Amylolysis of Pearl Millet Starch and Its Fractions by Pearl Millet Alpha-Amylase[1]

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ABSTRACT

α-Amylase was isolated from germinated pearl millet and purified by glycogen-amylase complex formation. Germination resulted in a 120-fold increase in specific activity of the enzyme over that of α-amylase from mature grain. Amylolysis of millet starch and its fractions by purified α-amylase was studied by gel filtration. Raw millet starch was resistant to attack by α-amylase from germinated millet. Only limited degradation (26%) occurred after starch was incubated with the enzyme for 24 hr at 35° C. Millet amylose was readily hydrolyzed by purified millet α-amylase. During a 10-min reaction period at 35° C, 36% of the molecule was degraded to molecular weights of 40,000-10,000. Conversely, some portions of millet amylopectin were hydrolyzed slowly by α-amylase.

As indicated by several recent reviews on the chemistry and nutrition of pearl millet (*Pennisetum americanum* (L.) Leeke), few data are available on millet amylases or the action of amylases on millet starch (Hoseney and Varriano-Marston 1980, Hoseney et al 1981, Hulse et al 1980).

Germinated pearl millet is used for preparing processed foods for children, sweet goods, malt vinegar, and beer (Asha et al 1976a, Vogel and Graham 1978). During grain germination, the α-amylase activity increases dramatically, as much as 1,000-fold for some grains (Barrett 1975). As with other cereal grains, α-amylase is likely to play an important role in determining the quality of millet-based products. Therefore, the objectives of this study were to isolate and purify α-amylase from germinated pearl millet and to determine the molecular size of millet starch and starch fractions after hydrolysis by purified millet α-amylase.

MATERIALS AND METHODS

Materials

The pearl millet used in this study was HMP 700 (Hays millet population) grown in Hays, Kansas, in 1980.

Germination

Pearl millet (120-150 g) was cleaned and sieved (40-mesh sieve) to obtain uniform kernel size. Broken kernels were removed by hand. Sound grains were steeped for 12-14 hr at room temperature in deionized water containing 0.3% chlorox to prevent microbial growth. The chlorox solution was changed three times during the steeping stage. Samples (30-g) were placed in Pyrex test tubes (6 × 1 in.) and stoppered with a cork having a 3/16-in.-diameter hole, or 80-100-g samples were placed on wet filter paper in large trays. The grains were germinated at room temperature until sprouts were visible (1-3 days). After sprouting, samples were frozen and freeze-dried. Rootlets were removed from the dry samples by rubbing the sprouted grains between the hands and sifting the material through a 40-mesh screen. Samples were then milled on a Udy Cyclone Mill.

Purification of Glycogen

Protein contaminating shellfish glycogen was removed by the method of Loyter and Schram (1962). The reagent was kept frozen until used.

Isolation of α-Amylase from Germinated Pearl Millet

α-Amylase was extracted from germinated millet using 0.05*M* sodium acetate buffer (pH 5.0) containing 0.01*M* $CaCl_2$. The ratio of buffer to meal was 2.5:1; extraction time was 2 hr. The slurry was centrifuged for 15 min at 4,000 rpm and the precipitate discarded.

The filtered supernatant was made to 0.2% with calcium acetate and the pH was adjusted to 6.0. The extract was divided into 15-ml fractions and heated at 70° C for 15 min to inactivate β-amylase, followed by rapid cooling in an ice bath and filtration. The filtrate was dialyzed overnight at 4° C against 0.2% calcium acetate, the insoluble material was removed by filtration, and the filtrate was freeze-dried.

The freeze-dried sample was dissolved in the same acetate buffer that was used in the extraction step. Purification of α-amylase was completed by forming an amylase-glycogen complex according to the method of Loyter and Schramm (1962). Details of the isolation procedure are diagrammed in Fig. 1.

α-Amylase Activity

A modification of the Barnes and Blakeney (1974) method was used to determine α-amylase activity in crude and purified extracts. Commercial Phadebas tablets were not used. Forty milligrams of cross-linked potato amylose covalently labeled with Cibachron blue (Pharmacia, Piscataway, NJ) was added to 5 ml of enzyme-buffer solution (0.05*M* acetate, pH 5.0, containing 0.01*M* $CaCl_2$) held at 35° C. The digest was incubated for 15 min, shaken every 5 min, and then 1 ml of 0.5*N* NaOH was added to terminate the reaction. The volume of the digest was diluted to 10 ml with water, filtered (Whatman No. 4 filter paper), and the absorbance was read at 620 nm. The absorbance reading was converted to units of α-amylase activity by use of a standard curve prepared from data obtained by the reaction of various concentrations of purified millet α-amylase with the amylose-Cibachron blue substrate under the described conditions. Enzyme activity (units of activity) was expressed as μmol of apparent maltose produced per milliliter of α-amylase solution per minute. Specific activity was expressed as units (U) of α-amylase per milligram of protein. Protein content of the enzyme extracts was determined by Miller's (1959) method.

In the starch, amylose, and amylopectin digestion studies, the activity of purified α-amylase used in the digest was determined by the method of Robyt and Whelan (1968). After enzyme digestion, reducing sugars in the digest were determined by Nelson's colorimetric copper method (1944) using a maltose standard curve.

Sodium Dodecyl Sulfate (SDS) Gel Electrophoresis

The method of Weber and Osborn (1969) was used to determine the molecular weight of purified α-amylase.

pH and Temperature Optima

Optimal pH for activity of purified α-amylases from millet was determined using a soluble starch substrate and acetate or tris buffers (containing 0.01*M* $CaCl_2$) of pH 3.0-7.2. The method of Robyt and Whelan (1968) was used, except that the temperature was maintained at 35° C. The temperature optimum was determined at the optimal pH using 0.02*M* acetate buffer containing 0.01*M* $CaCl_2$.

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Starch Isolation and Fractionation

Starch was isolated from pearl millet as described by Beleia et al (1980). Millet starch was fractionated into amylose and amylopectin by the methods of Montgomery and Senti (1958). Iodine affinities of pearl millet amylose and amylopectin, determined according to Schoch (1964), were 18.7 and 1.42%, respectively.

Hydrolysis of Starch and Starch Fractions

Raw Starch. Fifty milligrams of defatted millet starch was suspended in 5 ml of 0.05*M* acetate buffer, pH 5.0, containing 0.01*M* $CaCl_2$. Purified α-amylase (270 or 540 U) was added, and the suspension was incubated for 24 hr at 35° C. After incubation, the suspension was centrifuged at 5,000 rpm for 10 min, the supernatant was poured off, the starch pellet was dissolved in 3 ml of 1*N* NaOH, and an aliquot containing 10 mg/ml was applied to a Sepharose 2B Cl column and eluted.

After the supernatant was filtered through a 0.45-μm filter and boiled to inactivate the enzyme, reducing sugars were determined in the filtrate by Nelson's (1944) method. Maltose was used as a standard curve. Percent hydrolysis was calculated as follows:

$$\text{Percent hydrolysis} = \frac{\text{Reducing sugars (maltose equiv.) in digest}}{\text{Total CHO (maltose equiv.) in digest}} \times 100.$$

Amylopectin and Amylose. Millet starch or its fractions (20 mg) were dissolved in 1*N* NaOH, and the pH of the solutions was adjusted to 6.0 with 1*N* HCl. Buffer (0.05*M* acetate, pH 5.0, 0.01*M* $CaCl_2$) was added to make a total volume of 5 ml, purified millet α-amylase was added (27 U), and the mixture was incubated for 5 to 15 min at 35° C. After incubation, the mixture was boiled to inactivate the enzyme, and an aliquot containing 10 mg was applied to a Sepharose 4B Cl column (amylose), a Sepharose 2B Cl column (amylopectin), or Bio-Gel P10 (amylopectin).

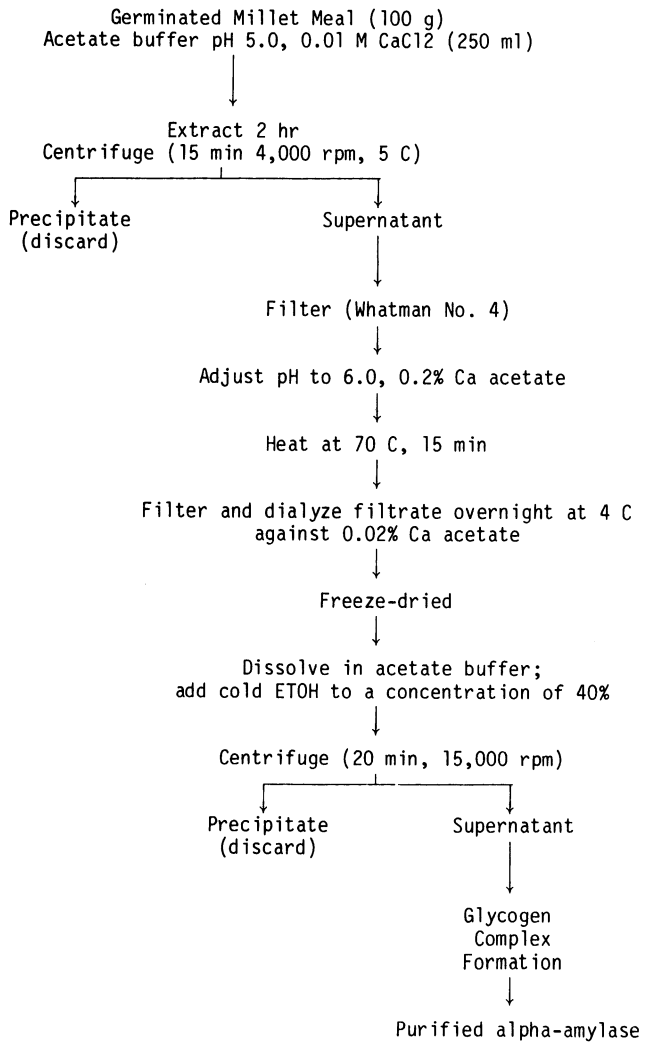

Fig. 1. α-Amylase isolation procedure.

TABLE I
α-Amylase Purified from Germinated Pearl Millet

Fraction	U/ml[a]	Protein mg/ml	Specific Activity U/mg	Volume (ml)	Recovery[b] (%)
Buffer extract	1,235	10.8	114	123	100
Heat-treated extract	1,025	9.4	109	85	57
Glycogen complex	1,359	0.7	1,928	12	11

[a] $U = \frac{\mu g \text{ maltose/ml/min}}{342} \times 2.$

[b] $\frac{U \text{ ml}-1 \times \text{volume}}{\text{Total activity of buffer extract}} \times 100.$

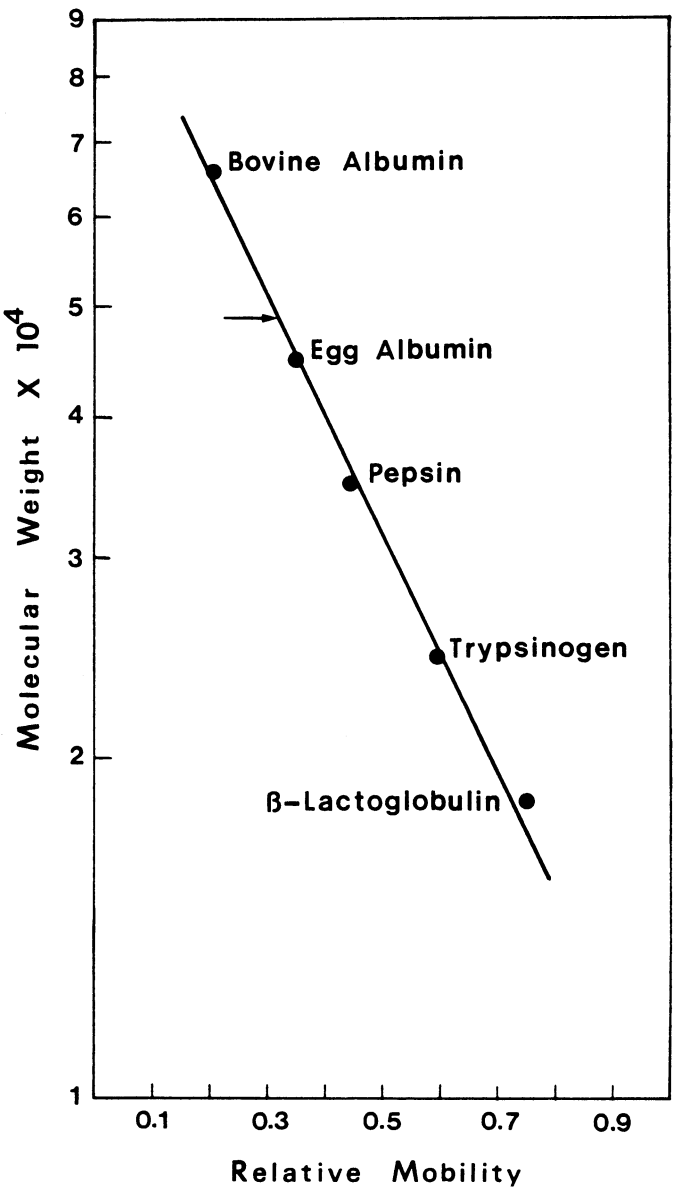

Fig. 2. Molecular weight of purified α-amylase (arrow) as determined by sodium dodecyl sulfate gel electrophoresis. Standards are labeled.

Gel Filtration Chromatography

Columns (2.6 × 76 cm) were packed with Sepharose 2B Cl, Sepharose 4B Cl (Pharmacia, Uppsala, Sweden) or Bio-Gel P10 (Bio-Rad, Richmond, CA) in 0.01*N* NaOH containing 0.02% sodium azide. Ten milligrams of carbohydrate was loaded onto the column, the column was eluted in an ascending direction with 0.01*N* NaOH containing 0.02% sodium azide, and 5-ml fractions were collected at a flow rate of 25 ml/hr. Total carbohydrate in the fractions was determined by the phenol-sulfuric acid method (Dubois et al 1956) using a maltose standard curve. Molecular weight calibrations on all columns were determined using dextran standards (Sigma Chemical Co., St. Louis, MO).

RESULTS AND DISCUSSION

Properties of Purified α-Amylase

The α-amylase activity of the crude extract was substantially reduced as a result of the heat treatment (Table I); only 57% of the activity was recovered. At the glycogen complex stage, 11% of the enzyme was recovered with a 17-fold increase in specific activity. Germination caused a 120-fold increase in specific activity over that observed for α-amylase purified from mature pearl millet (Beleia and Varriano-Marston 1981a).

SDS gels of α-amylase from germinated pearl millet purified by glycogen complex formation showed one protein band at a molecular weight of 48,000 (Fig. 2). That value falls within the range reported for α-amylases from malted grains (Greenwood and Milne 1968, MacGregor 1978, Tkachuk and Kruger 1974).

Optimum pH for α-amylase from malted millet was 4.0–4.5 (Fig. 3), or slightly lower than the 4.4–4.8 range reported for α-amylases from mature millet (Beleia and Varriano-Marston 1981a). The acid tolerance of millet α-amylase may be important in preparing some fermented millet foods, eg, *kisra*, in which the pH drops to about 4 in 18 hr (El Tinay et al 1979).

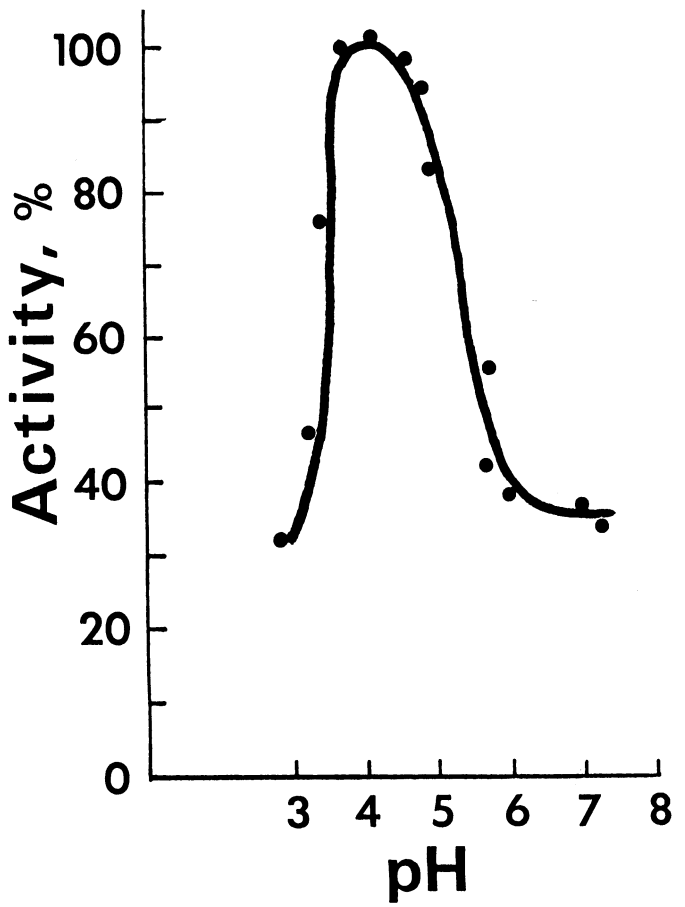

Fig. 3. pH optima for pearl millet α-amylase, expressed as percent of maximum activity.

Temperature optimum for α-amylase from malted millet was determined at pH 4.2 with 0.02*M* acetate buffer containing 0.01*M* $CaCl_2$. Enzyme activity reached maximum at 45–55°C (Fig. 4). Only 75% of the activity remained after heating to 65°C.

α-Amylolysis of Millet Starch and Starch Fractions

Raw Starch. The molecular weight of millet starch (dissolved in 1*N* NaOH) is so large (more than 20×10^6) that most of the sample elutes at the void volume (V_o) of the Sepharose 2B Cl column (Fig. 5). Calibration of the column with standard dextrans showed that the number of starch fragments having molecular weights between 70,000 and 500,000 increased after 24-hr hydrolysis of raw starch by millet α-amylase. In addition, starch fragments appeared at the V_t (total volume) of the column and probably consisted of the lower molecular weight products formed during the initial stages of amylolysis. The limited starch hydrolysis (26%) after 24-hr digestion indicated that raw millet starch is quite resistant to degradation by pearl millet α-amylase. Beleia and Varriano-

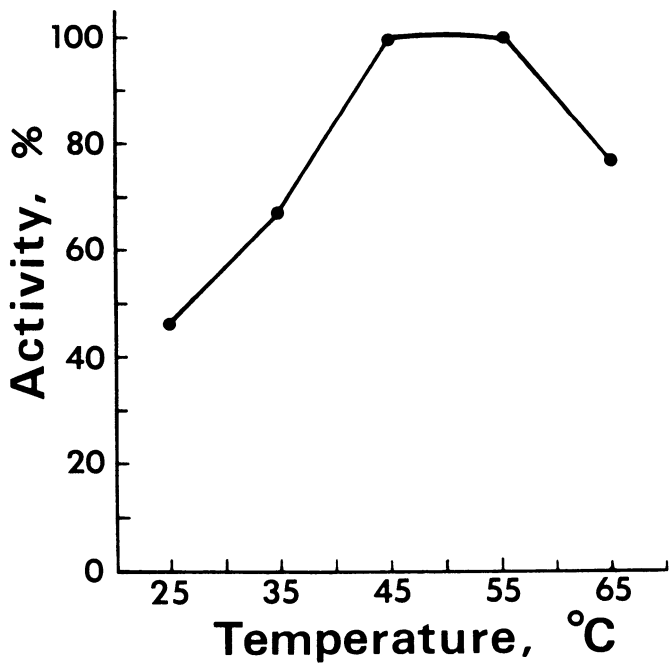

Fig. 4. Temperature optima for pearl millet α-amylase.

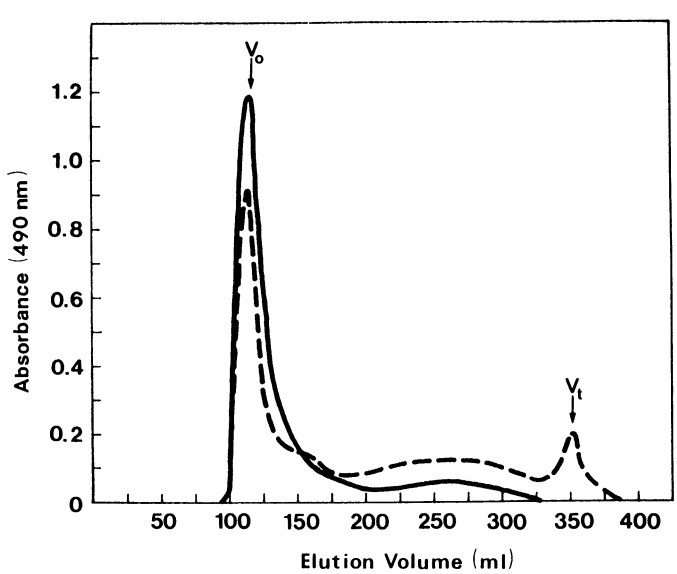

Fig. 5. Elution profile of untreated millet starch (——) and starch hydrolyzed (26%) by millet alpha-amylase (----). Sepharose 2B Cl column.

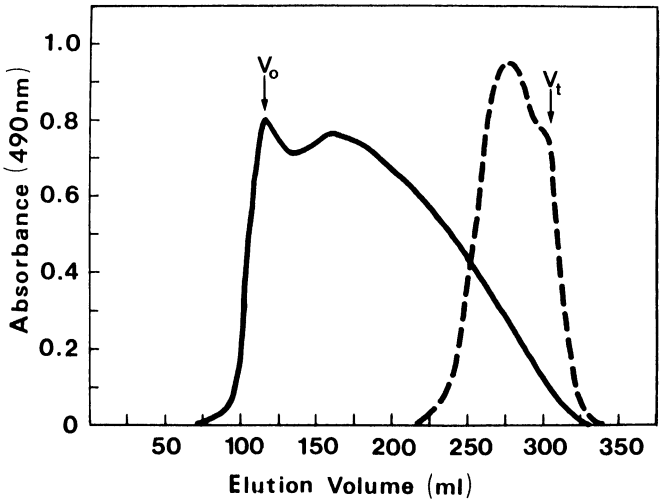

Fig. 6. Sepharose 4B Cl elution profile of millet amylose before (——) and after 36% hydrolysis by millet α-amylase (----).

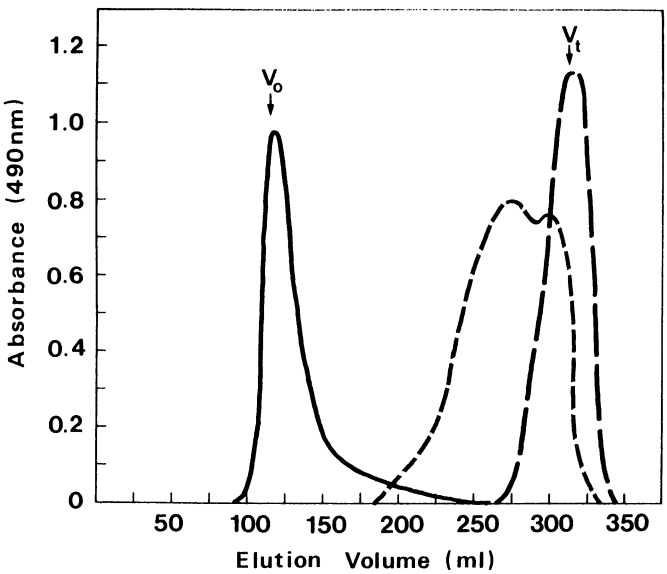

Fig. 7. Sepharose 2B Cl elution patterns of millet amylopectin before (——) and after hydrolyzed 12% (----) and 35% (— —) by millet α-amylase.

Marston (1981b) found that raw wheat starch was more easily digested by millet amylases than was raw millet starch, suggesting that the latter starch has a more ordered arrangement of molecules.

Amylose. The elution pattern of millet amylose from Sepharose 4B Cl is shown in Fig. 6. A portion of the amylose eluted at the V_o, indicating that those particles have molecular weights greater than 5×10^6 (Pharmacia 1975). Because millet amylose had a high iodine affinity (18.7%), the sample probably was not contaminated with enough amylopectin to produce such a large peak at the V_o. Millet amylose may have a limited amount of branching, such as that observed for other starches (Banks and Greenwood 1966). The β-amylolysis limit of millet amylose as determined by Whelan's (1964) method averaged 73.9% compared with 67.2% for a wheat amylose fractionated from wheat starch by the same procedure. The incomplete conversion of amylose to maltose by β-amylase might be evidence of branching in amylose.

When millet amylose was incubated for 10 min at 35° C with 27 U of pearl millet α-amylase, 36% of the amylose was hydrolyzed. Fragments having molecular sizes ranging from 10,000 to 40,000 (Fig. 6) were produced, but the majority had molecular weights of about 10,000 as estimated by column calibration with standard dextrans.

Amylopectin. Millet amylopectin had an average chain length

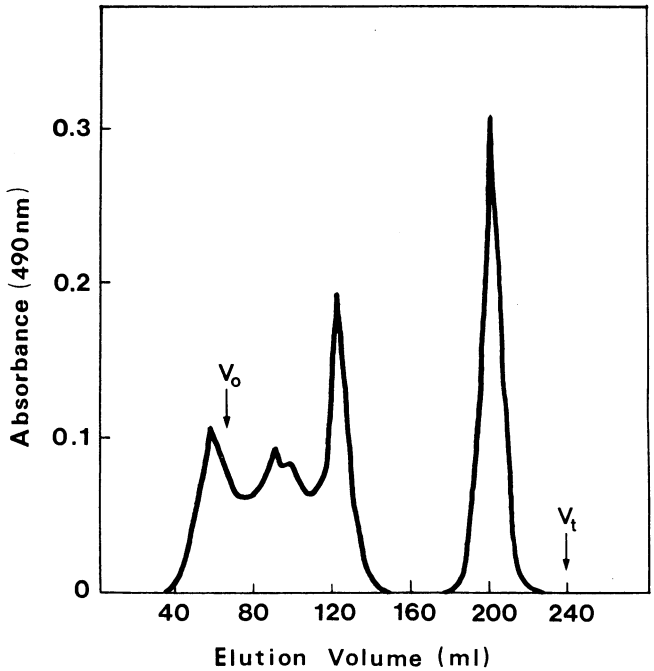

Fig. 8. Bio-Gel P10 elution profile of millet amylopectin hydrolyzed 35% by millet α-amylase.

($\overline{C.L.}$) of 28, as determined by the method of Gunja-Smith et al (1970). This is larger than the $\overline{C.L.}$ of 17 we obtained for wheat amylopectin prepared by the method of Montgomery and Senti (1958).

Millet amylopectin eluted as a single peak at the V_o of a Sepharose 2B Cl column (Fig. 7). Hydrolysis by pearl millet α-amylase (27 U, 15 min) produced particle sizes ranging from 70,000 to 500,000. Longer digestion (35%) produced one peak eluting at the V_t of the column (Fig. 7). The fractions in that peak were pooled and applied to a Bio-Gel P10 column (Fig. 8). Part of the millet amylopectin was resistant to amylolysis because some large molecular weight products still eluted at the V_o of the column (>17,000 mol wt). The majority of fragments had molecular weights ranging from 5,000 to 10,000. Digestion to 70% gave much smaller fragments, so the resistant bonds in millet amylopectin appear to be broken with longer digestion.

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