Debranching of Wheat Amylopectin[1]

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ABSTRACT

Gel chromatographic evidence indicates that the bimodal distribution of chains obtained from debranching wheat amylopectin probably results from a single, normal population of amylopectin molecules. Amylopectin β-limit dextrin has a normal distribution when eluted from a Sepharose CL-4B column. When aliquots from the leading and trailing edges of this distribution were debranched with pullulanase and subjected to Bio-Gel P-10 chromatography, debranched amylopectin's bimodal distribution was preserved. A third peak, composed of maltose and maltotriose derived from β-amylase-hydrolyzed A chains, was also observed. In addition, comparison of the chain profiles of native amylopectin and β-limit dextrin showed that the peak corresponding to the shorter average chain length material (amylopectin) was hydrolyzed more than the peak corresponding to the longer average chain length material (β-limit dextrin). The modes of attack of pullulanase and isoamylase on amylopectin also differed. Pullulanase yields the bimodal distribution of chains throughout the degradation of amylopectin. Isoamylase, however, quickly reduces the polymer to intermediate fractions, which are in turn further debranched to form the bimodal distribution.

Incubation of wheat amylopectin with pullulanase (pullulan 6-glucanohydrolase, EC 3.2.1.41) or isoamylase (glycogen 6-glucanohydrolase, EC 3.2.1.68) followed by gel filtration of the debranced fragments yields a bimodal distribution of carbohydrate. The smaller, leading peak contains material with a degree of polymerization (DP) of 52–60, and the second peak contains material of DP 11–25 (Lii and Lineback 1977). This bimodal distribution is generally assumed to derive from a single, completely debranched, amylopectin population. Several proposed models of amylopectin account for two populations of chains by displaying the short chains arranged in clusters throughout a basic structure composed of long chains (French 1975; Robin et al 1974, 1975).

Other explanations for the origins of this bimodal distribution are feasible. Incomplete debranching, not detected earlier, is one possible cause. A bimodal distribution also might arise from the debranching of two distinct populations of polysaccharide. The latter was suggested (Marshall 1974) but never proved. Consequently, one purpose of this study was to investigate possible explanations for the bimodal distribution of glucose chains obtained when wheat amylopectin is incubated with pullulanase or isoamylase.

Pullulanase and isoamylase have different specificities towards certain substrates (Gunja-Smith et al 1970b, Marshall 1974). The actions of the two enzymes on amylopectin have been compared (Harada et al 1972), but the differences in their modes of attack are not fully understood. Consequently, a second purpose of this study was to determine specifically the modes in which pullulanase and isoamylase debranch wheat amylopectin.

MATERIALS AND METHODS

Starch Isolation

After Scout hard wheat flour was milled from grain on a Buhler experimental mill, prime starch was isolated from the flour by the dough-kneading procedure (Wolf 1964). The aqueous slurry obtained from handwashing the dough ball was centrifuged (2,000 × *g*, 15 min) and the tailings layer was removed by scraping with a spatula. The prime starch was reslurried in water and recentrifuged three additional times. Recovered prime starch was dried in a forced air oven at 40–45° C.

Starch Fractionation

Scout prime starch was separated into amylose and amylopectin by Schoch's procedure (1957). Amylose was initially precipitated with *n*-pentanol and collected with a Sharples centrifuge. Amylopectin then was collected by precipitating the supernatant with methanol. The iodine affinity values of the amylopectin as determined by potentiometric titration compared well with those of Lii and Lineback (1977).

Preparation of Amylopectin β-limit Dextrin

The β-limit dextrin was prepared by treating 1 g of amylopectin dispersed in 50 ml of acetate buffer (0.02*M*, pH 5.0) with 4,500 units (one unit will hydrolyze 1.0 mg of maltose from starch in 3 min at pH 4.8 at 20° C) of sweet potato β-amylase (crystalline, Type 1-B, Sigma Chemical Co., St. Louis, MO) at 37° C with dialysis to remove maltose (Marshall and Whelan 1974, Whelan 1964).

Debranching with Isoamylase

Cytophaga isoamylase (Lytic enzyme, L1, BDH Biochemicals Ltd., Poole, England) was partially purified by the method of Gunja-Smith et al (1970b) by dispersing the commercial preparation in cold distilled water (4° C). The mixture was centrifuged at 40,000 × *g* and the supernatant was lyophilized. The resulting powder was void of any α-1,4-glucosidase activity (Drummond et al 1971) and had an activity of 0.0078 units per milligram, measured as reported by Gunja-Smith et al (1970b).

Digests contained 50 mg of amylopectin and 0.27 unit of isoamylase in 10 ml of acetate buffer (0.1*M*, pH 5.5). Total debranching was achieved by incubating this digest at 37° C for 24 hr. To stop the reaction, the digest was immersed in a boiling water bath for 20 min and filtered to remove the coagulated enzyme (Lii and Lineback 1977).

Debranching with Pullulanase

Enterobacter aerogenes pullulanase, obtained from the Sigma Chemical Co., St. Louis, MO, as a suspension in 3.2*M* ammonium sulfate, was used without modification. Analysis indicated no α-1,4-glucosidase activity (Drummond et al 1971).

Digests contained 20 mg of amylopectin or β-limit dextrin and 3.2 units of pullulanase (one unit will liberate 1.0 μmol of maltotriose from pullulan per minute at pH 5.0 at 25° C) in 2 ml of acetate buffer (0.1*M*, pH 5.5). Complete and partial debranching were performed as described for isoamylase (Lii and Lineback 1977).

Gel Filtration of Polysaccharides

Pharmacia K26/70 columns were packed with Sepharose CL-2B (amylopectin) and Sepharose CL-4B (β-limit dextrin) and then eluted in an ascending direction with 0.01*M* sodium hydroxide containing 0.02% sodium azide. The columns had a V_t of about 290 ml. Five milligrams of carbohydrates were loaded on the column, and 8-ml fractions were collected at 30-min intervals (Boyer et al

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1976, Eberman and Schwarz 1975). Carbohydrates were located in the effluent fractions by assaying an aliquot of each fraction by the phenol sulfuric acid procedure (Dubois et al 1956).

Gel Filtration of Enzyme Digests

Pharmacia K26/70 columns were packed with Bio-Gel P-10 (200–400 mesh) for pullulanase-incubated amylopectin and β-limit dextrin and isoamylase-incubated amylopectin. Bio-Gel P-4 (−400 mesh) was used for pullulanase- and-β-amylase-incubated amylopectin. The digests were eluted in an ascending direction with distilled water containing 0.02% sodium azide (Lii and Lineback 1977). Fractions (4 ml) were collected on the drop mode at flow rates of approximately 15 ml/hr. Effluent fractions were analyzed as described for the gel filtration of the polysaccharides.

RESULTS AND DISCUSSION

Origins of Bimodal Distribution

We considered three possible explanations for the origins of the bimodal distribution of carbohydrate obtained from debranching wheat amylopectin: 1) incomplete debranching, due to areas of dense branching in the amylopectin molecule, results in one population of linear chains and one population of branched fragments; 2) the two distributions of carbohydrate result from debranching two distinct populations of amylopectin; 3) two populations of chains exist in a single amylopectin molecule.

Gunja-Smith et al (1970a), Lee et al (1968) Lii and Lineback (1977), and Marshall (1974) indicated that bimodal distribution occurs after total debranching of amylopectin (Fig. 1). Pullulanase-incubated or isoamylase-incubated amylopectin was further incubated with β-amylase to determine the completeness of debranching. Typical values of conversion to maltose were 95% for pullulanase and 100% for isoamylase. A small quantity of pullulanase-resistant linkages therefore remained. However, the resultant branched material appears in the void volume of a Sephadex G-50 gel chromatogram (Marshall 1974, Whelan 1971). The carbohydrate eluted after the void volume has the same bimodal distribution regardless of the debraching enzyme used and is generally assumed to be composed of totally linear chains (Robin et al 1974).

Despite this data, we considered the possibility that the leading peak (peak 1 of Fig. 1) was composed of undebranched material. Peak 1 contains material with a $\overline{DP}$ roughly three times that of peak 2 (Lii and Lineback 1977). Thus, peak 1 could result from three linear chains bound together by two close branch points unsusceptible to pullulanase and isoamylase. Further support for this hypothesis comes from the isolation of doubly and triply branched oligosaccharides after rigorous treatment of amylopectin with α-amylase (French 1975). The close proximity of these branch points perhaps inhibits the action of the debranching enzymes. The 100% β-amylolysis limit for isoamylase-debranched amylopectin is explainable. The carbohydrate comprising peak 1 represents approximately 25% of the total. If peak 1 were composed of branched material, the smallest fragment that could remain after β-amylase degradation would have a $\overline{DP}$ of approximately 10. Therefore, the $\overline{DP}$ of peak 1 could conceivably be reduced more than 80% from about 60 to 10. Consequently, if all of peak 2 and 80% of peak 1 were totally converted to maltose, then 95% of the carbohydrates in the bimodal distribution would be converted to maltose. This percentage was considered close enough to the reported values to warrant examination.

To test that hypothesis, isolated peak 1 material was subjected to β-amylase degradation. This digest was subjected to chromatography on Bio-Gel P-4 to separate the resultant maltose from any possible branched oligosaccharides (Fig. 2). Only maltose was obtained. Chromatography of glucose and maltotetraose assured that the separation would have been made. Hence, it is apparent that incomplete debranching is not the cause of the bimodal distribution.

Another explanation for the origin of the bimodal distribution is that two separate populations of starch components are debranched. Possibly only peak 2 of Fig. 1 is derived from amylopectin. Debranching of a starch component intermediate in structural properties between amylose and amylopectin may account for peak 1. Evidence for the existence of this type of species is limited, although intermediate material has been isolated from wheat starch (Banks and Greenwood 1975, Perlin 1958).

Chromatography on agarose gels was attempted in order to display the molecular weight distribution of Scout amylopectin. An intermediate material responsible for peak 1 would have a longer average chain length and perhaps different gel chromatographic behavior than would normal amylopectin. Unfortunately the exclusion limit of Sepharose 2B was too low to provide a true representation of the molecular weight distribution because most of the carbohydrate was eluted in the void volume of the column.

An indirect method of testing the intermediate material hypothesis was therefore devised. Scout β-limit dextrin was debranched with pullulanase and subjected to Bio-Gel P-10 gel filtration. The resultant chromatogram shows a trimodal distribution of carbohydrate (Fig. 3). In addition to peaks 1 and 2, a third peak (peak 3), corresponding to the maltose and maltotriose derived from the β-amylase-hydrolyzed A chains, is also present.

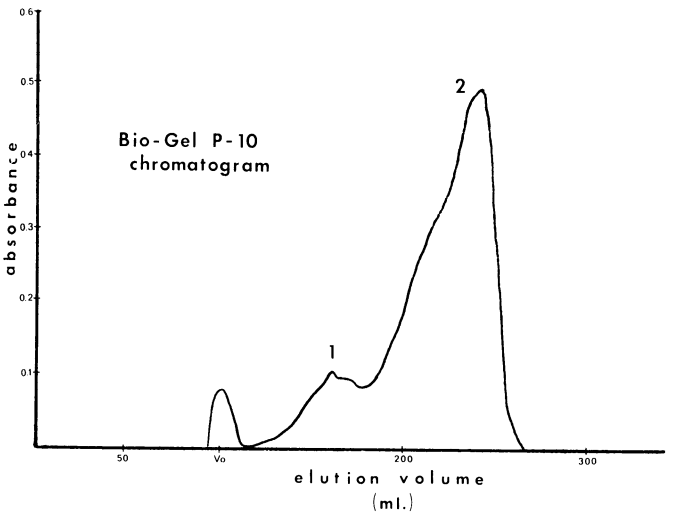

Fig. 1. Bio-Gel P-10 fractionation of the products of pullulanase debranching of amylopectin.

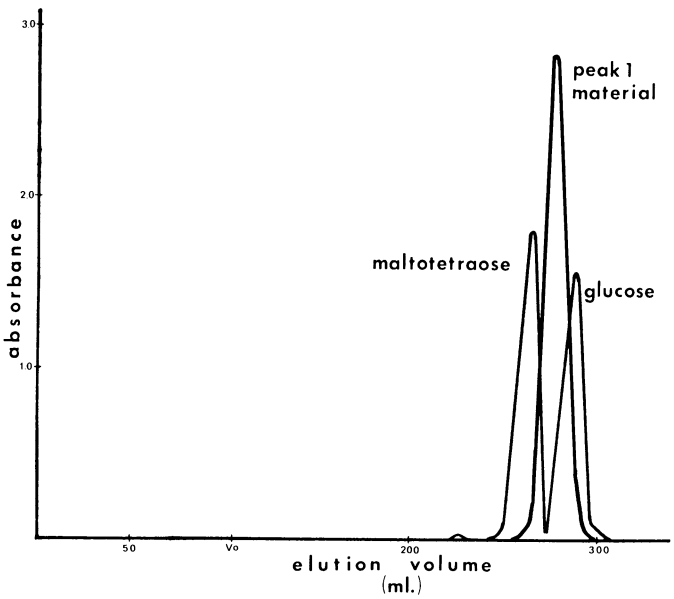

Fig. 2. Bio-Gel P-4 fractionation of the products of β-amylolysis of isolated peak 1 carbohydrates (shown in Fig. 1). Glucose and maltotetraose standards are also shown.

Further differences in the chain profiles of amylopectin and its β-limit dextrin may be observed. Peak 1 is larger relative to peak 2 for the β-limit dextrin than it is for amylopectin. The chain profile of the β-limit dextrin is essentially representative of the interior chains of amylopectin, which suggests that peak 2 of the bimodal distribution of amylopectin contains most of the exterior chains. Akai et al (1971) also drew this conclusion from similar data.

Chromatography of the β-limit dextrin on Sepharose CL-4B yielded a normal distribution (Fig. 4, top). The resolution of Sepharose columns may not be high in this case, so leading and trailing edge material obtained from this normal distribution was incubated with pullulanase and subjected to Bio-Gel P-10 chromatogrpahy (Fig. 4, bottom). Almost identical distributions were obtained. This is strong though not conclusive proof that both peaks of the bimodal distribution arise from the same polysaccharide. Therefore, the bimodal distribution of chains obtained from debranching wheat amylopectin probably results from two populations of chains existing within the amylopectin molecule.

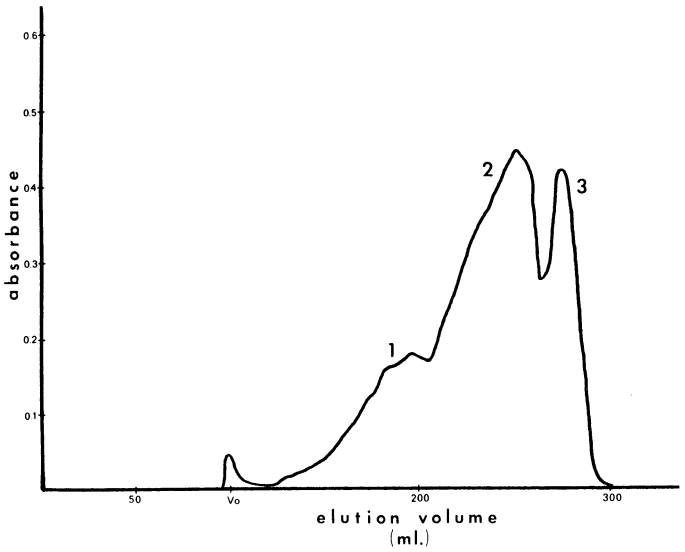

Fig. 3. Bio-Gel P-10 fractionation of the products of pullulanase debranching of amylopectin β-limit dextrin.

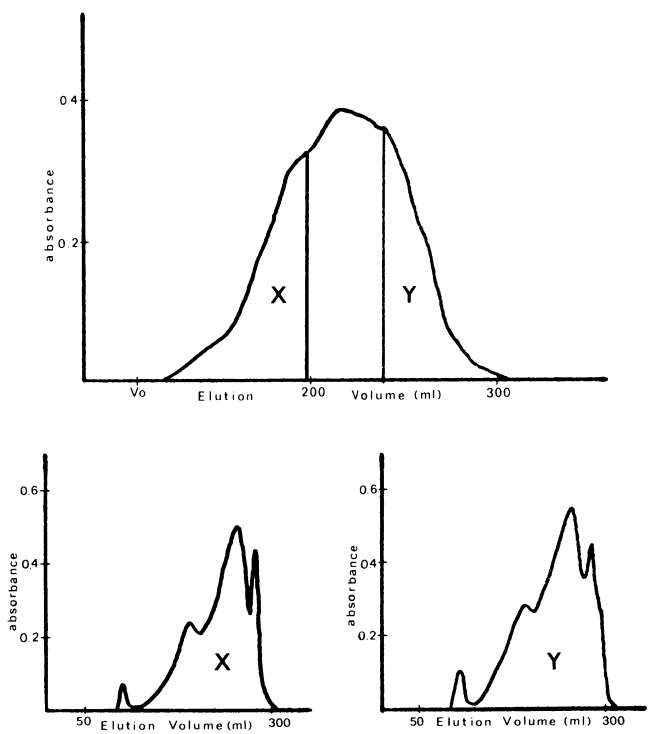

Fig. 4. Elution profile of amylopectin β-limit dextrin (intact) on a Sepharose CL-4B column **(top)**. Bio-Gel P-10 fractionations of the products of pullulanase debranching of areas X **(lower left)** and Y **(lower right)**.

Mode of Attack of Debranching Enzymes

To study the actions of the debranching enzymes on amylopectin, we obtained chromatograms of digests at various points in the degradations (Figs. 5 and 6). Because several different modes of attack by these enzymes are possible, such data must be interpreted cautiously. One possibility is that one enzyme molecule completely debranches one amylopectin molecule before proceeding to the next one. If so, Bio-Gel P-10 chromatograms of the debranched fragments would show only the products of total debranching, and the bimodal distribution would be obtained. Sepharose chromatograms of the undigested amylopectin would show no shifts toward smaller sizes. A second mode of attack could be a stepwise degradation of all amylopectin molecules from the exteriors to the centers. In this case, the chromatograms of the debranched fragments would show abundant exterior chains, peak 2 material, in the early stages of degradation. Furthermore, because all amylopectin molecules would be affected equally, a shift towards lower molecular size would be observed in the Sepharose chromatograms of the incompletely debranched amylopectin. Consequently, by examining shifts in the Sepharose chromatograms of the undigested amylopectin, the mode of attack with respect to different molecules of amylopectin could be determined. Similarly, by examining Bio-Gel P-10 chromatograms of the debranched fragment, the mode of attack with respect to different

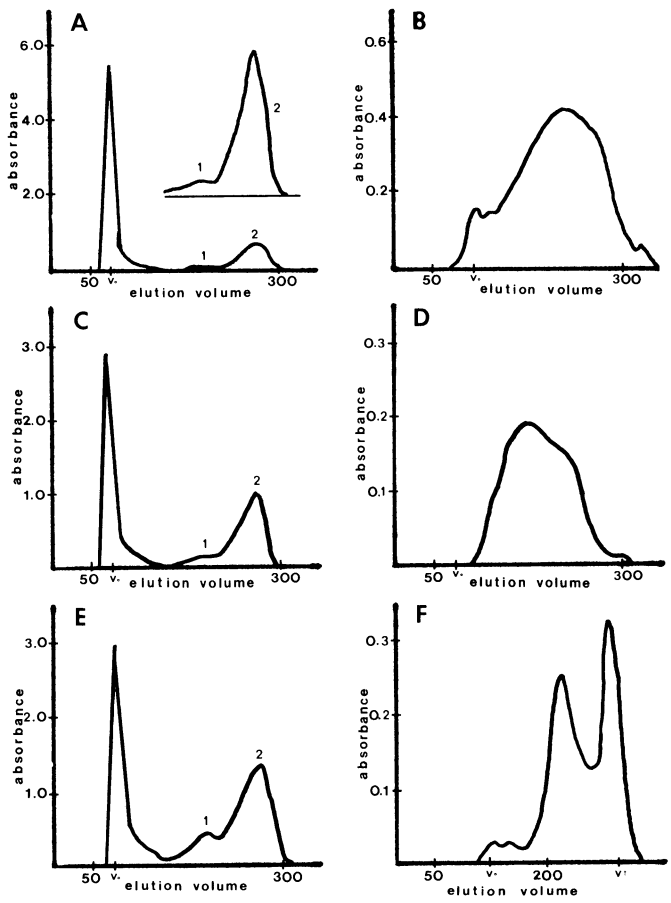

Fig. 5. Bio-Gel P-10 fractionations of the products of pullulanase debranching of amylopectin at 30% **(A)**, 47% **(C)**, and 70% **(E)** degradation. Elution profiles of the corresponding void volume carbohydrates on Sepharose CL-2B **(B)**, Sepharose CL-4B **(D)**, and Sepharose CL-6B **(F)**.

bonds in the amylopectin molecule could be determined. Therefore, by carefully interpreting the chromatograms of the carbohydrates obtained from amylopectin-debranching enzyme digests at different stages in the degradations, different modes of attack could be differentiated.

Figures 5A, C, and E are Bio-Gel P-10 chromatograms of carbohydrates obtained from pullulanase-amylopectin digests at different stages of degradation. The material in the void volumes of the chromatograms is amylopectin that has not been totally debranched. The debranched material appears as the bimodal distribution. Figures 5B, D, and F are Sepharose chromatograms of the void volume material from the corresponding Bio-Gel P-10 chromatogram.

In Fig. 5A, the bimodal distribution comprises about 30% of the total carbohydrate eluted. The partially digested amylopectin from the void volume of this chromatogram yields a roughly normal distribution on Sepharose CL-2B (Fig. 5B). Native amylopectin was eluted in the void volume of this column. Consequently, the amylopectin had shifted toward a smaller molecular size distribution, which indicates that pullulanase was attacking all amylopectin molecules equally under the conditions employed. We determined the percent of debranching of amylopectin by taking the total carbohydrate recovered from Bio-Gel P-10 minus the carbohydrate eluted at the void volume of Bio-Gel P-10 divided by the total carbohydrate recovered from Bio-Gel P-10 times 100. When the amylopectin was 47% debranched (Fig. 5C), the corresponding void volume material yielded a normal distribution on Sepharose CL-4B (Fig. 5D). Because Sepharose CL-4B has a lower exclusion limit than Sepharose CL-2B has, we knew that the shift toward lower molecular size had continued. Sizes of this material and of the β-limit dextrin are comparable; both represent about 50% of the amylopectin molecule and both yielded normal distributions on Sepharose CL-4B. The trend toward smaller size continued in the late stages of degradation. When the bimodal distribution comprised 70% of the Bio-Gel P-10 chromatogram (Fig. 5E), the size of the corresponding void volume material was greatly reduced. In Fig. 5F some of the undebranched amylopectin was so small that it was eluted in the V_t of a Sepharose CL-6B column.

The ratio of the two peaks in the bimodal distribution was not constant throughout the degradation. In the early stages peak 1 was smaller, relative to peak 2, than in the later stages (Figs. 5A and E). These data are consistent with data that indicated that peak 2 contained most of the exterior chains. If pullulanase attacks in a stepwise manner from the exteriors to the cores, peak 2 should, and does, predominate in the early stages of debranching. Therefore, under the conditions in this experiment, pullulanase apparently has a random attack with respect to the different molecules and an orderly attack with respect to the different bonds within those molecules.

Figure 6 shows that the carbohydrates obtained from isoamylase-amylopectin digests differ from those obtained from pullulanase-amylopectin digests. Void volume material is rapidly lost, and an intermediate peak with $\overline{DP}$ larger than that of peak 1 is formed. This intermediate fraction is slowly debranched and finally merges with peak 2 material to form the bimodal distribution. Sepharose chromatograms of void volume material were not obtained, and therefore the mode of attack on the different molecules of amylopectin was not ascertained. However, isoamylase has a more random mode of attack than does pullulanase toward bonds within the amylopectin molecule. Branched intermediates could not be formed unless the interior of the amylopectin molecule was open to attack by isoamylase.

These chromatograms also indicate that isoamylase has difficulty severing certain bonds. The large amylopectin molecule is rapidly converted to partially or completely debranched fragments, but the branched intermediate peak is slowly degraded by the enzyme. The cause of this difference in the susceptibility of different bonds in the amylopectin molecule to hydrolysis by isoamylase is not known at this time. Possibilities include the proximity of the branch points and the lengths of the chains involved.

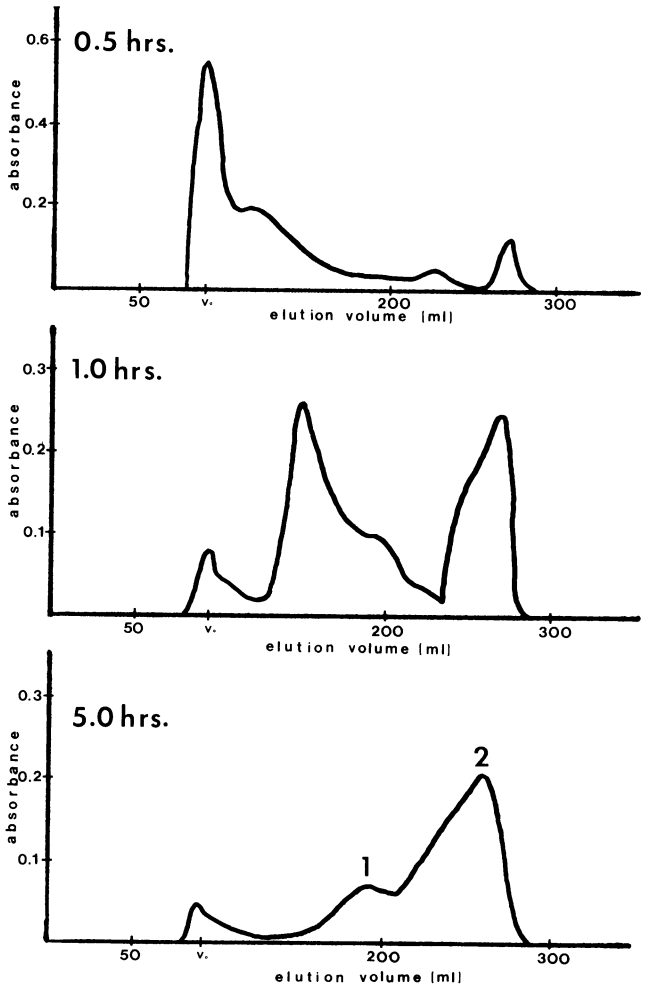

Fig. 6. Bio-Gel P-10 fractionations of the products of isoamylase debranching of amylopectin after 0.5 hr **(top)**, 1.0 hr **(center)**, and 5.0 hr **(bottom)**.

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