References

Beaufay, H., and DeDuve, C. (1959), *Biochem. J. 73*, 604.

DeDuve, C., and Beaufay, H. (1959), *Biochem. J. 73*, 610.

DeDuve, C., Pressman, B. C., Gianetto, R., Wattiaux, R., and Appelmans, F. (1955), *Biochem. J. 60*, 604.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956), *Anal. Chem. 28*, 350.

Fishman, W. H., and Goldman, S. S. (1965), *J. Histochem. Cytochem. 13*, 441.

Fishman, W. H., Goldman, S. S., and DeLellis, R. (1967), *Nature 213*, 457.

Furth, A. J., and Robinson, D. (1965), *Biochem. J. 97*, 59.

Koenig, H., and Jibril, A. (1962), *Biochim. Biophys. Acta 65*, 543.

Levvy, G. A., and Marsh, C. A. (1960), *Enzymes 4*, 397.

Maver, M. E., Peterson, E. A., Sober, H. A., and Greco, A. E. (1959), *Ann. N. Y. Acad. Sci. 81*, 599.

Mills, G. T., Paul, J., and Smith, E. E. B. (1953), *Biochem. J. 53*, 232.

Moore, B. W., and Angeletti, P. U. (1961), *Ann. N. Y. Acad. Sci. 94*, 659.

Moore, B. W., and Lee, R. H. (1960), *J. Biol. Chem. 235*, 1359.

Moore, S., and Stein, W. H. (1963), *Methods Enzymol. 6*, 819.

Oshima, G. (1936), *J. Biochem. (Tokyo) 23*, 305.

Paigen, K. (1961), *Exptl. Cell Res. 25*, 286.

Peterson, E. A., and Chiazze, E. A. (1962), *Arch. Biochem. Biophys. 99*, 136.

Plapp, B. V., and Cole, R. D. (1966), *Arch. Biochem. Biophys. 116*, 193.

Plapp, B. V., Hopkins, T. R., and Cole, R. D. (1963), *J. Biol. Chem. 238*, 3315.

Press, E. M., Porter, R. R., and Cebra, J. (1960), *Biochem. J. 74*, 501.

Roy, A. B. (1958), *Biochem. J. 68*, 519.

Sadahiro, R., Takanashi, S., and Kawada, M. (1965), *J. Biochem. (Tokyo) 58*, 104.

Smith, E. E. B., and Mills, G. T. (1953), *Biochem. J. 54*, 164.

Wakabayashi, M., Fujita, T., Ninomiya, H., Nakamura, K., and Shirai, Y. (1966), *Kobe J. Med. Sci. 12*, 71.

Wakabayashi, M., Fujita, T., Ninomiya, H., Nakamura, K., and Shirai, Y. (1967), *Chem. Abstr. 66*, 91872.

Crystallization and Properties of α-Amylase from Five Strains of *Bacillus amyloliquefaciens**

N. E. Welker† and L. Leon Campbell

ABSTRACT: A method for the crystallization of the α-amylase (α-1,4-glucan:4-glucanohydrolase, EC 3.2.1.1) of five strains of *Bacillus amyloliquefaciens* is described. The enzymes were found to have optimal activity and stability at pH 5.9 and 25°. The enzymes were not significantly different with respect to their electrophoretic mobility in polyacrylamide gel (pH 8.6), pH optimum, ultraviolet absorption spectrum, and immunological properties. Quantitative but not qualitative differences were noted in their activity on starch, amylopectin, glycogen, and amylose. Differences were found among the five enzymes with respect to pH and temperature stability and K_m and energy of activation values.

The α-amylase (α-1,4-glucan:4-glucanohydrolase, EC 3.2.1.1) of *Bacillus subtilis* has been studied extensively by several investigators (see review of Fischer and Stein, 1960). Owing to the diversity of strains of the organism and the experimental conditions employed by different investigators the literature on this subject is very confused. Welker and Campbell (1967a) have shown that the highly amylolytic strains of *B. subtilis* used by most investigators for the production of α-amylase are not genetically related to *B. subtilis* but are strains of *Bacillus amyloliquefaciens* (Fukumoto, 1943). We have also demonstrated that the α-amylase of authentic strains of *B. subtilis* differs from that of *B. amyloliquefaciens* in its electrophoretic and immunological properties (Welker and Campbell, 1967b).

This report is concerned with the purification, crystallization, and comparison of some general properties of the α-amylase from five strains of *B. amyloliquefaciens*.

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TABLE I: Purification and Crystallization of α-Amylase of *Bacillus amyloliquefaciens*, Strain T.

Procedure	Vol. (ml)	Total Units[a]	Sp Act. (units/mg of protein)	Yield (%)
Crude liquor	10,000	6,120,000	215	100
Step 1	1,430	4,633,200	880	75.7
Step 2	1,640	4,526,400	1,000	74.0
Step 3	62	3,520,500	1,318	57.5
Step 4	20	3,060,000	1,561	50.0
Step 5	(1143.5 mg)	1,781,573	1,558	29.1

[a] One unit of α-amylase activity is equal to 1 mg of reducing sugar, measured as maltose, released in 3 min at 25°, pH 5.9. Similar values were obtained in the purification of the α-amylase from strains F, N, P, and SB.

faciens. Subsequent papers will present data on the physical and chemical properties of the five enzymes.

Materials and Methods

Amylose and amylopectin (amylose free) were obtained from Calbiochem, Los Angeles, Calif. Rabbit liver glycogen was obtained from Nutritional Biochemical Corp., Cleveland, Ohio, and Lintner soluble starch was obtained from Pfanstiehl Laboratories, Inc., Waukegan, Ill.

Organisms. The organisms used in this investigation were *B. amyloliquefaciens*, strains SB, P, T, N, and F (Welker and Campbell, 1967a). Stock cultures of these organisms were maintained on potato agar slants (Spizizen, 1958) and stored at ambient room temperature.

Assay of α-Amylase. The saccharogenic assay of Fischer and Stein (1961) and Stein and Fischer (1961) was used to measure enzyme activity. This procedure makes use of the colorimetric determination (3,5-dinitrosalicylic acid) of reducing groups released from starch as a result of enzyme activity. All readings were made in a Bausch and Lomb Spectronic 20 colorimeter at 540 mμ. Protein concentration was determined by the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as a standard. One unit of α-amylase activity is defined as 1 mg of reducing sugar (calculated from a standard curve previously calibrated against maltose) released in 3 min at 25°, pH 5.9.

Production of Enzyme. The medium used for enzyme production was that described by Okunuki (1960) supplemented with 1×10^{-5} M $CaCl_2$. The medium was prepared in 10-l. quantities as follows. The $(NH_4)_2HPO_4$, sodium citrate, KCl, and yeast extract were dissolved in 5 l. of distilled water and the pH was adjusted to 7.2 with 2 N HCl. The potato starch was dissolved in 4.85 l. of distilled water as follows. A trace of commercial α-amylase (Biddle Sawyer and Co., New York, N. Y.) was added and the starch solution was heated at 70–80° until clearing occurred. Calcium chloride was made up as a stock solution (0.5 g/100 ml) and the medium components were autoclaved at 121° for 20 min. The salts–yeast extract solution was combined with the starch solution; the ethanol and $CaCl_2$ were added aseptically. The pH of the medium was 7.1.

The medium was inoculated with 1 l. of a culture prepared in the following manner. Cells from an 18-hr growth on a Tryptose blood agar base plate (Difco) were used to inoculate a 2800-ml Fernbach flask, containing 1-l. of Pen-Assay broth (Difco). The flask was shaken on a rotary shaker for 3–3.5 hr at 37°. The inoculum was thoroughly mixed with the medium and 1 l. was poured into each of ten sterile Pyrex baking dishes (2 × 9 × 13.5 in.) covered with aluminum foil. After 120 hr of incubation at 37° the pellicle of cells on the surface of each dish was removed by pouring the contents through 12 layers of cheesecloth. Each dish was washed with 150–200 ml of water and the water washes were combined with the supernatant fluids. The resultant cloudy liquor contains the α-amylase.

Purification and Crystallization of α-Amylase. The enzyme was purified by a modification of the procedure described by Stein and Fischer (1961).

STEP 1. The pH of the liquor was adjusted to 6.2 with 1 N acetic acid and 420 g/l. of anhydrous ammonium sulfate was added slowly with stirring at ambient room temperature. The mixture was stirred for 1 hr and placed at 4° for 12–16 hr. The precipitate was collected on a coarse sintered-glass funnel containing 40–50 g of Hyflo Super-Cel (Johns-Manville Co.) as a filter aid. The filter aid, containing the enzyme, was suspended in 500–600 ml of 0.1 M calcium acetate and the pH was adjusted between 8 and 10. The mixture was stirred for 1 hr and left at 4° for 12–16 hr. The filter aid was removed by suction filtration through a Büchner funnel containing one sheet of Whatman No. 1 filter paper. The filter aid was suspended in 200–300 ml of 0.1 M calcium acetate, stirred for 1 hr at 4°, and filtered as before. The filter aid was treated twice more in the same manner and the washes were combined and centrifuged to remove the remaining filter aid.

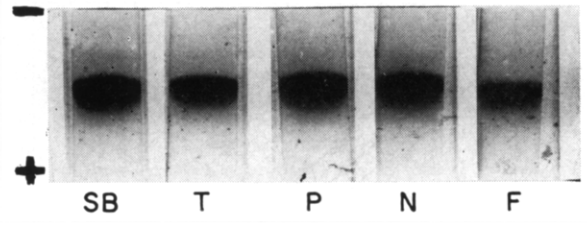

FIGURE 1: Disc electrophoretic pattern of a crystalline α-amylase of *B. amyloliquefaciens*, strains SB, P, T, N, and F. The upper gel contained 2.5% acrylamide and 0.6% bisacrylamide; the lower gel contained 7% acrylamide and 0.18% bisacrylamide. Electrophoresis was conducted for 1 hr; 3 ma/column in 2.5×10^{-3} M Tris–1.9×10^{-2} M glycine buffer (pH 8.6) at room temperature. The enzymes moved approximately 1.5 cm under these conditions.

STEP 2. The dark brown enzyme solution was dialyzed for 3 days against several changes of 0.01 M calcium acetate at 4°.

STEP 3. The dialyzed enzyme solution was slowly added, with stirring, to 1.7 volumes of cold acetone (−10°), and then placed at −20° for 12–16 hr. The precipitate was removed by centrifugation and the residual acetone was removed by directing a jet of air on the precipitate. The precipitate was dissolved in 50–70 ml of 0.01 M $CaCl_2$ and centrifuged at 17,000 rpm for 25 min to remove insoluble material. The insoluble material was suspended in 0.01 M calcium acetate (10–20 ml) and centrifuged as before. The supernatant fluids, containing the α-amylase, were combined.

STEP 4. The dark brown enzyme solution was placed in a screw-cap tube (20 × 180 mm) and the pH adjusted to 6.6 with 0.1 N acetic acid. Crystallization was initiated by adding 0.01 volume of 0.02 M zinc acetate as described by Stein and Fischer (1961). The tube containing the enzyme was placed on a tube rotator at 4° and crystallization was allowed to proceed for 1 week. The needle-shape crystals were removed by centrifugation, washed once with two to three volumes of cold 0.01 M calcium acetate, and centrifuged. The crystals were suspended in 15–25 ml of 0.1 N NH_4OH and the tube containing the enzyme was placed on the tube rotator at 4°. Additional NH_4OH was added if the crystals did not dissolve after 2–3 hr.

STEP 5. The pH was carefully adjusted to 6.6 with 0.1 N acetic acid and 0.01 volume of 0.02 M zinc acetate and 1.0 M calcium acetate was added. The tube was placed on the tube rotator until crystallization was complete (approximately 1 week). The crystals were removed by centrifugation at 17,000 rpm for 25 min, suspended in distilled water, and lyophilized in a Virtis freeze-drying apparatus. The lyophilized crystals were stored at −10° until used. Table I summarizes a typical purification procedure. α-Amylase from the five strains was obtained in yields ranging from 20 to 34%. The five enzymes were judged to be homogeneous by the appearance of a single band in disc electrophoresis (Figure 1) and by ultracentrifugational and electrophoretic data (N. E. Welker and L. L. Campbell, unpublished results).

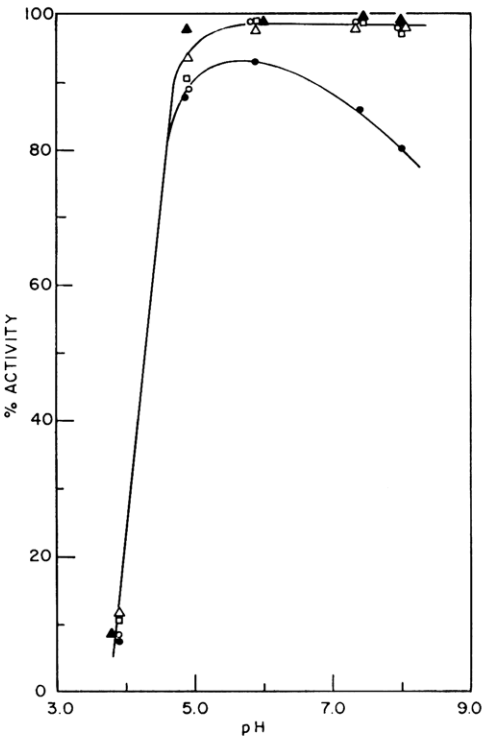

FIGURE 2: Stability of an α-amylase of *B. amyloliquefaciens*, strains SB, P, T, N, and F, as a function of pH. Samples of the enzymes (100 μg/ml) were exposed to the indicated pH values for 2 hr at 25° and diluted to a final concentration of 1 μg/ml with 0.02 M sodium glycerol phosphate buffer (pH 5.9), and the saccharogenic activity was measured. α-Amylase from strains SB (○), N (△), P (□), F (●), and T (▲).

Immunological Procedures. The following procedure was used for obtaining rabbit sera against the five enzymes. Each of the enzymes was alum precipitated by mixing a 1% aqueous enzyme solution with an equal volume of 10% aluminum potassium sulfate and adjusting the pH to 6.7. The precipitate was centrifuged, washed with, and suspended in 10 ml of 0.03 M potassium phosphate–0.15 M NaCl buffer (pH 7.2). The rabbits were injected intramuscularly over a period of 4 weeks (four injections per week), with increasing amounts of alum-precipitated enzyme (3.0 mg/injection for the first week, 4.5 mg/injection for the second week, 6.0 mg/injection for the third week, and 7.5 mg/injection for the fourth week). One week after the last injection the rabbits were bled from the heart and the sera were stored at −20°.

Ouchterlony plates were prepared as described by Postgate and Campbell (1963). The Ouchterlony plates contained 0.2 ml of the sera to be tested in the outer wells and 40 μg of enzyme (dissolved in 0.03 M potassium

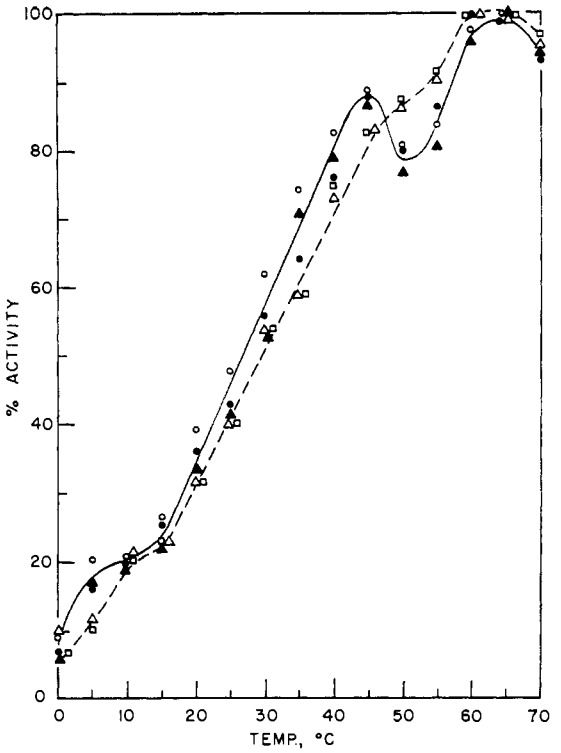

FIGURE 3: Effect of temperature on α-amylase activity of *B. amyloliquefaciens*, strains SB, P, T, N, and F. Starch substrates buffered at pH 5.9 were incubated at the appropriate temperatures for 2 min and the saccharogenic activity was measured. α-Amylase from strains SB (○), N (△), P (□), F (●) and T (▲).

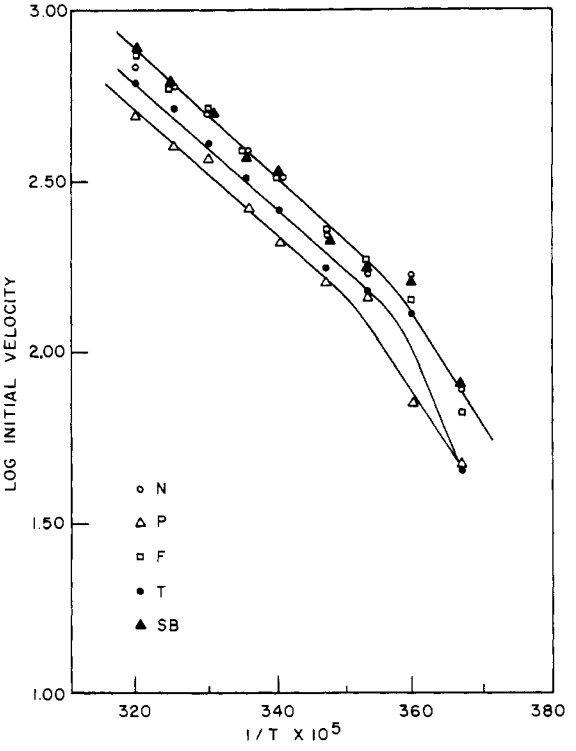

FIGURE 4: Arrhenius plots of the activity of an α-amylase of *B. amyloliquefaciens*, strains SB, P, T, N, and F, measured at different temperatures.

phosphate–0.15 M NaCl buffer, pH 7.2) in the center well. The plates were left at 16° until precipitate lines were clear.

Results

Effect of Enzyme Concentration on α-Amylase Activity. Enzyme activity was found to be linearly related to enzyme concentration over the range studied (0.1–10 μg/ml) for each of the five enzymes. Enzyme activity (1 μg/ml) is not linear with time after 3 min. This is probably due to a limitation in the assay (colorimetric determination of maltose is not linear above 1.5 mg of maltose/ml) and to end-product inhibition of the enzymes by maltodextrins, as postulated by Robyt and French (1963). All subsequent studies were carried out using 1–2 μg/ml of the crystalline enzymes with an assay time of 1–2 min.

Effect of pH on Enzyme Activity. The effect of pH on enzyme activity at 25° was determined by the use of buffered starch solutions (1%) ranging from pH 3.6 to 9.0. Buffers were prepared according to Gomori (1955). The pH range of maximal enzyme activity for the five enzymes was found to be 5.5–6.5 with a maximum at pH 5.9.

The stability of the five enzymes, as a function of pH, was examined by exposing samples of the enzymes (100 μg/ml) to buffers at various pH values for 2 hr and then diluting into pH 5.9 buffer for assay (final concentration of enzyme was 1 μg/ml). Figure 2 shows that the enzymes obtained from strains SB, N, P, and T are stable between pH 4.5 and 8.0. The α-amylase obtained from strain F is less stable over this pH range and has an optimum pH of stability at 5.9.

Effect of Temperature on Enzyme Activity. Activity measurements of the five α-amylases were carried out at pH 5.9 over a temperature range of 0–70° at 5° increments. As shown in Figure 3, three of the enzymes (α-amylase obtained from strains SB, T, and F) exhibit two optima, one at 45° and the other at 65°, while the other two enzymes (α-amylase obtained from strains N and P) showed a single optimum at 65°. The thermal stability of the five enzymes was examined by equilibrating samples in a water bath at the desired temperatures for 1 hr and then assaying under optimal conditions (25°, pH 5.9). All five enzymes are rapidly inactivated above 45°. The enzymes showed varying degrees of relative stability between 25 and 45°. The α-amylase from strains P, SB, N, and T was inactivated at 35° by 6, 9, 12, and 15%, respectively, while the α-amylase from strain F was not inactivated at 35°. The highest temperature (25°) at which the five enzymes are not inactivated was selected as the temperature for assay in subsequent studies.

TABLE II: Temperature Coefficients (Q_{10}) of an α-Amylase of *B. amyloliquefaciens*, Strains SB, P, T, N, and F.[a]

Enzyme	Temperature Coefficient (°C)					
	0–10	5–15	10–20	15–25	20–30	25–35
SB	2.13	1.28	1.92	1.82	1.57	1.55
T	3.21	1.36	1.76	1.84	1.56	1.71
F	3.09	1.62	1.74	1.68	1.58	1.52
N	2.13	2.50	1.73	1.47	1.58	1.58
P	3.09	1.99	1.46	1.70	1.71	1.48

[a] α-Amylase activity measurements were carried out at pH 5.9 over the temperature range of 0–35°. Protein concentration, 1 μg/ml; starch concentration, 1%.

A plot of the logarithm of the initial enzyme velocity as a function of the reciprocal of the absolute temperature is shown in Figure 4. The five enzymes exhibit a diphasic Arrhenius curve. Using the Arrhenius equation, the energy of activation of the reaction was calculated to be: 13,900 ($s_{\bar{x}}$ = ±105) cal from 0 to 7° and 7850 ($s_{\bar{x}}$ = ±29) cal from 7 to 40° for the α-amylase obtained from strains SB, F, and N; 13,300 ($s_{\bar{x}}$ = ±150) cal from 0 to 12° and 7570 ($s_{\bar{x}}$ = ±49) cal from 12 to 40° for the α-amylase obtained from strain P; and 21,800 ($s_{\bar{x}}$ = ±160) cal from 0 to 7° and 7360 ($s_{\bar{x}}$ = ±52) cal from 12 to 40° for the α-amylase obtained from strain T. The coefficients of temperature (Q_{10}) for each of the five α-amylases are shown in Table II.

Effect of Starch Concentration on α-Amylase Activity. The effect of starch concentration on α-amylase activity was plotted according to Lineweaver and Burk (1934). The calculated K_m values are shown in Table III.

Enzyme Activity on Starch Fractions and Glycogen. Table IV shows that enzyme activity was greater with the branched-chain substrate, amylopectin, than with the straight-chain substrate, amylose. Enzyme activity with glycogen, a substrate having approximately twice as many branching points as amylopectin (Meyer and Gibbons, 1951), was similar to that with amylose. The

TABLE III: Effect of Starch Concentration on the Activity of the α-Amylase of *B. amyloliquefaciens*, Strains SB, T, F, N, and P.

Enzyme	K_m (g/l.)[a]	$s_{\bar{x}}$
SB	2.63	±0.05
T	2.22	+0.08
F	3.57	±0.04
N	2.03	±0.01
P	3.44	±0.09

[a] K_m values calculated from Lineweaver–Burk plots.

TABLE IV: Hydrolysis of Starch Fractions and Glycogen by the α-Amylase of *B. amyloliquefaciens*, Strains SB, T, F, N, and P.

Enzyme	Per Cent Activity[a]			
	Soluble Starch	Amylopectin	Glycogen	Amylose
SB	100	79.0	34.3	38.0
T	100	79.8	26.6	25.0
F	100	82.0	29.6	29.6
N	100	87.7	27.7	22.0
P	100	84.0	29.7	32.3

[a] Milligrams of maltose formed per microgram of protein per 3 min as measured by the saccharogenic assay. Substrates were dissolved in 0.02 M sodium glycerol phosphate buffer (pH 5.9). Reaction temperature was 25°. All activities are reported relative to that on starch.

TABLE V: Specific Activity on Starch of an α-Amylase of *B. amyloliquefaciens*, Strains SB, T, P, F, and N.

α-Amylase	Sp Act. (units/mg of protein)[a]	
	Av	$S\bar{x}$[b]
SB	1705	35
T	1614	16
P	1412	28
F	1947	48
N	1939	16

[a] One unit of α-amylase activity is equal to 1 mg of reducing sugar, measured as maltose, released from starch (1%) in 3 min at pH 5.9, 25°. The values are an average of twelve different samples (1 μg/ml) for each enzyme. [b] Standard deviation of the mean.

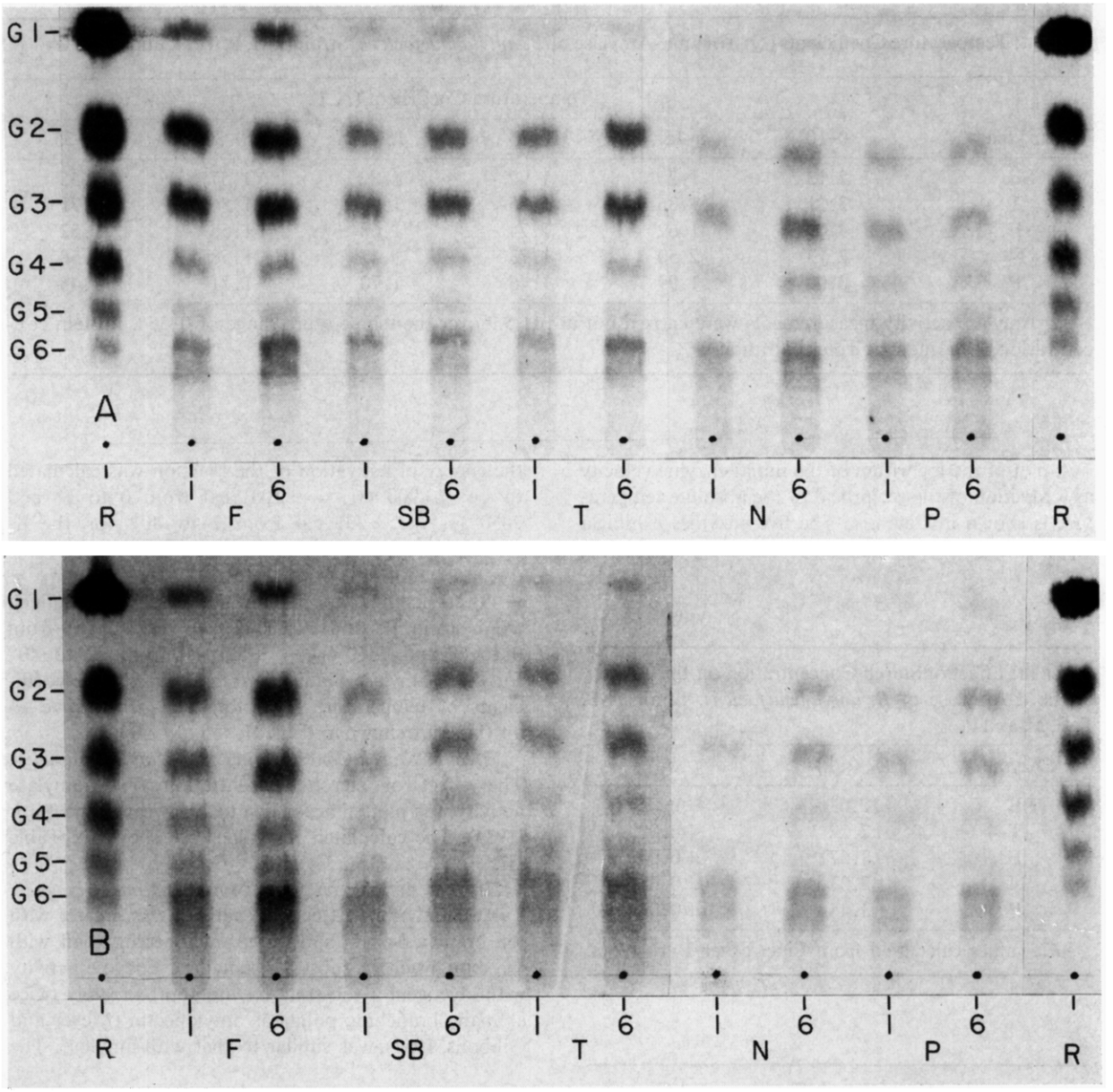

FIGURE 5: Chromatographic analysis of the digestion of starch, amylopectin, glycogen, and amylose by an α-amylase of *B. amyloliquefaciens*, strains SB, P, T, N, and F. Four ascents were made on Schleicher & Schuell No. 598 filter paper with 1-butanol–pyridine–water (6:4:3, v/v) as the solvent. Substrates were: (A) starch, (B) amylopectin, (C) amylose, and (D) glycogen. Samples of amylopectin and glycogen (0.1 ml of the 1- and 6-min digest),

five enzymes varied in their activity on amylopectin, glycogen, and amylose by 8.8, 7.7, and 16%, respectively. The specific activities of the five enzymes on starch are shown in Table V. The specific activities of the α-amylase obtained from strains F and N are similar and higher than the specific activities for the α-amylase obtained from strains SB, T, and P. The order of magnitude of the activities of the five enzymes on starch does not hold when amylopectin, glycogen, and amylose are the substrates.

A chromatographic analysis of the digestion of these substrates by the five enzymes was made to determine if the observed differences in enzyme activity on each substrate were due to a difference in the hydrolysis products released. The substrates (1%) were dissolved in 0.02 M sodium glycerol phosphate buffer (pH 5.9), equilibrated at 25° for 30 min, and mixed with the enzyme (1.6, 1.3, 1.4, 1.1, and 1.2 μg/ml of the α-amylase obtained from strains F, SB, T, N, and P, respectively). Samples (1 ml) were removed

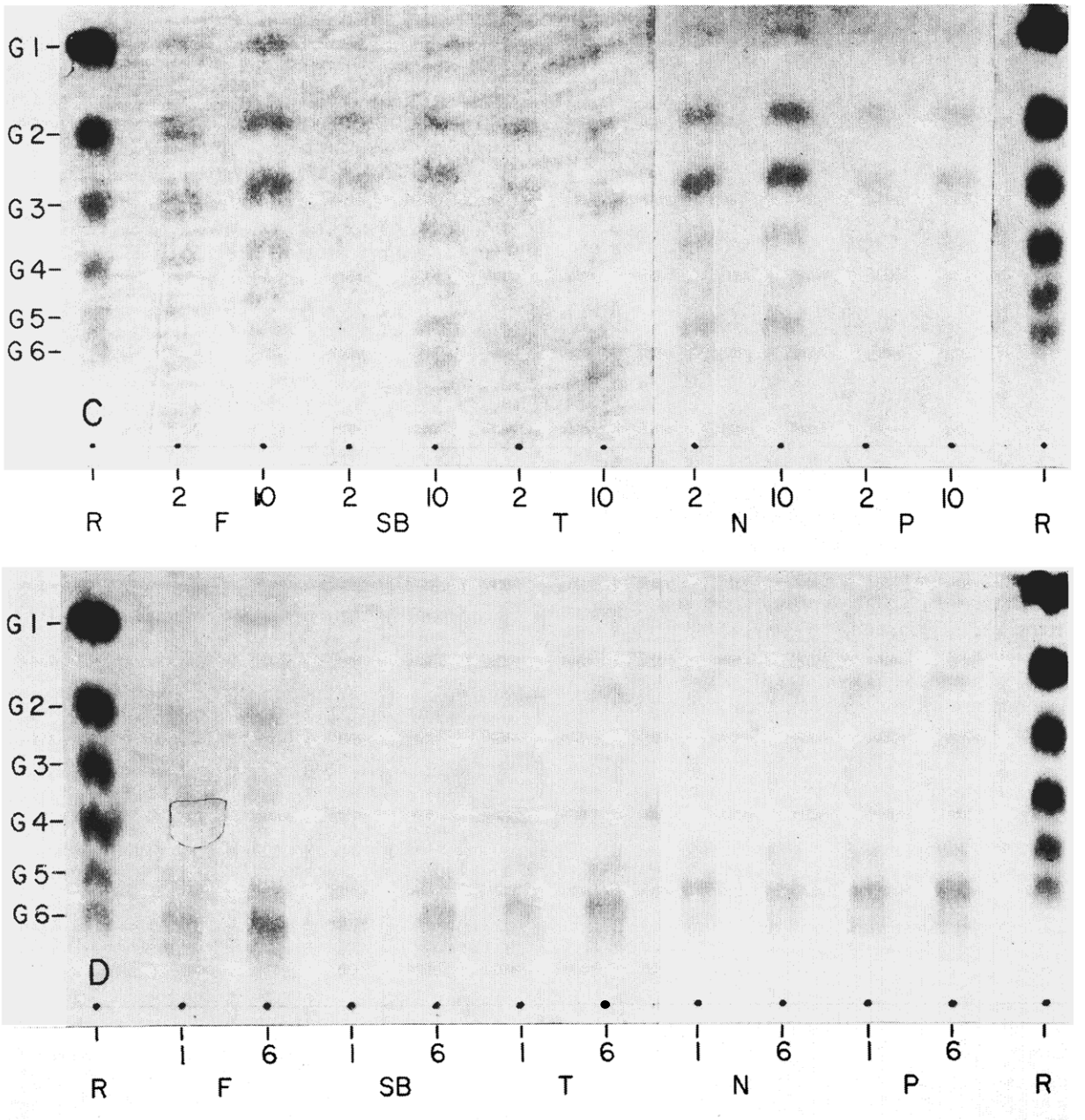

FIGURE 5 (*Continued*)
amylose (0.1 ml of the 2- and 10-min digest), and starch (0.08 ml of the 1- and 6-min digest) were spotted along the bottom of the paper. The reference sugars (R) at each side of the chromatogram consist of glucose (G1), maltose (G2), maltotriose (G3), maltotetraose (G4), maltopentaose (G5), and maltohexaose (G6).

at 1-min intervals, placed in tubes precooled (−20°) in a Dry Ice–Methyl Cellosolve bath, and stored at −20° until used. The samples were thawed and heated in a boiling water bath for 7 min, deionized with a small quantity of Amberlite MB-3 mixed-bed resin, and placed at 4° overnight. The resin was removed by filtration through Whatman No. 1 filter paper and each tube was washed twice with 0.5 ml of distilled water. The samples were lyophilized in a Virtis freeze-drying apparatus and stored at −20° until used. Each lyophilized sample was dissolved in 0.5 ml of distilled water and the sugar components were separated using multiple ascending paper chromatography, as described by Welker and Campbell (1963). The developed chromatograms are shown in Figure 5. There is no qualitative difference between the five enzymes with respect to their action on starch (Figure 5A), amylopectin (Figure 5B), amylose (Figure 5C), and glycogen (Figure 5D). The predominant initial products, with amylose and amylopectin as

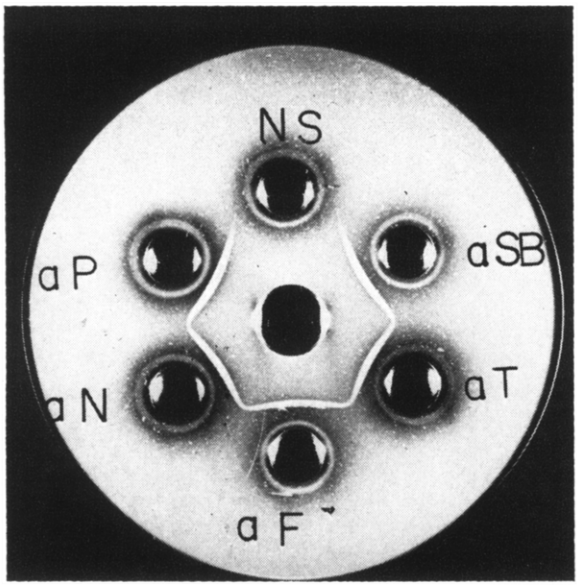

FIGURE 6: Ouchterlony plate showing immunologic relationship between the α-amylase of *B. amyloliquefaciens*, strain F, and sera against the α-amylase of strains SB, P, T, N, and F. α-Amylase (40 μg) and sera were incubated at 16° until precipitate lines appeared. NS, nonimmune sera; aSB, aT, aF, aN, and aP designate sera against the α-amylase from strains SB, T, F, N, and P, respectively.

the substrates, are G_2, G_3, G_6, and G_7. The immediate products with glycogen are G_6 and G_7.

Ultraviolet Absorption Spectrum. A sample of each of the five crystalline enzymes was dissolved in 0.02 M sodium glycerol phosphate buffer (pH 7.0, 100 μg/ml) and the ultraviolet absorption spectrum was measured in a Gilford spectrophotometer. The absorption spectra of the five enzymes were essentially identical and showed a maximum at 280 mμ with an inflection point at 290 mμ.

Immunological Comparison of the Five Enzymes. The α-amylase obtained from each of the five strains of *B. amyloliquefaciens* showed a single line of identity with each of the five immune sera. No precipitate line was observed with the nonimmune sera. A photograph of a typical Ouchterlony plate showing a line of identity for the α-amylase obtained from strain F and the five immune sera is shown in Figure 6. These results indicate that the five enzymes have a common precipitating antigenic determinant.

Discussion

A study of the crystalline α-amylase of five strains of *B. amyloliquefaciens* was conducted in order to compare the properties of the enzyme from strains of different origins as well as those reported for other bacterial amylases. The general properties of the α-amylases reported here are similar to those found by Fischer and co-workers (Menzi *et al.*, 1957; Stein and Fischer, 1960; Fischer and Stein, 1960) for the α-amylase of the Takamine strain of *B. subtilis* and by Akabori *et al.* (1956) for *B. subtilis*, strain N. This is not surprising in view of our study (Welker and Campbell, 1967a) which established that these two organisms are strains of *B. amyloliquefaciens* (designated in this study as strains T and N, respectively). In many of the properties examined (*e.g.*, electrophoretic mobility in polyacrylamide gel, pH optimum, ultraviolet absorption spectrum, and immunological properties) no significant differences were noted between the five enzymes studied. While the five enzymes appear to differ quantitatively in their activity on starch, amylopectin, glycogen, and amylose no qualitative differences were noted (Figure 5). These results are similar to those reported by Robyt and French (1963) for a commercial crystalline α-amylase of *B. subtilis*. The organism used for the commercial production of the enzyme employed by Robyt and French (1963) has been identified as *B. amyloliquefaciens*, strain N by Welker and Campbell (1967a). Pazur and Okada (1966) have demonstrated that crystalline α-amylase from the Takamine strain of *B. subtilis* is an endo-α-1,4-glucanohydrolase (liquefying α-amylase) while the α-amylase of the K-2 strain of *B. subtilis* is an exo-α-1,4-glucanohydrolase (saccharifying α-amylase). We have established that both of these organisms are strains of *B. amyloliquefaciens* (Welker and Campbell, 1967a) but we have not studied the α-amylase of the K-2 strain.

The five enzymes appear to have a common precipitating antigenic determinant as judged by Ouchterlony immunodiffusion data (Figure 6). Inoue *et al.* (1965) have reported that the α-amylases of *B. subtilis*, strains H, N, and K show cross reactions with the antiserum prepared against the α-amylase from strain H; they also reported that the α-amylase from *B. subtilis* var. *globigi* does not cross-react with strain H α-amylase antiserum. We have established that the H, N, and K strains of *B. subtilis* are actually strains of *B. amyloliquefaciens* (Welker and Campbell, 1967a). We have further shown that the α-amylase of an authentic strain of *B. subtilis* (strain W-23) does not cross-react with the antiserum prepared against the α-amylase of the five strains of *B. amyloliquefaciens* (Welker and Campbell, 1967b).

The differences reported here on the pH and temperature stability, the temperature optima, and the energy of activation between the five enzymes might be a reflection of slight alterations in the primary structure or in the conformational state of the enzymes. Owing to the fact that these enzymes are calcium metalloproteins and that they are subject to dimerization by zinc ions (Fischer and Stein, 1960; N. E. Welker and L. L. Campbell, unpublished data) and that the organisms are proteolytic, the differences noted might possibly be attributable to differences in trace-metal content or to contamination by trace amounts of protease. Studies to clarify these points are in progress.

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References

Akabori, S., Okada, Y., Fujiwana, S., and Sugae, K. (1956), *J. Biochem. (Tokyo) 43*, 741.
Fischer, E. H., and Stein, E. A. (1960), *Enzymes 4*, 313.
Fischer, E. H., and Stein, E. A. (1961), *Biochem. Prepn. 8*, 27.
Fukumoto, J. (1943), *J. Agr. Chem. Soc. 19*, 634.
Gomori, G. (1955), *Methods Enzymol. 1*, 3.
Inoue, Y., Sutou, T., and Vemura, T. (1965), *Agr. Biol. Chem. 29*, 1146.
Lineweaver, H., and Burk, D. (1934), *J. Am. Chem. Soc. 56*, 658.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Menzi, R., Stein, E. A., and Fischer, E. H. (1957), *Helv. Chim. Acta 40*, 534.
Meyer, K. H., and Gibbons, G. C. (1951), *Advan. Enzymol. 12*, 341.
Okunuki, K. (1960), *in* The Separation and Isolation of Proteins, Vol. 1, Alexander, P., and Block, R. J., Ed., New York, N. Y., Pergamon, p 32.
Pazur, J. H., and Okada, S. (1966), *J. Biol. Chem. 241*, 4146.
Postgate, J. R., and Campbell, L. L. (1963), *J. Bacteriol. 86*, 274.
Robyt, J., and French, D. (1963), *Arch. Biochem. Biophys. 150*, 451.
Stein, E. A., and Fischer, E. H. (1960), *Biochim. Biophys. Acta 39*, 287.
Stein, E. A., and Fischer, E. H. (1961), *Biochem. Prepn. 8*, 34.
Spizizen, J. (1958), *Proc. Natl. Acad. Sci. U. S. 44*, 1072.
Welker, N. E., and Campbell, L. L. (1963), *J. Bacteriol. 86*, 681.
Welker, N. E., and Campbell, L. L. (1967a), *J. Bacteriol. 94*, 1124.
Welker, N. E., and Campbell, L. L. (1967b), *J. Bacteriol. 94*, 1131.

A Phosphodiesterase from the Carrot*

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ABSTRACT: A method is given for the purification of a phosphodiesterase from carrots. The characteristics of the partially purified enzyme have been studied. The enzyme possesses exonucleolytic activity which degrades both oligodeoxyribonucleotides and oligoribonucleotides, starting from a free hydroxyl on the 3′ end and producing 5′-mononucleotides. Native deoxyribonucleic acid (DNA) is resistant while denatured DNA and transfer ribonucleic acid (tRNA) are hydrolyzed. Divalent ions (Mg^{2+}, Mn^{2+}, Ca^{2+}, and Zn^{2+}) increase activity while EDTA inhibits. The pH optimum for assay is 9.0–9.5 and the enzyme is most heat stable at a pH of 8.5–9.0. Some phosphatase activity remains after purification, but does not seem to be associated with phosphodiesterase. The similarity of this enzyme to phosphodiesterase I of animal origin is noted.

We recently required fairly large amounts of a phosphotransferase (Tunis and Chargaff, 1960) for the phosphorylation of 5-fluoropyrimidine arabinosides (Strider *et al.*, 1967). During the course of this work, we noticed the presence of appreciable quantities of phosphodiesterase activity in the carrot (*Daucus carota sativa*) extracts which were being used as a source of enzyme.

In the present paper, we describe a purification procedure leading to a 300-fold enrichment of this phosphodiesterase activity, and some of its properties. Enzymes of this type are potentially useful in nucleotide sequence studies (Lehman *et al.*, 1965), once their specificities have been established. The present carrot phosphodiesterase appears to be an exonuclease which degrades small oligonucleotides and nucleic acids lacking secondary structure, while double-stranded DNA is hardly attacked. Model oligonucleotides are degraded completely to 5′-mononucleotides, the mode of attack being 3′→5′. In this respect, the enzyme resembles such phosphodiesterases which are found in snake venom (Razzell and Khorana, 1959), hog kidney (Razzell, 1961), *Escherichia coli* (Lehman, 1963), and an enzyme recently discovered in malt (Holbrook *et al.*, 1966). Similar activities seem to have been encountered, in peas, corn, and potatoes (Razzell, 1966), but the active principles have not been purified.

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