Purification and Properties of the Amylase of *Bacillus macerans**

John A. DePinto† and L. Leon Campbell

ABSTRACT: The amylase (α-1,4-glucan 4-glycosyltransferase, cyclizing, EC 2.4.1.19) of *Bacillus macerans* was purified 140-fold and shown to be a single, homogeneous protein by disc electrophoresis and by ultracentrifugation. The molecular weight as calculated from sedimentation and diffusion data is 139,300. The protein has a partial specific volume of 0.712 ml/g, a frictional ratio of 1.79, an extinction coefficient of 9.9×10^4 at 280 mμ, a K_m of 3.33×10^{-3} g/ml on soluble starch, and a pH optimum of 6.1–6.2. Amino acid analysis showed no cystine. The energy of activation between 25 and 55° is 12,250 cal/mole. The enzyme produces cyclodextrins from starch and starch derivatives.

The amylase of *Bacillus macerans* (BMA)[1] differs from other amylases by its ability to form Schardinger dextrins from starch and starchlike substrates (French, 1957). Schardinger dextrins are cycloamylose molecules ranging in size from the α-dextrin, containing six glucosyl residues, to twelve glucosyl ring structures and beyond (French, 1957; French *et al.*, 1965). The existence of branched Schardinger dextrins has been reported by French *et al.* (1965). Although the properties of Schardinger dextrins have been extensively studied, almost all of the studies on BMA have been performed with crude enzyme preparations (French *et al.*, 1954). Schwimmer and Garibaldi purified BMA approximately 60-fold, but lack of sufficient material limited their studies (Schwimmer and Garibaldi, 1952; Schwimmer, 1953). The fact that the organism had to be grown in an oatmeal–$CaCO_3$ medium for enzyme production and generally required a 1-week or longer incubation period[2] before the enzyme appeared in the culture filtrate (Tilden and Hudson, 1942) may have been one of the reasons for the few attempts to purify the enzyme.

The purification of the enzyme became feasible with our demonstration that BMA can be produced in a starch–casein hydrolysate–yeast extract medium and that it was an intracellular rather than an extracellular enzyme (DePinto and Campbell, 1964). It became important to reinvestigate the action patterns of BMA since we also found another intracellular enzyme in *B. macerans* which can degrade Schardinger dextrins (DePinto and Campbell, 1964).

This paper concerns the purification and some properties of BMA. The accompanying paper presents similar studies on the cyclodextrinase (DePinto and Campbell, 1968). Our studies on the action patterns of BMA and the cyclodextrinase will be reported elsewhere.

Materials and Methods

Stock cultures of *B. macerans* ATCC 8514 were maintained on potato agar slants (Spizizen, 1958) and stored at ambient room temperature. The organism was grown 8–10 hr at 40° in a Model FS-314 batch fermentor assembly (New Brunswick Scientific Co., N. J.) using a 1% inoculum in the medium described by DePinto and Campbell (1964). Agitation was at 300 rpm and aeration was at the rate of 10 l./min. Cell-free extracts were prepared by sonic disruption using a Model S-110 Branson Sonifier (Heat systems, Great Neck, Long Island, N. Y.) at a power setting of eight.

BMA activity was measured by the dextrinogenic assay described by DePinto and Campbell (1964) except that the buffer was pH 6.2. Protein was estimated by the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as a standard. One dextrinogenic unit is defined as that amount of protein which will degrade 10 mg of starch/min at 40° under the standard assay conditions. The formation of cyclic dextrins was monitored during the purification of the enzyme by the specific slide test of Tilden and Hudson (1942).

Standard capacity DEAE-cellulose (Mann Research Laboratories) was washed twice with 1 N NaOH,

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† Part of the dissertation presented to the Graduate Faculty of the University of Illinois in partial fulfillment of requirements for the Ph.D. degree. Predoctoral trainee (GM-510) of the National Institute of General Medical Sciences, U. S. Public Health Service, during the tenure of this work. Present address: Department of Biology, Bradley University, Peoria, Ill. 61606.

[1] Abbreviation used: BMA, amylase of *B. macerans*.

[2] Schwimmer and Garibaldi (1952) were able to reduce the incubation time in an oatmeal medium to 12–15 hr employing an aeration rate of 150 l. of air/min.

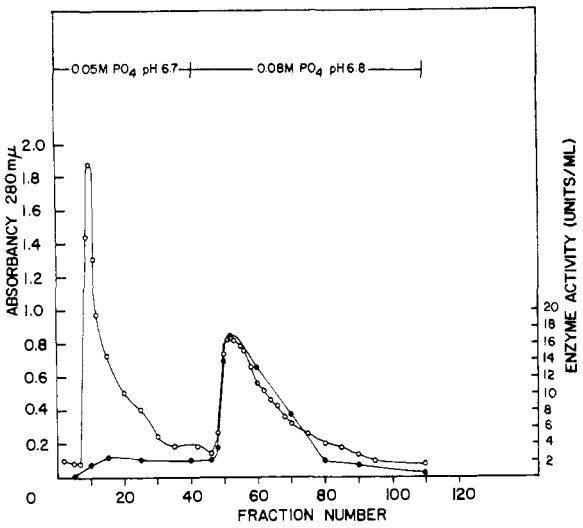

FIGURE 1: DEAE-cellulose chromatography of BMA. Protein (○) and BMA (●). Each fraction represents approximately 10 ml.

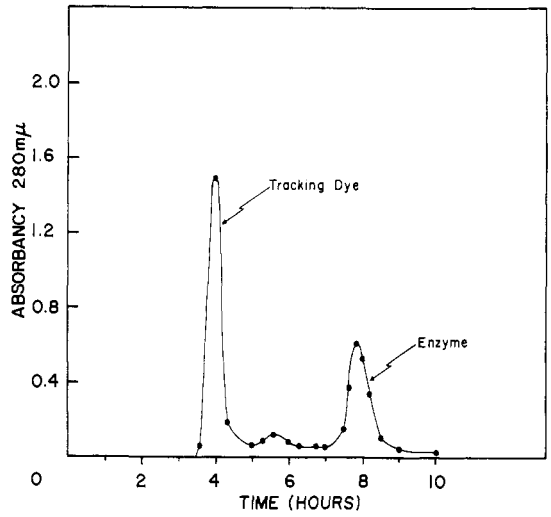

FIGURE 2: Preparative polyacrylamide electrophoresis of BMA.

three times with demineralized water, neutralized with concentrated phosphoric acid, and equilibrated overnight with 1 M KH_2PO_4. Excess phosphate was removed by repeated washing with demineralized water.

Analytical acrylamide gel disc electrophoresis was performed in a Model 12 Canalco disc electrophoresis instrument using their premixed standard gel kit. Photographs were taken using Polaroid type 55 P/N film in a Graflex Crown Graphic special camera with a Polaroid backplate adaptor at a setting of f/13 and 0.1-sec exposure. The resulting negatives were traced with a Joyce-Loebl double-beam recording microdensitometer Model III B (Joyce Loebl and Co., Ltd., Gateshead-on-Tyne, England). Preparative disc electrophoresis was performed using a preparative electrophoresis instrument (Buchler Instruments, Inc., Fort Lee, N. J.) maintained at 4°. Samples (5–10 ml) containing 50–150 mg of protein in 5% sucrose were run at 50 ma using a 50-ml lower gel and a 10-ml upper gel in a Tris buffer system (Jovin *et al.*, 1964). Fractions were collected at 4° with a Buchler-refrigerated fraction collector.

Sedimentation velocity studies were carried out in a Spinco Model E analytical ultracentrifuge at 59,780 rpm using a standard cell with a Kel-F centerpiece. Diffusion coefficients were determined in the ultracentrifuge at 9341 rpm using a double-sectored synthetic boundary cell (Ehrenberg, 1957).

Amino acid analyses were performed using a Beckman–Spinco amino acid analyzer. Samples were dissolved in 1 ml of 6 N HCl and sealed under reduced pressure in Pyrex tubes. Duplicate samples were hydrolyzed at 110° for 24, 48, and 72 hr. Half-cystine was determined as cysteic acid after performic acid oxidation (Hirs, 1956). Tryptophan was estimated by two spectrophotometric methods (Bencze and Schmid, 1957; Goodwin and Morton, 1946). Total nitrogen was determined by duplicate micro-Kjeldahl analyses.

Partial specific volume was calculated from the amino acid analyses (Cohn and Edsall, 1943). The partial specific volume was also determined experimentally by weighing protein solutions in calibrated 100-μl capillary pipets. The slope of the line relating weight fraction of protein to the mass of the contents of the pipet was determined by least squares and used to calculate the partial specific volume (Bull, 1943).

Results

Purification of BMA. Unless otherwise noted all operations were carried out at 4°.

1. PREPARATION OF EXTRACT. Cells (250 g wet wt) were suspended in cold Sørenson's phosphate buffer (0.01 M, pH 6.2) to a volume of 500 ml. The suspension was divided into five parts and each part was disrupted, while kept cold, with a Branson Sonifer for two 5-min intervals. The crude extract was centrifuged for 90 min at 17,500 rpm in a Servall centrifuge (SS-34 head) and the pellet was discarded.

2. MANGANOUS CHLORIDE PRECIPITATION. $MnCl_2$ (5 ml of a 1 M solution) was added with stirring at room temperature for every 100 ml of extract. The extract became milky in appearance and was stirred for 10 min. The white precipitate was removed by centrifugation for 30 min at 17,500 rpm (SS-34 head) and was discarded since it lacked BMA and cyclodextrinase activity. The clear supernatant fraction was dialyzed for 12 hr against 14 l. of 0.01 M phosphate buffer at pH 6.2. During dialysis, a precipitate formed in the bag. Centrifugation was repeated and the clear supernatant fraction was redialyzed against fresh buffer. The procedure of dialysis and centrifugation was repeated until a precipitate no longer formed in the dialysis bag. Usually a total of three buffer changes

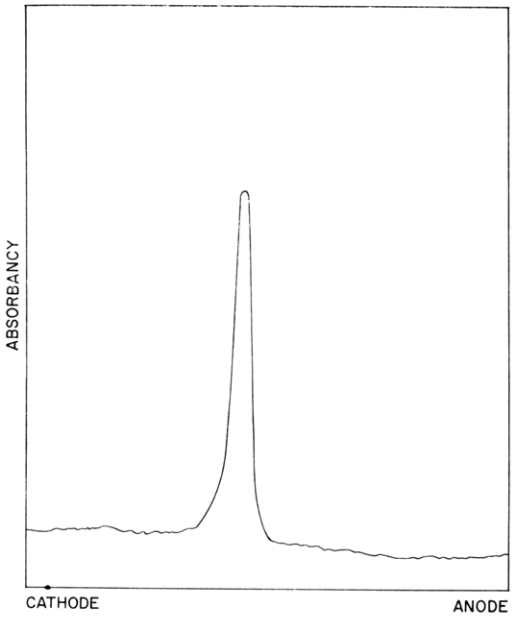

FIGURE 3: Densitometer tracing of analytical acrylamide disc electrophoresis of BMA.

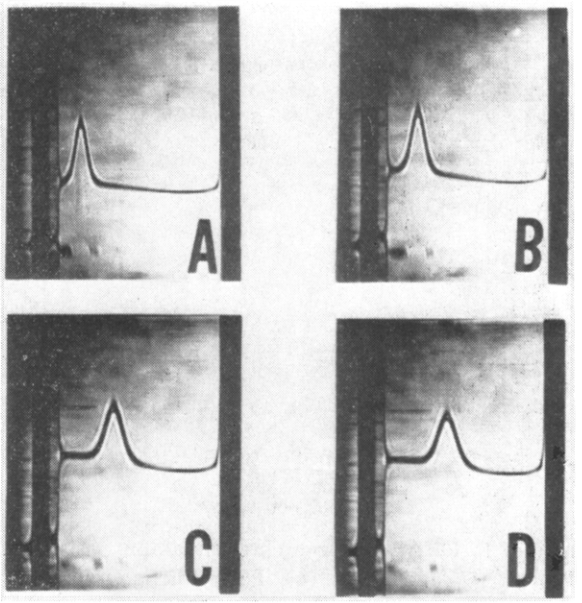

FIGURE 4: Sedimentation velocity pattern of BMA at 59,780 rpm. The enzyme (6 mg/ml; specific activity, 80) was dissolved in 0.01 M phosphate buffer (pH 6.2). The temperature was 16.6°. Direction of sedimentation is to the right. Time (minutes) after attaining speed: (A) 16, (B) 24, (C) 56, and (D) 64. The bar angle was 50°.

accomplished this. This procedure apparently inactivates 90–95% of the cyclodextrinase since it could never be detected in the precipitates.

3. AMMONIUM SULFATE PRECIPITATION. The supernatant liquid was adjusted to a protein concentration of approximately 20 mg/ml and solid ammonium sulfate (enzyme grade) was slowly added with stirring at room temperature to 30% saturation. Stirring continued for 30 min after the addition of the salt. The precipitate was collected by centrifugation for 30 min at 17,500 rpm (SS-34 head) and was discarded. Ammonium sulfate was then added to 45% saturation and the precipitate was collected in the same manner. It was dissolved in 0.01 M phosphate buffer at pH 6.7 and dialyzed overnight against 12 l. of the same buffer.

4. DEAE-CELLULOSE CHROMATOGRAPHY. A column (2.5 × 40 cm) was packed using standard capacity DEAE-cellulose in the phosphate form. It was equilibrated with 0.01 M phosphate buffer at pH 6.7 and the sample was applied. The column was washed with 500 ml of 0.05 M phosphate buffer at pH 6.7 with a flow rate of approximately 1 ml/min. Under these conditions BMA adsorbs to the column. The enzyme was then eluted from the column with 0.08 M phosphate at pH 6.8. The results of a typical run are shown in Figure 1.

5. AMMONIUM SULFATE PRECIPITATION. The fractions from the DEAE-cellulose column that contained activity were pooled and adjusted to pH 6.2 with 1.0 N HCl, and solid ammonium sulfate was added to 35% saturation. The precipitate was discarded. Additional ammonium sulfate was added to 45% saturation and the precipitate was dissolved in approximately 10 ml of 0.01 M phosphate buffer at pH 6.2 and dialyzed overnight against 10 l. of the buffer.

6. PREPARATIVE ACRYLAMIDE DISC ELECTROPHORESIS. The final step in the purification of BMA was preparative disc electrophoresis. Identical results were obtained using samples containing 50–150 mg of protein. The results of a run are shown in Figure 2. Since the Tris buffer at the pH used interfered with the assay, the

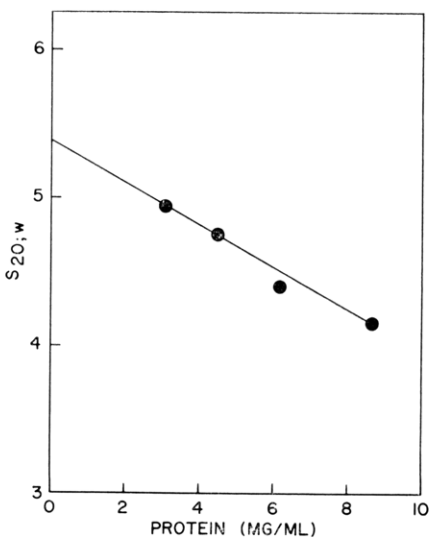

FIGURE 5: Extrapolation of $s_{20,w}$ values of BMA to infinite dilution.

TABLE I: Purification Summary of the Amylase of *B. macerans*.[a]

Step	Vol (ml)	Protein		Amylase of *B. macerans*				
		mg/ml	Total mg	Units/ml	Total Units	Sp Act.	% Recov	Purifcn (-fold)
Crude extract	500	54.2	26,100	31.6	15,800	0.58	100	1.0
17,500 rpm supernatant fraction	370	59.4	21,978	39.4	14,578	0.66	92.3	1.1
$MnCl_2$	300	24.5	7,350	35.3	10,594	1.44	87.7	2.5
$(NH_4)_2SO_4$, 30–45%	50	52.8	2,640	181.1	9,055	3.43	57.3	5.9
DEAE	450	1.9	855	12.2	5,490	6.42	34.8	11.1
$(NH_4)_2SO_4$, 35–45%	11.5	15.2	175	265.2	3,050	17.45	19.3	30.1
Electrophoresis and lyophilization	7.5	3.1	23.3	252.8	1,896	81.55	12.0	140.1

[a] The specific slide test of Tilden and Hudson (1942) for cyclodextrins was positive at each step in the purification.

peak fractions were combined and dialyzed against 5 l. of 10^{-3} M phosphate buffer at pH 6.2 for 24 hr with two complete buffer changes before they were assayed. The enzyme was then lyophilized, dissolved in a minimal volume of 0.01 M phosphate buffer (pH 6.2), and dialyzed overnight against the same buffer before being used for the various physical and chemical studies. A typical purification summary is given in Table I.

Properties of Purified BMA. ANALYTICAL ACRYLAMIDE DISC ELECTROPHORESIS. The purified enzyme was examined in the range of 45–450 μg by analytical disc electrophoresis. Only one band could be detected over this concentration range. A densitometer tracing of a Polaroid negative is shown in Figure 3. The absorbancy of the tracking dye which was at the bottom of the sample is not shown.

Molecular Weight. The molecular weight of BMA was calculated from sedimentation and diffusion data. The enzyme moved as a single, uniform boundary as seen in Figure 4. The extrapolation of four protein concentrations to infinite dilution gave an $s_{20,w}$ of 5.40 as shown in Figure 5. The diffusion was measured at four different concentrations. Some diffusion patterns are shown in Figure 6, and Figure 7 shows the $D_{20,w}$

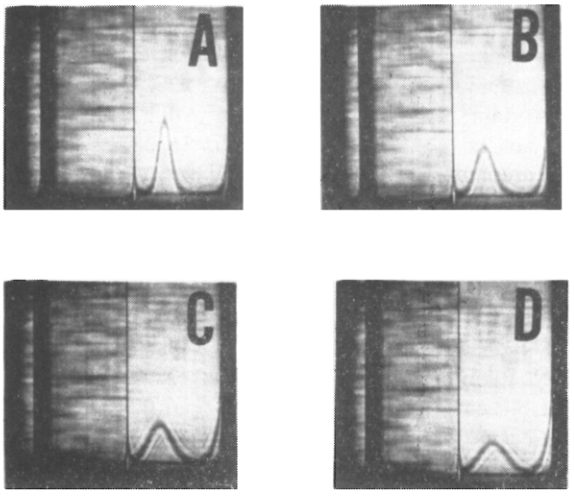

FIGURE 6: Diffusion pattern of BMA in the ultracentrifuge at 9341 rpm and 20°. The enzyme (6 mg/ml; specific activity, 80) was dissolved in 0.01 M phosphate buffer (pH 6.2). Time (minutes) after attaining speed: (A) 16, (B) 48, (C) 86, and (D) 134. The bar angle was 50°.

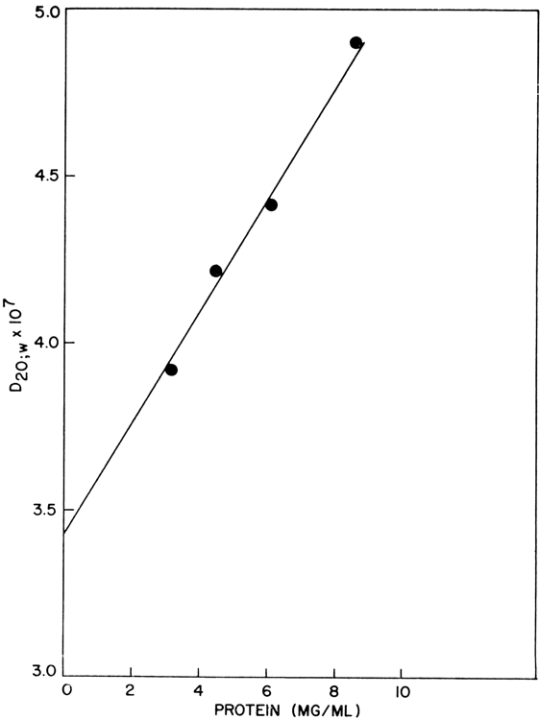

FIGURE 7: Extrapolation of $D_{20,w}$ values of BMA to infinite dilution.

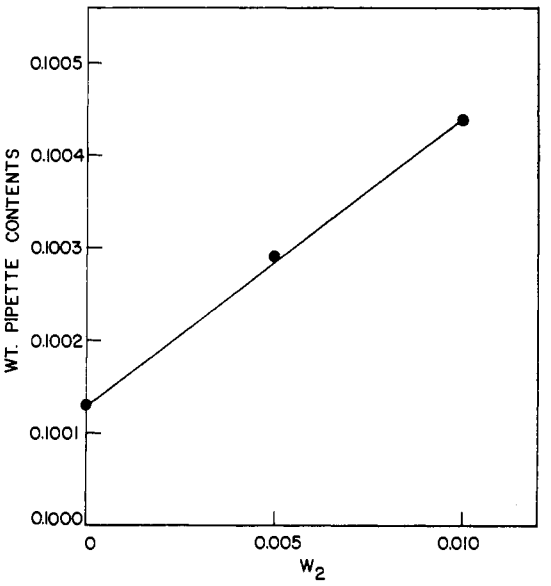

FIGURE 8: Partial specific volume of BMA. The weight of the contents of the capillary microliter pipet was plotted against the weight fraction (W_2) of the enzyme. The slope of the line was calculated by the least-square method to be 0.029.

values extrapolated to infinite dilution. The $D_{20,w}$ was 3.43×10^{-7} cm^2 sec^{-1}. The partial specific volume of the enzyme was calculated from its amino acid composition to be 0.724 ml/g. The actual determination of $\bar{V}$, using the slope of the line shown in Figure 8,

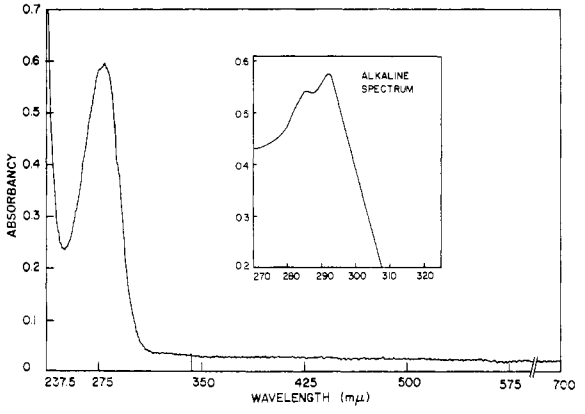

FIGURE 9: Absorption spectrum of BMA. The enzyme (8.3 μg/ml; specific activity, 80) was dissolved in 0.01 M phosphate buffer (pH 6.2) for the normal spectrum and in 0.1 N NaOH for the alkaline spectrum. The slope of the tangent to the two peaks in the alkaline spectrum was +8.68 This value was used for one of the tryptophan estimation methods (Bencze and Schmid, 1957). The molar extinction coefficient at 280 mμ was 9.9×10^4.

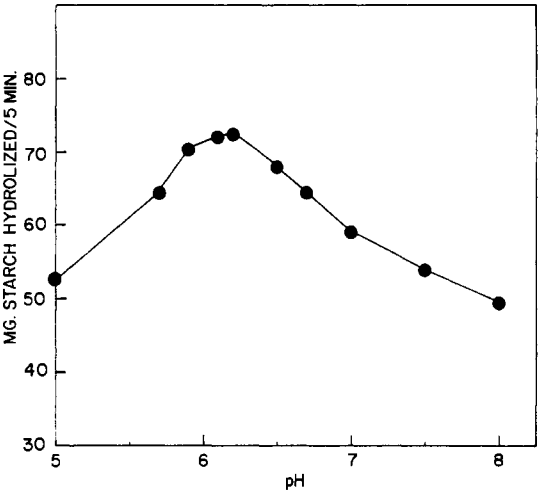

FIGURE 10: The effect of pH on the activity of BMA. Soluble starch, in 0.01 M phosphate buffer above pH 5.8 and in 0.01 M citrate below pH 5.8 was incubated with 1.5 units of enzyme for 5 min at 40°.

gave a value of 0.712 ml/g. From these data, the molecular weight was calculated to be 139,300. The frictional ratio calculated from these data, by the method of Edsall (1953) was 1.79.

Absorption Spectrum. The absorption spectra of

TABLE II: Amino Acid Composition of the Amylase of *B. macerans*.[a]

Amino Acid	Time of Hydrolysis (hr) 24	48	72	Av
Lysine	5.55	5.55	5.34	5.48
Histidine	2.23	2.19	2.23	2.22
Arginine	4.40	4.15	4.20	4.25
Aspartic	17.78	17.40	16.38	17.19
Threonine	9.50	8.37	7.64	10.37[b]
Serine	5.79	4.77	4.02	6.64[b]
Glutamic	8.70	8.49	8.35	8.51
Proline	3.75	4.03	3.69	3.82
Glycine	7.92	7.67	7.34	7.64
Alanine	7.61	7.45	7.13	7.40
Valine	7.82	7.79	7.52	7.71
Methionine	2.60	2.50	2.29	2.46
Isoleucine	6.14	6.33	6.02	6.16
Leucine	7.07	6.95	6.61	6.88
Tyrosine	7.74	7.10	7.94	7.26
Phenylalanine	7.49	7.34	6.86	7.23
Tryptophan[c]				3.19
Half-cystine[d]	0			0

[a] Values are in g of amino acid/100 g of protein. [b] Extrapolated values. [c] Determined from two spectrophotometric methods. [d] Determined as cysteic acid.

TABLE III: Amino Acid Composition and Molecular Weight of the Amylase of *B. macerans*.

Amino Acid	Amino Acid Residues/100 g of Protein	N % Total	Min Mol Wt[a]	Residues/Molecule[b] Calcd	Residues/Molecule[b] Nearest Integer	Calcd Mol Wt[c]
Lysine	4.80	6.91	2,668	52.2	52	138,736
Histidine	1.96	3.95	6,994	19.9	20	139,880
Arginine	3.81	8.99	4,097	34.0	34	139,298
Aspartic	14.86	11.90	774	180.0	180	139,320
Threonine	8.80	8.03	1,149	121.2	121	139,029
Serine	5.50	5.74	1,583	88.0	88	139,304
Glutamic	7.47	5.33	1,728	80.6	81	139,968
Proline	3.22	3.06	3,015	46.2	46	138,690
Glycine	5.80	9.38	983	141.7	142	139,586
Alanine	5.90	7.65	1,204	115.7	116	139,664
Valine	6.52	6.07	1,520	91.6	92	139,840
Methionine	2.16	1.52	6,073	22.9	23	139,679
Isoleucine	5.31	4.33	2,130	65.4	65	138,450
Leucine	5.93	4.84	1,907	73.0	73	139,211
Tyrosine	6.53	3.69	2,497	55.8	56	139,832
Phenylalanine	6.44	4.03	2,284	61.0	61	139,324
Tryptophan	2.91	2.88	6,395	21.8	22	140,690
Half-cystine	0	0				
Total	97.92	98.30			1272	139,441 (av)

[a] (Molecular weight amino acid residues × 100)/% amino acid residue in protein. [b] The number of residues per molecule was calculated using a molecular weight of 139,300. [c] Minimal molecular weight times the nearest integral number of residues.

purified BMA in 0.01 M phosphate buffer (pH 6.2) and in 0.1 N NaOH are shown in Figure 9. Maximum absorption was at 280 mμ. The molar extinction coefficient was calculated to be 9.9×10^4.

Amino Acid Composition. Tables II and III show the results of the amino acid analyses of BMA. The values for serine and threonine were extrapolated to zero-time hydrolysis. Duplicate micro-Kjeldahl analyses gave a value of 15.2% nitrogen. No cystine was detected by the method employed. The alkaline spectrum shown in Figure 9 was used to estimate the tryptophan content. One method (Bencze and Schmid, 1957) gave a molar ratio of tyrosine:tryptophan of 2.6–2.9. The other method (Goodwin and Morton, 1946) resulted in a molar ratio for tyrosine:tryptophan of 2.6. The ratio of 2.6 was used in the calculations for Tables II and III. An anthrone test for bound carbohydrate was negative.

The Effect of pH, Temperature, and Substrate Concentration on BMA. The pH optimum of the purified enzyme is shown in Figure 10. Under these conditions the optimum pH was 6.1–6.2. A Lineweaver–Burk plot of the enzymatic reaction is shown in Figure 11. The slope of the line as calculated by the method of least squares was 0.5615. This resulted in a calculated K_m for soluble starch of 3.33 mg/ml. The effect of temperature on the enzyme is shown in Figure 12. The energy of activation as calculated using the Arrhenius equation was 12,250 cal/mole.

Discussion

The purification method presented is a considerable improvement over other methods, in both time and degree of purity of the enzyme. Heretofore, the most highly purified material was considered to be at least 90% pure (Schwimmer and Garibaldi, 1952). In our case, since only one band was detectable by analytical acrylamide disc electrophoresis up to a protein concentration of 450 μg and since this method is reportedly sensitive enough to detect 5 μg or less, this would represent a degree of purity of at least 99%. The $s_{20,w}$ obtained for BMA was identical with a value reported by Schwimmer (1953). The frictional ratio suggests that the shape of the enzyme deviates significantly from a hydrated sphere. The K_m value of 3.33×10^{-3} g/ml is somewhat higher than K_m values of other kinds of amylases. These have ranged from 1×10^{-3} to 6.5×10^{-4} g/ml (Manning and Campbell, 1961; Bernfeld, 1951; Markovitz *et al.*, 1956; Menzi *et al.*, 1957). A calculated value of 12,250 cal/mole for the energy of activation is in the range of that found for other types of amylase (Fischer and Stein, 1960). Except for higher threonine and alanine values (Table II)

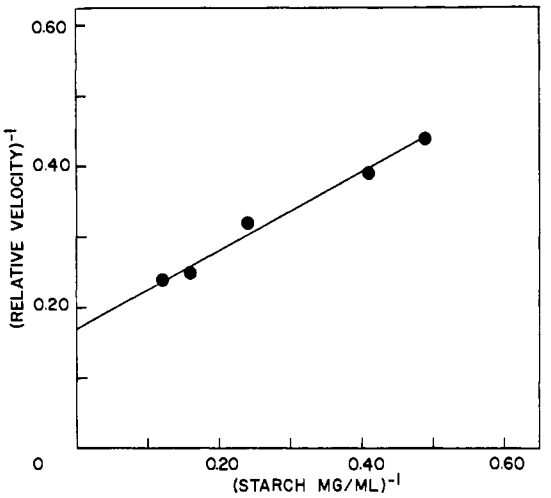

FIGURE 11: Lineweaver–Burk plot of starch hydrolysis by BMA.

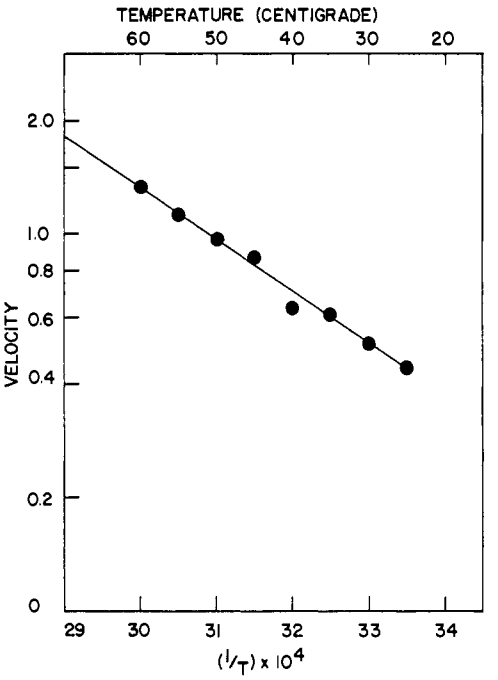

FIGURE 12: Effect of temperature on BMA activity. Activity measurements were carried out at pH 6.2 over the temperature range 25–60°. Approximately 0.6 unit of enzyme was used. The slope of the straight line, used to calculate the energy of activation, was -2.77×10^3.

the amino acid composition of BMA is similar to that reported for the α-amylases of *Bacillus subtilis*, hog pancreas, and human saliva (Fischer and Stein, 1960). The ratio (2.6) of aspartic plus glutamic acids to lysine plus arginine may account for the relatively low isoelectric point (pH 4.5) reported for BMA by Schwimmer (1953). The levels of threonine and alanine are similar to those reported for the amylase of *Aspergillus oryzae* (Fischer and Stein, 1960). Cystine is present in the amylase of *A. oryzae* and in all α-amylases except *B. subtilis* (Fischer and Stein, 1960; Campbell and Manning, 1961). The absence of cystine in BMA points up another difference between this enzyme and most other amylases.

References

Bencze, W. L., and Schmid, K. (1957), *Anal. Chem. 29*, 1193.

Bernfeld, P. (1951), *Advan. Enzymol. 12*, 379.

Bull, H. B. (1943), Physical Biochemistry, New York, N. Y., Wiley, pp 272–292.

Campbell, L. L., and Manning, G. B. (1961), *J. Biol. Chem. 236*, 2962.

Cohn, E. J., and Edsall, J. T. (1943), Proteins, Amino Acids and Peptides, New York, N. Y., Reinhold, p 375.

DePinto, J. A., and Campbell, L. L. (1964), *Science 146*, 1064.

DePinto, J. A., and Campbell, L. L. (1968), *Biochemistry 7*, 121 (this issue; following paper).

Edsall, J. T. (1953), *Proteins 1*, 549.

Ehrenberg, A. (1957), *Acta Chem. Scand. 11*, 1257.

Fischer, E. H., and Stein, E. A. (1960), *Enzymes 4*, 313.

French, D. (1957), *Advan. Carbohydrate Chem. 12*, 189.

French, D., Levine, M. L., Norberg, E., Nordin, P., Pazur, J. H., and Wild, G. (1954), *J. Am. Chem. Soc. 76*, 2387.

French, D., Pulley, A. O., Effenberger, J. A., Rougvie, M. A., and Abdullah, M. (1965), *Arch. Biochem. Biophys. 111*, 153.

Goodwin, T. W., and Morton, R. A. (1946), *Biochem. J. 40*, 628.

Hirs, C. H. W. (1956), *J. Biol. Chem. 219*, 611.

Jovin, T., Chrambach, A., and Naughton, M. A. (1964), *Anal. Biochem. 9*, 351.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Manning, G. B., and Campbell, L. L. (1961), *J. Biol. Chem. 236*, 2952.

Markovitz, A., Klein, H. P., and Fischer, E. H. (1956), *Biochim. Biophys. Acta 19*, 267.

Menzi, R., Stein, E. A., and Fischer, E. H. (1957), *Helv. Chim. Acta 40*, 534.

Schwimmer, S. (1953), *Arch. Biochem. Biophys. 43*, 108.

Schwimmer, S., and Garibaldi, J. A. (1952), *Cereal Chem. 29*, 108.

Spizizen, J. (1958), *Proc. Natl. Acad. Sci. U. S. 44*, 1072.

Tilden, E. B., and Hudson, C. B. (1942), *J. Bacteriol. 43*, 527.