The Major Alkaline Proteinase of *Aspergillus oryzae*, Aspergillopeptidase B. II. Partial Specific Volume, Molecular Weight, and Amino Acid Composition*

A. R. SUBRAMANIAN AND GEORGE KALNITSKY

From the Department of Biochemistry, State University of Iowa, Iowa City

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The nitrogen content, amino acid composition, partial specific volume, and molecular weight of aspergillopeptidase B, the major alkaline proteinase of *Aspergillus oryzae*, has been determined using an apparently homogeneous enzyme preparation. The nitrogen content, determined on a Coleman nitrogen analyzer, is 16.5%. The amino acids were analyzed on a commercial amino acid analyzer after hydrolysis of the enzyme at 110° with constant-boiling hydrochloric acid for 20, 40, 70, and 110 hours. Tryptophan was determined in an alkali hydrolysate and also spectrophotometrically. Cystine and cysteine were determined as cysteic acid. Amide was determined separately after hydrolysis with 1 N H_2SO_4. The analysis accounted for 98% of the dry weight of the enzyme protein as amino acid residues and 101% of its total nitrogen. Interesting features of the amino acid composition are the preponderance of glycine, alanine, and serine, and the complete absence of sulfur-containing amino acids. The partial specific volume of the enzyme, determined from density measurements of a series of solutions of concentrations between 2.7 and 0.5%, is 0.682 ml/g. The molecular weight, determined by the approach-to-equilibrium method using enzyme solutions of 0.5 and 1% concentration, gives an average value of 18,000. From the molecular weight and the amino acid content, the following amino acid composition has been calculated for the enzyme: Asp_{21} Glu_{12} $Gly_{19}Ala_{23}$ Val_{15} Leu_9 $Ileu_{9-10}$ Ser_{19} Thr_{11} Pro_4 Phe_5 Tyr_5 Try_2 His_4 Lys_{11-12} Arg_2 $(—CONH_2)_{15}$. There are 171–173 amino acid residues per molecule of enzyme, and the molecular weight calculated from the amino acid composition lies between 17,561 and 17,802.

The major alkaline proteinase of the common mold *Aspergillus oryzae* has been crystallized by four groups of workers (Crewther and Lennox, 1950; Akabori *et al.*, 1953; Miura and Motonaga, 1954; Bergkvist, 1963). However, the crystalline preparation was not homogeneous (Crewther and Lennox, 1953), and little is known concerning the physical, chemical, and enzymatic properties of this enzyme. In the preceding paper (Subramanian and Kalnitsky, 1964) a procedure has been described for isolating the major alkaline proteinase of *Aspergillus oryzae* in an apparently homogeneous form. In this paper we report the nitrogen content, partial specific volume, molecular weight, and amino acid composition of this enzyme.

Materials and Methods

Enzyme Preparation.—Aspergillopeptidase B used in these studies was isolated from a commercial enzyme concentrate (Fungal Protease, batch F. 8954, 80,400 hemoglobin units/g, purchased from Miles Chemical Co., Clifton, N. J.) and purified as described in the preceding paper (Subramanian and Kalnitsky, 1964). Briefly, the purification consisted of five steps: dialysis, batchwise treatment with ECTEOLA-cellulose, fractional precipitation with ammonium sulfate (from 75 to 85% saturation), chromatography on Amberlite CG-50 (*p*H 6.5), and rechromatography on either cellulose phosphate (*p*H 6.5) or on Amberlite CG-50 (*p*H 7.5). The enzyme-containing fractions obtained on chromatography were then dialyzed extensively against distilled water and lyophilized. Enzymes made by both the final chromatographic steps were used; cellulose phosphate–purified material was used for nitrogen determination, amino acid analysis, and for one series of measurement of partial specific volume, and the Amberlite-purified (*p*H 7.5) preparation was used in the determination of molecular weight.

Buffer Solution.—For determination of physical properties, the enzyme was dissolved in 0.1 M sodium acetate buffer, *p*H 5.0. This *p*H was chosen because it is high enough to prevent denaturation and yet low enough to keep self-digestion at a minimum. The buffer was prepared by diluting to 1 liter 5.78 g (0.0704 mole) of anhydrous sodium acetate and 28.5 ml (0.0296 mole) of 1.04 N acetic acid (Gomori, 1955). Its actual *p*H, measured on a Radiometer Model 25 *p*H meter, using National Bureau of Standards *p*H 4 buffer, was 5.02.

Determination of Moisture.—The percentage of moisture in the enzyme preparation was determined by weighing the material before and after drying at 100° in an Abderhalden apparatus (using water as the boiling liquid) under vacuum (*ca.* 1 mm Hg) in the presence of a desiccant (Drierite). Approximately 4–6 mg of the enzyme in an aluminum boat was weighed on a Cahn electrobalance (Cahn Instrument Co., Paramount, Calif.). The aluminum boat and its contents were then placed inside a larger porcelain boat, which was pushed into the piston of the Abderhalden apparatus. The enzyme was kept at 100° for varying lengths of time to determine the rate of moisture loss. In the meantime, some desiccant (Drierite) was placed in the balance chamber about 2 hours before the anhydrous protein was weighed. Notwithstanding this precaution, moisture absorption during weighing was noticeable and it was corrected for as follows: A stopwatch was started as the sample was being transferred from desiccator to balance pan. The first weighing was done as quickly as possible, and the weighing was continued at 1-minute intervals, for 5 minutes. The weights were then plotted against time and a smooth line drawn

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through the points was extrapolated to obtain the weight at zero time. A 4-mg sample of the protein gained 10–20 μg during each 1-minute interval.

Determination of Nitrogen.—Nitrogen analysis was carried out on 3 to 4-mg samples of the enzyme, using a Coleman nitrogen analyzer (Model 29, Coleman Instruments, Inc.), an automated instrument employing the micro-Dumas method. The accuracy and reproducibility of the method was checked by carrying out several determinations of the nitrogen content of urea and casein.

Determination of Partial Specific Volume.—The apparent specific volume of the enzyme was obtained from the density of a series of enzyme solutions of known weight concentration determined at constant temperature (Kraemer, 1940; Schachman, 1957). The temperature, measured with a National Bureau of Standards certified thermometer, was kept at 25 ± 0.02° with a Sargent Thermonitor. A specific-gravity bottle of 5-ml capacity, provided with a cap, was used for density determination. It was calibrated with water obtained by redistilling 1 liter of distilled tap water, to which 0.2 g $KMnO_4$ and 0.5 g NaOH (both reagent grade) had been added, and collecting only the middle 400-ml fraction (Bauer and Lewin, 1959). The enzyme solution having the highest concentration was prepared directly from lyophilized material in each series of experiments; other solutions were made stepwise by taking a weighed amount of the solution whose density has been measured, and diluting it to the desired concentration with a weighed amount of the buffer. The weight of the enzyme taken was corrected for its moisture content. For density determination of each solution, the specific-gravity bottle was filled with solution and weighed at least three times. In almost all cases the triplicate weights were within 0.1 mg of the average. When they differed by more than 0.1 mg, the experiment was repeated until three weights differing no more than 0.1 mg from the average were obtained.

Determination of Molecular Weight by Approach-to-Equilibrium Method.—The molecular weight of the enzyme was determined by the approach-to-sedimentation-equilibrium method (Archibald, 1947; Schachman, 1957) using a Spinco Model E ultracentrifuge at 12,590 rpm. The solutions used contained 1 and 0.5% enzyme in 0.1 M sodium acetate buffer, *p*H 5.0 The procedure and the method of calculation described at length by Schachman (1957) was used with the following modifications: (1) The distances on the plate were measured with a two-dimensional traveling microscope graduated in 0.001-cm divisions (W. G. Pye and Co., Ltd., England). (2) The initial concentration, c_o, was obtained from the area of the peak (obtained with the synthetic-boundary cell) calculated by summing up the heights of the peak measured at 0.025-cm intervals. Calculations of the area, made in this way from several exposures in a single run, agreed within 1% with one another. (3) Direct measurement of the concentration gradient at the meniscus, $(dc/dx)m$, presented some uncertainty, because it was difficult to judge exactly where the curvature met the meniscus. This was satisfactorily solved by a graphical procedure: The dc/dx values were measured at 0.01-cm intervals beginning from the plateau region up to as close to the meniscus as possible; these values were then plotted on a graph paper and a smooth line drawn through the points was extrapolated to obtain the dc/dx value at the meniscus. The measured dc/dx values were also used in correcting for the small concentration depletion at the meniscus caused by the centrifugal field (Klainer and Kegeles, 1955). This procedure was checked by determining the molecular weight of bovine pancreatic ribonuclease A. Three experiments gave values of 15,070, 14,950, and 14,540; the molecular weight calculated from its amino acid composition is 13,683 (Hirs *et al.*, 1956).

Analysis of Amino Acids.—After hydrolysis of the enzyme with acid or alkali, the amino acids in the hydrolysate were analyzed in a Phoenix amino acid analyzer (serial K 5000-13), using the procedure of Spackman *et al.*, (1958). The reproducibility of the instrument was checked by three analyses of a standard amino acid mixture supplied by the instrument manufacturer. The integration constants (*C* values) for each amino acid obtained from these analyses remained fairly constant; the variation was 1.5% or less for 10 amino acids, and 2% or below for all, except for glycine (3%), cystine (4%), and arginine (5%). The baseline remained stable in these and in the subsequent analyses, which were all carried out with one batch of ninhydrin-reagent solution. Standard solutions (about 1 μmole/ml) of cysteic acid and tryptophan in 0.2 M sodium citrate buffer, *p*H 2.2, were prepared from chromatographically pure materials dried to constant weight *in vacuo* at 100°.

Acid Hydrolysis.—The enzyme was hydrolyzed with constant-boiling hydrochloric acid at 110° for 20, 40, 70, and 110 hours. Four to 8 mg of the enzyme was weighed directly into Pyrex ampules, and 5 ml of the HCl was added to each. The ampules were cooled in an acetone–dry ice mixture to freeze their contents and were then sealed after evacuation (*ca.* 1 mm Hg). They were then kept for varying periods in an Abderhalden apparatus heated by vapors from boiling toluene. After the ampules were removed their contents were evaporated to dryness at room temperature in a vacuum desiccator containing NaOH pellets. The residue was washed down the sides of the ampules with distilled water once, and evaporated to dryness again. Finally the residue was transferred quantitatively to a 5-ml volumetric flask using 0.2 M sodium citrate buffer, *p*H 2.2. The solution was then diluted to the mark with the same buffer and, after filtering through a tiny plastic funnel plugged with Pyrex wool, aliquots were analyzed for amino acids.

Alkali Hydrolysis.—For the analysis of tryptophan, the enzyme was hydrolyzed with 3.7 N barium hydroxide at 100° for 12 hours (Warner, 1942). Approximately 5 mg of the enzyme was weighed directly into a Pyrex ampule, and enough solid $Ba(OH)_2$ was added to yield 2.5 ml of a 3.7 N solution. Distilled water was then added up to a previously made 2.5-ml mark. After evacuation and sealing, the ampule was maintained at 100° in an Abderhalden apparatus (boiling water). The $Ba(OH)_2$ entered solution at the elevated temperature. After 12 hours the contents of the ampule was transferred (with distilled water) into a 100-ml centrifuge tube. CO_2 was passed through the solution to precipitate barium, and the solution was then centrifuged. The supernatant was filtered through a quantitative filter paper (to remove a few floating $BaCO_3$ particles) and evaporated to dryness over a steam bath, and the residue was then transferred quantitatively to a 5-ml volumetric flask using the 0.2 M citrate buffer. After dilution of the solution to the mark with the same buffer, aliquots were analyzed on the short column of the amino acid analyzer.

Performic Acid Oxidation and Analysis of Cysteic Acid.—For separate determinations of cysteine and cystine as cysteic acid, the enzyme was oxidized with performic acid at 0° for 5 hours (Schram *et al.*, 1954), the solution was evaporated in a rotary evaporator at 40°, and the residue then was refluxed with constant-boiling HCl for 20 hours. After evaporation of the hy-

drolysate on a rotary evaporator at 60°, the dry residue was transferred quantitatively to a 5-ml volumetric flask with the citrate buffer and diluted to the mark with the same buffer, and aliquots were analyzed for cysteic acid on the long column of the amino acid analyzer.

Amide Analysis.—The amide-ammonia content was separately obtained by hydrolyzing the enzyme with 1 N H_2SO_4 at 100° and estimating the ammonia formed by the ninhydrin method (Laki *et al.*, 1954). One-mg samples of the enzyme, weighed on the Cahn electrobalance, were dissolved in 1 ml of 1 N H_2SO_4, and heated (well-stoppered) in a boiling-water bath for 4, 8, and 20 hours. After removal of the solutions from the bath 200-μl aliquots (in quadruplicate) were placed in the outer chambers of Conway microdiffusion cells, and the ammonia, set free by 1 ml of 10% NaOH, was allowed to diffuse overnight (10–12 hours) into 0.2 ml of 0.1 N H_2SO_4 contained in the central well. The contents of the central well were then transferred quantitatively (using four 0.2-ml portions of distilled water) into test tubes, and the NH_3 was estimated by the ninhydrin method of Rosen (1957), measuring the absorbance at 570 mμ (in 1-cm cells) on a Beckman DU spectrophotometer. A B-D Cornwall continuous-pipetting device was used to rapidly dispense the 5-ml portions of the diluent (isopropyl alcohol–water, 1:1) into the raction tubes. The ninhydrin method was standardized using a solution of ammonium sulfate (1 mM) prepared from analytical reagent grade material. Blanks (0.2 ml of 1 N H_2SO_4) and standards (0.2 ml of 1 mM $(NH_4)_2SO_4$) were carried through the diffusion step along with the experimental determinations.

Spectrophotometric Estimation of Tyrosine and Tryptophan.—These estimations were made from the absorption spectrum of the enzyme in 0.1 N NaOH obtained (with 1-cm cells) on a Cary recording spectrophotometer (Model 14 PM). Two different methods of calculation were used. One was the method of mixtures of Goodwin and Morton (1946), which yields the mole fraction of tyrosine and tryptophan from the optical density values at the two wavelengths of 294.4 mμ and 280 mμ (Beaven and Holiday, 1952). The other was the method of Bencze and Schmid (1957), which enables one to obtain the total tyrosine and tryptophan content and their ratio from the slope of a tangent to the two absorption maxima (one due to tryptophan and the other due to tyrosine), and the optical density of the larger maximum.

Results

Moisture and Ash Content.—The per cent weight of the enzyme, after different periods of drying, is shown in Table I. Moisture appears to be driven off completely by 2 hours' drying at 100° *in vacuo;* no further loss of moisture was observed for about 11 hours under these conditions of drying. Further, within a period of 20 weeks, the moisture content of the preparation remained fairly constant; the enzyme was stored refrigerated in a tightly stoppered vial, kept in a desiccator containing Drierite.

The ash content of the preparation was not determined, since an accurate estimation would require an unreasonably large amount of material. However, it is unlikely to be more than a very small percentage for the following reasons: (1) the enzyme preparation had been dialyzed extensively; (2) spectrographic examination of the preparation revealed no metals, except traces of iron and calcium; and (3) analysis for inorganic phosphate (used in the last two steps of the purification procedure) by the method of Fiske and Subbarow (Hawk *et al.*, 1954) showed that the enzyme preparation contained 0.2% phosphorus (or 0.58% as phosphate). The ash content is unlikely to be much larger than the phosphate content, since the latter represents the major inorganic anion.

Table I
Per Cent Weight of the Enzyme Preparation after Different Periods of Drying[a]

Time of Drying at 100° (hr)	Per Cent of Weight
2	93.8
4.4	93.7
6.4	93.6
8.5	93.8
10.7	94.1
Average	93.8 ± 0.3
2[b]	93.4

[a] The protein was dried *in vacuo* (*ca.* 1 mm Hg) at 100° [b] Dry weight of a sample of the same enzyme preparation determined 20 weeks later.

The moist enzyme was used for all the analyses described below, and the weight of anhydrous enzyme was calculated by correcting for the moisture content. A correction for the phosphate content was also made in the nitrogen and amino acid analyses.

Per Cent of Nitrogen.—Three determinations yielded 16.31, 16.56, and 16.56%, giving an average value of 16.5% nitrogen in aspergillopeptidase B.

Partial Specific Volume.—Table II presents the results of the determination of partial specific volume. The apparent partial specific volume remains constant within the precision of the experimental method in the concentration range 0.5–2.7% of enzyme. The partial specific volume of the enzyme obtained from the average of eight determinations is 0.682 ml/g.

Table II
Partial Specific Volume of Aspergillopeptidase B

Expt	Protein Concentration (g/100 g soln) n	Density (g/ml) d	Apparent Partial Specific Volume[a] (ml/g)
I[b]	0	1.00034	
	0.471	1.00180	0.690
	0.697	1.00258	0.679
	1.365	1.00472	0.680
	1.783	1.00610	0.679
	2.256	1.00756	0.682
	2.701	1.00884	0.688
II[c]	0	1.00031	
	0.990	1.00334	0.664[d]
	1.463	1.00480	0.673
	1.879	1.00592	0.687
		Average	0.682

[a] $V_{app} = \frac{100/d - (100 - n)/do}{n}$, where V_{app} = apparent partial specific volume, d = density of protein solution, do = density of solvent buffer, n = percentage of solute, g/100 g soln. [b] Enzyme, finally purified on Amberlite CG-50, *p*H 7.5. [c] Enzyme, finally purified on cellulose phosphate. [d] Disregarded in calculating the average.

After determination of the amino acid composition of the enzyme, the partial specific volume was calculated from the known specific volumes of the constituent amino acids, using the data provided by Cohn and Edsall (1943). The calculation yielded a value of 0.72 ml/g.

Molecular Weight.—Table III presents the results of molecular-weight determinations. The average value of 12 measurements is 18,000. In one experiment, run no. 545, the measurements were continued over an extended period to examine any change in molecular weight with time, which could result if the sample were heterogeneous. The molecular weight, however, remained constant, within the reproducibility of the method.

TABLE III
MOLECULAR WEIGHT OF ASPERGILLOPEPTIDASE B[a]

Run No. and Protein Concn	Time after Attaining Speed (min)	Mol Wt
545; 1%	12	16,480
	28	17,460
	44	17,990
	60	18,060
	76	18,250
475; 1%	9	18,330
	18	17,110
	26	16,880
536; 1%	15	17,460
	31	18,550
538; 0.5%	22	19,010
	30	20,070
		Average 17,970

[a] All centrifugal runs were made at 21° in a Spinco Model E ultracentrifuge at 12,590 rpm, with protein solutions made up in 0.1 M sodium acetate buffer, *p*H 5.02.

Amino Acids in the Acid Hydrolysate.—The amino acid composition of the hydrolysate obtained after 20, 40, 70, and 110 hours of acid hydrolysis is shown in Table IV. The values of isoleucine and valine increase continuously with time. Most of the isoleucine is released by 70 hours, but surprisingly, the upward trend of valine does not appear to level off even at 110 hours. Tryptophan is completely destroyed. Serine and threonine, and tyrosine to a smaller extent, are progressively decomposed, while the amount of NH_3 increases with time.

TABLE IV
AMINO ACID COMPOSITION OF HYDROLYSATES OF ASPERGILLOPEPTIDASE B[a]

Amino Acid	Grams of Amino Acid/100 g Anhydrous Protein			
	20 hr	40 hr	70 hr	110 hr
Aspartic acid	15.32	15.50	14.73	15.10
Glutamic acid	9.75	9.40	9.67	9.80
Glycine	8.00	7.72	7.95	7.99
Alanine	11.62	11.37	11.23	11.37
Valine	7.86	8.44	9.06	9.66
Leucine	6.63	6.49	6.56	6.60
Isoleucine	6.36	6.57	6.83	6.97
Serine	10.46	10.10	9.24	8.09
Threonine	7.20	6.91	6.52	6.27
Half cystine	0	0	0	0
Methionine[b]	0.36	0.29	0.21	0.22
Proline	2.61	2.44	2.52	2.65
Phenylalanine	4.34	4.26	4.41	4.46
Tyrosine	5.24	5.11	5.22	4.75
Histidine	3.08	3.25	3.38	3.23
Lysine	9.23	9.57	9.44	9.32
Arginine	2.12	2.30	2.47	2.25
Ammonia	1.56	1.80	1.97	2.27

[a] Hydrolyses were carried out with constant-boiling hydrochloric acid at 110° for the indicated periods. [b] Not corrected for the approximately 10% loss found to occur during chromatography (Moore and Stein, 1954).

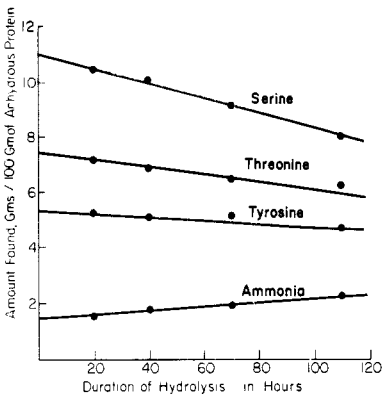

FIG. 1.—Extrapolation of serine, threonine, tyrosine, and NH_3 content to zero time of hydrolysis. The straight lines are the best-fit lines calculated by the method of least squares.

The amount of methionine is small, its optical density measuring 0.01–0.02 at the peaks. The significance of such a small optical density value is rendered rather uncertain by the slight change in baseline which occurs with the emergence, just before methionine, of the second buffer of the analyzer. Therefore the value for methionine shown in the table might be rather too high. The rest of the amino acids are all completely released by 20 hours and are stable under the hydrolytic conditions.

Figure 1 shows the amounts of serine, threonine, tyrosine, and NH_3 liberated, plotted against the time of hydrolysis. The straight lines are the best-fit lines calculated by the method of least squares. The concentrations of these three amino acids and ammonia at zero time of hydrolysis was obtained in this fashion. The increase in NH_3 during hydrolysis of proteins has been shown to arise from the decomposition of the labile amino acids (Rees, 1946).

No cystine appeared on the chromatogram of any of the acid hydrolysates. However, cystine is known to be labile under conditions of acid hydrolysis, and any cysteine remaining unoxidized would go unnoticed, since it would emerge with proline. Analysis of these two residues as cysteic acid is known to be free from these pitfalls. The analysis of the acid hydrolysate of the performic acid–oxidized enzyme, however, confirmed the complete absence of cystine or cysteine in the enzyme; no cysteic acid peak appeared in the chromatogram. About 0.1 residue of cysteic acid per mole of enzyme could have been estimated easily.

Amide Content.—The results of the amide estimation are shown in Table V. The enzyme dissolves in the acid with difficulty and a few shiny particles could be seen even after 4 hours of heating at 100°. This was why some samples were hydrolyzed for longer periods. The gradual increase in NH_3 observed with longer periods of hydrolysis probably results from the decomposition of labile amino acids (Hamilton, 1960). Extrapolation of 8-hour and 20-hour data to zero time yields 1.45 g of NH_3 per 100 g of enzyme. Interestingly, hydrolysis for 4 hours gave the same result. The results from the acid hydrolysates, determined on the amino acid analyzer and corrected to zero time, agree well with the direct estimation.

Tryptophan and Tyrosine Content.—The ultraviolet-absorption spectra of the enzyme in 0.1 M NaOH, 0.1 M HCl, and 0.1 M sodium acetate buffer, *p*H 5, are presented in Figure 2. The absorption spectrum in

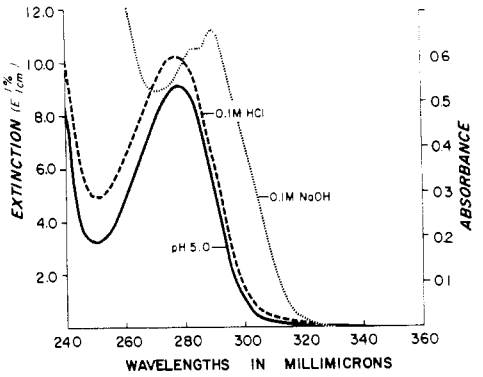

FIG. 2.—The ultraviolet-absorption spectra of aspergillopeptidase B. At *p*H 5.02 in 0.1 M sodium acetate buffer, ———; in 0.1 M HCl, ---------; in 0.1 M NaOH, · · · · · The data from this spectrum were used to calculate tyrosine and tryptophan contents. The protein concentration was kept the same (0.59 mg/ml) in the three solutions. Measurements were carried out on a Cary recording spectrophotometer.

TABLE V
AMIDE-NH_3 CONTENT OF ASPERGILLOPEPTIDASE B[a]

Procedure	Time (hr)	NH_3 Found (g/100 g dry protein)
Hydrolysis with 1 N H_2SO_4 at 100°	4	1.45
	8	1.50
	20	1.58
Extrapolated to zero time from 8- and 20-hr data	0	1.45
Hydrolysis with 6 N HCl at 110° corrected to zero time		1.48

[a] After hydrolysis with 1 N H_2SO_4, NH_3 was separated by micro-Conway diffusion and estimated spectrophotometrically at 570 mμ after reaction with ninhydrin reagent. The hydrolysates with constant-boiling HCl were analyzed on the amino acid analyzer.

TABLE VI
TRYPTOPHAN AND TYROSINE CONTENT OF ASPERGILLOPEPTIDASE B

Procedure	Tyrosine (g/100 g dry protein)	Tryptophan (g/100 g dry protein)
Absorption in 0.1 M NaOH at 2944 and 2800 A	5.2	2.1
Slope of tangent to absorption maxima in 0.1 M NaOH	5.1	1.9
Hydrolysis with 6 N HCl after correction for decomposition	5.3	
Hydrolysis with 3.7 N $Ba(OH)_2$		1.2

alkali distinctly reveals the presence of tyrosine and tryptophan; the maximum at 290 mμ is due to ionized tyrosine and that at 283 mμ is due to tryptophan. The native enzyme (*p*H 5) displays a single absorption band having a maximum at 278 mμ. The $E_{1\,cm}^{1\%}$ value of the native enzyme at the wavelength of maximum absorption is 9.08; at 280 mμ, 9.0.

Table VI shows the tryptophan and tyrosine contents obtained from the absorption spectrum. Also shown in that table are the tryptophan content from the alkali hydrolysate and the corrected tyrosine content from the acid hydrolysates. The two physical methods give nearly the same results, which in the case of tyrosine is in good agreement with the results obtained from acid hydrolysis. The tryptophan content obtained by alkali hydrolysis, however, is considerably less than that obtained with the physical methods on the intact protein. The reason for this discrepancy has not been investigated. The results from alkali hydrolysis, nonetheless, serve to confirm by chemical identification the presence of tryptophan in the enzyme.

Amino Acid Composition.—The amino acid composition of aspergillopeptidase B obtained from the results of the foregoing analyses is presented in Table VII. For most of the stable amino acids, the values represent the average of four determinations. Valine and isoleucine are calculated on the basis of the results after 110 hours' hydrolysis. The values of serine, threonine, and tyrosine are the extrapolated figures at zero time of hydrolysis. Tryptophan is represented by the value obtained spectrophotometrically, according to the procedure of Goodwin and Morton (1946). The amide-NH_3 is represented by the direct estimation after hydrolysis with 1 N H_2SO_4.

The third and fourth columns in Table VII present, respectively, the grams of amino acid residues per 100 g of enzyme and the nitrogen content contributed by each amino acid as the percentage of the total nitrogen. The analytical results for the amino acid residues account for 98.1% of the dry weight of the enzyme taken for hydrolysis, and 101% of its nitrogen content. The satisfactory accounting of the weight of the enzyme by amino acid residues confirms the belief that the ash content of the enzyme preparation was negligible.

Column 5 shows the number of amino acid residues in 18,000 g of enzyme. An inspection of the figures reveals that, with two exceptions, all are integers or very close to integers. The exceptions are isoleucine and lysine. Isoleucyl peptides are, it is known, rather resistant to acid hydrolysis (Light and Smith, 1963). The results of the amino acid analysis in Table IV bear out this general observation; small amounts of isoleucine are released continuously over the entire 110-hour period of hydrolysis. For lysine, the values from the individual analyses are 11.4, 11.8, 11.6, and 11.5, making the assignment of a number uncertain. Thus, there appear to be 171–173 amino acid residues per molecule of the enzyme. The molecular weight, calculated from the integral number of residues, taking into account terminal water and amide groups, lies between 17,561 and 17,802.

DISCUSSION

The experimentally determined partial specific volume of aspergillopeptidase B is relatively low; for many proteins the partial specific volume lies between 0.70 and 0.75 (Edsall, 1953). A small amount of carbohydrate (1–2%, equivalent of mannose) has been persistently found to be present in the different batches of the purified enzyme. This amount, however, is too small to affect the partial specific volume significantly. The calculated partial specific volume remains somewhat higher than the experimental value.

Among the amino acids comprising the aspergillopeptidase B, alanine, aspartic acid (or asparagine), serine, and glycine account for 23, 21, 19, and 19 residues, respectively, per molecule of the enzyme. Omitting aspartic acid for the moment (which would be present as aspartic acid and as asparagine), the three other amino acids represent 35% of the total number of residues. On the other hand, proline, phenylalanine, tyrosine, tryptophan, histidine, and arginine are each represented by a few residues; together they account for only 13% of the total residues.

The amino acid composition presents an additional point of interest. The absence of the sulfur-containing amino acids cystine, cysteine, and methionine is par-

TABLE VII
AMINO ACID COMPOSITION OF ASPERGILLOPEPTIDASE B[a]

Amino Acid	Amino Acid (g/100 g protein)	Amino Acid Residues (g/100 g protein)	N Content[b] (%)	Residues in 18,000 g[c]	Residues to Nearest Integer
Aspartic acid[d]	15.31	13.24	9.67	20.7	21
Glutamic acid	9.66	8.48	5.57	11.8	12
Glycine	7.92	6.02	8.96	19.0	19
Alanine	11.40	9.09	10.87	23.0	23
Valine[e]	9.66	8.17	6.99	14.8	15
Leucine	6.57	5.67	4.25	9.0	9
Isoleucine[e]	6.97	6.01	4.51	9.6	9–10
Serine[f]	11.08	9.18	8.95	19.0	19
Threonine[f]	7.46	6.33	5.32	11.3	11
Half Cystine[g]	0	0	0	0	0
Methionine	0.27	0.24	0.15	0.3	0
Proline	2.56	2.16	1.88	4.0	4
Phenylalanine	4.37	3.89	2.25	4.8	5
Tyrosine[f]	5.34	4.81	2.50	5.3	5
Tryptophan[h]	2.14	1.95	1.62	1.9	2
Histidine	3.24	2.86	5.32	3.8	4
Lysine	9.39	8.24	10.91	11.6	11–12
Arginine[d]	2.22	1.99	4.32	2.3	2
Amide-NH_3[i]	1.45		7.23	15.3	15[j]
Total		98.1	101.3		171–173[k]

[a] Obtained from the data in Tables IV, V, and VI. [b] Nitrogen content as percentage, calculated on the basis of 16.5% nitrogen in the anhydrous protein. [c] The molecular weight of the enzyme by the approach to equilibrium method. [d] Value for 70 hr. hydrolysis disregarded because it departed from the mean more than other values. [e] The value at 110 hr. hydrolysis. [f] Extrapolated to zero time of hydrolysis. [g] Absence confirmed by analysis of cysteic acid in a performic acid-oxidized sample. [h] From absorption at 294.4 mμ and 280 mμ. [i] From separate estimation after hydrolysis with 1 N H_2SO_4. [j] Not counted in adding the number of residues. [k] Molecular weight obtained from integral number of residues taking into account terminal water and the amide groups = 17,561–17,802.

ticularly striking. The absence of cystine has been noticed in many bacterial exoproteins. Pollock and Richmond (1962) report that cystine was absent in ten bacterial exocellular proteins they examined. It has been suggested that this might be a feature of exoenzymes of bacteria and other microorganisms which are obligated to travel from their site of synthesis in the interior of the cells to the outer medium through the rigid cell wall. The hypha of *Aspergillus oryzae* has a surrounding cell wall structure.

In many proteins one or more disulfide bonds, prosthetic groups, or both act as guiding agents which direct the feeble side-chain interactions to engender the native molecule (Boyer, 1959). The complete absence of disulfide bonds in this enzyme indicates the importance of the relatively weak side-chain interactions (and possibly of the carbohydrate present) in maintaining the three-dimensional structure of the native molecule. The absence of disulfide bonds should therefore make this enzyme of interest in thermodynamic studies of factors conferring stability to the native protein structure and on the mechanism of denaturation.

The molecular weight of 18,000 places aspergillopeptidase B among the smallest proteolytic enzymes. To be sure, the well-characterized proteolytic enzymes (with the possible exception of leucine aminopeptidase) form a group that has low molecular weights lying within a narrow range (20,000–40,000) (Subramanian, 1964). The low molecular weight of aspergillopeptidase B coupled with the absence of any disulfide bond in the molecule would probably make the determination of its sequence somewhat less arduous than that of bovine trypsin (Walsh *et al.*, 1964) or bovine α-chymotrypsin A (Hartley, 1964), which have been announced recently.

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Metallocarboxypeptidase-Inhibitor Complexes*

JOSEPH E. COLEMAN AND BERT L. VALLEE

From the Biophysics Research Laboratory, Department of Biological Chemistry, Harvard Medical School and the Division of Medical Biology, Department of Medicine, Peter Bent Brigham Hospital, Boston, Mass.

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Previous gel-filtration and metal-ion-exchange studies have shown that apocarboxypeptidase A forms stable complexes with peptide substrates. Ester substrates, in contrast, form complexes only with the metalloenzyme. The present studies with [^{14}C]-β-phenylpropionate confirm the conclusions drawn from experiments employing gel-filtration and metal-ion exchange that a number of competitive inhibitors similarly form complexes only with the metalloenzyme. One mole of [^{14}C]-β-phenylpropionate binds to zinc carboxypeptidase when the concentration of this inhibitor is 10^{-3} M, while under the same conditions none binds to apocarboxypeptidase. Binding of the inhibitor increases the half-life for the exchange of $^{65}Zn^{2+} \rightleftharpoons Zn^{2+}$ at the active site from 5 to 576 hours. The stability of metalloenzyme-inhibitor complexes can be measured by their effects on metal-ion exchange. The stabilities are functions both of the species of metal ion at the active site and of the chemical structure of the inhibitors. Inhibitors bind progressively firmer in the order $Co^{2+} < Cd^{2+} < Zn^{2+}$. Substitutions of R groups in the basic acetate structure of inhibitors result in progressively greater affinities for the active site in the order $CH_3 <$ Br < I < phenyl < indole < benzyl. The obligatory stereospecificity for the C-terminal amino acid of the substrate is L; that required for binding these inhibitors is D. A model for inhibitor binding is proposed.

The effect of substrates on the association and dissociation of metal ions at the active site[1] of carboxypeptidase A[2] has provided a means of separating the catalytic event from substrate binding and has given insight into the chemical details of the latter process. Peptide substrates form stable apocarboxypeptidase-substrate complexes and thus do not require the metal ion for binding. In contrast, the metal is mandatory for the binding of ester substrates (Coleman and Vallee, 1962a,b).

These studies have now been extended to encompass the interaction of competitive inhibitors of carboxypeptidase including D-phenylalanine, D-tryptophan, D-leucine, iodoacetate, bromoacetate, phenylacetate, indole-3-acetate, propionate, β-iodopropionate, and β-phenylpropionate. [^{14}C]-β-Phenylpropionate has been employed to confirm directly the results obtained by the metal-ion-exchange methods. A preliminary account has been rendered (Coleman, 1963).

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[1] The designation "active site" will refer specifically to the nitrogen-metal-sulfur bond essential for hydrolysis. "Active center" will refer to all those features of primary, secondary, and tertiary structure of the enzyme, including the "active site," which are required for substrate binding, specificity, or hydrolysis of the substrate.

[2] In this paper "carboxypeptidase" refers to carboxypeptidase A only. All the experiments were carried out with δ-carboxypeptidase (for nomenclature see Bargetzi *et al.*, 1963) prepared from acetone powder of beef pancreas (Allan *et al.*, 1964) and containing N-terminal asparagine.

Experimental Procedures

Bovine Pancreatic Carboxypeptidase A [(CPD)Zn].[3]—Four-times-recrystallized δ-carboxypeptidase was prepared by the method of Allan *et al.* (1964). The ratio zinc/protein of the preparation was between 0.98 and 1.03 g-atoms/mole based on a molecular weight of 34,300 (Smith and Stockell, 1954; Vallee and Neurath, 1955; Brown *et al.*, 1961).

Apocarboxypeptidase, cadmium carboxypeptidase and cobalt carboxypeptidase were prepared as described (Coleman and Vallee, 1961; 1962a). *The inhibitors* D-phenylalanine, D-leucine, D-tryptophan, indole-3-acetic acid, *N*-acetylhistidine, *N*-acetyl-D-phenylalanine (Mann Research Laboratories) and β-phenylpropionic acid–[^{14}C] carboxyl (California Corp. for Biochemical Research) were chromatographically pure. The iodoacetic, β-iodopropionic, bromoacetic, propionic, and β-phenylpropionic acids (Eastman Organic Chemicals), iodoacetamide (K and K Laboratories), and phenyl-

[3] The abbreviations used are in formulations only and when required for differentiation: [(CPD)Zn], zinc carboxypeptidase, with (CPD) representing the apoenzyme and the brackets indicating the firm binding of zinc or other metals substituting for it; CGP, carbobenzoxyglycyl-L-phenylalanine; HPLA, hippuryl-DL-β-phenyllactic acid; Tris, tris(hydroxymethyl)aminomethane; β-ϕ-P, β-phenylpropionate,