeptidase B.

Homogeneity of the Enzyme Preparation.—Each of the four methods employed to examine homogeneity, namely, ultracentrifugation, paper and moving-boundary electrophoresis, and chromatography gave an unequivocal answer: no inhomogeneity could be detected by any of these methods. The ultracentrifugation and the moving-boundary-electrophoresis experiments were carried out in solutions having reasonably high ionic strength (0.07–0.2) to minimize any anomalous electrostatic effects. In the paper electrophoresis and the moving-boundary electrophoresis experiments, the protein concentrations employed were sufficiently high so that a small percentage of an impurity could have been detected if it were present. None was detected. Furthermore, the enzyme preparation maintains its homogeneous character in buffer solutions of widely different *p*H; at *p*H 5 and 8 on ultracentrifugation, *p*H 5 and 8 on free-boundary electrophoresis, and in the *p*H range 5–10 on paper electrophoresis.

ACKNOWLEDGMENT

We wish to acknowledge the participation of Dr. Leonard Friedman in some of the early stages of this work.

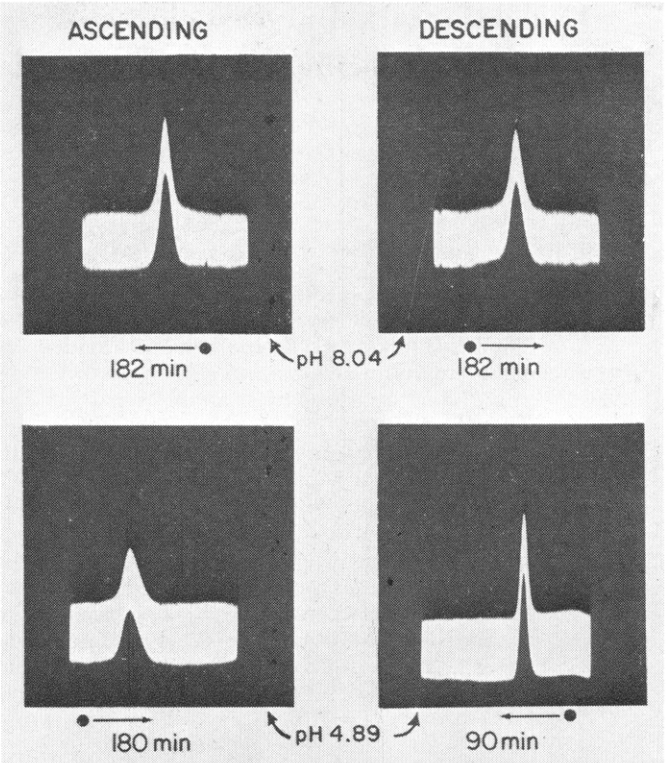

FIG. 11.—Moving-boundary electrophoretic pattern of aspergillopeptidase B at *p*H 8.04 and at *p*H 4.89. Electrophoresis was carried out in a 2-ml Tiselius cell in a Perkin-Elmer apparatus (Model 38) at 0°. Upper pair. 1.2% solution of aspergillopeptidase B in 0.02 M sodium barbital–0.18 M NaCl buffer (μ 0.2). *p*H 8.04. Lower pair. 1% aspergillopeptidase B in 0.02 M sodium acetate–0.08 M NaCl buffer (μ 0.1), *p*H 4.89. Arrows show the direction of movement of boundary, and the dots show its original position.

REFERENCE

Akabori, S., Hagihara, B., Ikenaka, T., and Sakota, N. (1953), *Symp. Enzyme Chem. (Tokyo) 8*, 49.
Bergkvist, R. (1963), *Acta Chem. Scand. 17*, 1541.
Crewther, W. G., and Lennox, F. G. (1950), *Nature 165*, 680.
Crewther, W. G., and Lennox, F. G. (1953), *Australian J. Biol. Sci. 6*, 428.
Dixon, M., and Webb, E. C. (1958), Enzymes, New York, Academic, pp. 35–61.
Feigl, F. (1954), Spot Tests, Vol. I, Oesper, R. E. (transl.), New York, Elsevier, p. 305.
Gomori, G. (1955), *Methods Enzymol. 1*, 138.
Green, A. A., and Hughes, W. L. (1955), *Methods Enzymol. 1*, 67.
Hagihara, B. (1960), *Enzymes 4*, 193.
Hoogerheide, J. C. (1954), *in* Industrial Fermentations, Vol. II, Underkofler, L. A., and Hickey, R. J., eds., New York, Chemical Publishing, p. 141.
Kunitz, M. (1947), *J. Gen. Physiol. 30*, 291.
Laskowsky, M. (1955), *Methods Enzymol. 2*, 19.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Mayer, S. W., and Tompkins, E. R. (1947), *J. Am. Chem. Soc. 69*, 2866.
Miura, Y., and Motonaga, W. (1954), *Ann. Rept. Takamine Lab. 6*, 14.
Mizunuma, T., and Iguchi, N. (1957), *Bull. Agr. Chem. Soc. Japan* (now *Agr. Biol. Chem.* [*Tokyo*]) *22*, 35.
Oshima, K. (1922), *Am. Food J. 17*, 30.
Peterson, E. A., and Sober, H. A. (1956), *J. Am. Chem. Soc. 78*, 751.
Thom, C., and Raper, K. B. (1945), A Manual of the Aspergilli, Baltimore, Williams and Wilkins.
Underkofler, L. A., Barton, R. R., and Rennert, S. S. (1958), *Appl. Microbiol. 6*, 212.
Yoshida, F. (1956), *Bull. Agr. Chem. Soc. Japan* (now *Agr. Biol. Chem.* [*Tokyo*]) *20*, 252.