High-Performance Aqueous Gel Permeation Chromatographic Analysis of Beta-Limit Dextrin Hydrolysis by Malted Hard Red Spring Wheat, Malted Durum Wheat, and Fungal (*Aspergillus oryzae*) Alpha-Amylases[1]

J. E. KRUGER and B. A. MARCHYLO, Grain Research Laboratory, Canadian Grain Commission, Winnipeg, Manitoba, Canada R3C 3G8

ABSTRACT

High-performance aqueous gel permeation chromatography using a Spherogel-TSK 3000 SW column with an exclusion limit of 1.5×10^5 daltons was used to analyze the molecular weight distribution of products formed by α-amylase catalyzed hydrolysis of β-limit dextrin. Products formed by α-amylases purified from malted hard red spring wheat (*Triticum aestivum* L. cv. Neepawa), malted durum wheat (*Triticum durum* cv. Wascana), and fungal (*Aspergillus oryzae*) sources were studied and compared. The breakdown of β-limit dextrin proceeded in stages. Initially, the β-limit dextrin solution exhibited a loss in turbidity concomitant with rapid production of high molecular weight dextrin products larger than 1.5×10^5 daltons and little production of low molecular weight dextrins and sugars. This was followed by formation of intermediate molecular weight products that slowly decreased in size concurrent with a progressive buildup of low molecular weight sugars and oligosaccharides. Malted hard red spring and durum wheat α-amylase hydrolysis appeared identical but differed from that effected by fungal α-amylase.

β-Limit dextrin is a starch-derived polymer formed by exhaustive hydrolysis of amylopectin by β-amylase. Because of its high content of amylopectin, waxy maize starch is commonly used in the preparation of this polymer. The resulting β-limit dextrin is a highly branched molecule composed of inner and outer chains in the approximate ratio of 1:1 (Manners and Matheson 1981).

One important use of β-limit dextrin is for the measurement of α-amylase in cereals. Activity analyses based on decreased iodine-binding ability of β-limit dextrin (ICC 1968) or the formation of dialyzable reaction products (Kruger and Marchylo 1972, Marchylo and Kruger 1978) from β-limit dextrin have been devised. In addition, a sensitive nephelometric α-amylase assay, which relies on the loss in turbidity of a β-limit dextrin substrate solution to measure activity, recently was introduced into the cereals industry (Campbell 1980a, 1980b; Kruger and Tipples 1981; O'Connell et al 1980; Osborne et al 1981).

β-Limit dextrin hydrolysis by α-amylase is characterized by the formation of a complex series of degradation products that produce a continually changing substrate for the enzyme. The size distribution of the various dextrin products and their rate of conversion undoubtedly influence the particular assay procedure. The present study was undertaken to provide a better understanding of such changes. The technique of high-pressure aqueous gel permeation chromatography was used to monitor dynamic changes in the molecular weight of degradation products formed during the hydrolytic breakdown of β-limit dextrin by α-amylase from malted hard red spring (HRS) wheat, malted durum wheat, and fungus (*Aspergillus oryzae*).

MATERIALS AND METHODS

α-Amylase Sources

Fungal α-amylase (*Aspergillus oryzae*) B grade, 5000 SKB units/g was purchased from Calbiochem, Los Angeles, CA. Wheat α-amylases were purified from malted hard red spring (HRS) (*Triticum aestivum* L. cv. Neepawa), and malted durum (*Triticum durum* cv. Wascana) wheat by a procedure involving extraction, heat-treatment at 70°C for 15 min, acetone fractionation, and glycogen-complex formation as described by Kruger and Tkachuk (1972).

Preparation of β-Limit Dextrin

The procedure followed was as described previously (Kruger and Marchylo 1972, Kruger and Tipples 1981). Waxy-maize starch (American Maize Products Co.) is solubilized in dimethylsulfoxide, diluted with buffer, and reacted for two days with sweet potato β-amylase (Sigma Chemical Co., St. Louis, MO) followed by concentration with an Amicon Ultrafiltration apparatus (Amicon Corp., Lexington, MA), dialysis, and freeze-drying.

High-Performance Aqueous Gel Permeation Chromatography

The equipment used was a Waters model ALC/GPC HPLC (Waters Associates, Milford, MA), consisting of a solvent reservoir, Wisp automatic sample injector, model 6000A pump, and a model R401 differential refractometer attached to a Spectrophysics SP 4000 computing integrator (Technical Marketing Assoc., Mississauga, Ont.) with digital interface and printer-plotter.

The column selected for analysis of the molecular weight distribution of dextrins was a 60 × 0.75 cm Spherogel-TSK 3000 SW column (Altex Scientific, Berkeley, CA) that contained a chemically modified silica gel-based support with an approximate dextrin exclusion limit of 150,000 daltons (Fukano et al 1978, Kato et al 1980). Water was used as eluent at a flow rate of 1.0 ml/min. The column was calibrated with the following dextrans obtained from Pharmacia (Canada) Ltd., Dorval, Quebec: T-110 (mol wt 1.1 × 10⁵), T-70 (mol wt 7.1 × 10⁴), T-40 (mol wt 4.1 × 10⁴), T-10 (mol wt 9.9 × 10³). A program supplied on the Spectrophysics SP 4000 computing integrator was used to give the molecular weight distribution.

Assay Conditions for β-Limit Dextrin Breakdown Products

A 1% suspension of β-limit dextrin in distilled water was prepared by bringing the mixture close to boiling, followed by cooling to room temperature, and centrifugation at 2,000 × g for 20 min to remove undissolved particulate matter. 0.05M acetate buffer (200 μl) at pH 5.5 was added to 4.0 ml of this suspension. Excess buffer must be avoided, or spurious chromatographic behavior will result. At zero time, 200 μl of suitably diluted α-amylase was added and the reaction monitored immediately and at timed intervals thereafter, using the Wisp automatic sampler at an injection volume of 200 μl. The temperature of the sampler compartment was 23 ± 0.5°C. Preliminary experiments were performed to select the enzyme concentration that would provide the best visualization of changes occurring during the hydrolytic reaction. The levels of enzyme used for HRS, durum, and fungal α-amylase were those amounts that catalyzed the hydrolysis of 4.8, 1.7, and 3.8 mg of maltose per min × 10⁻³ at 35°C as determined by the method of Kruger and Tipples (1981).

RESULTS AND DISCUSSION

Changes in molecular weight due to hydrolytic breakdown of a β-limit dextrin solution catalyzed by α-amylases from various sources were monitored automatically at timed intervals using an HPLC equipped with a gel permeation column having an approximate weight cut-off of 1.5 × 10⁵ daltons.

Figure 1 shows the changing molecular weight distribution of β-limit dextrin hydrolysis products effected by malted HRS wheat α-amylase.

The reaction proceeded in stages. At zero time, β-limit dextrin was eluted as a single peak with an apparent molecular weight above the exclusion limit of the column. During the early stages of the reaction, ie, up to 33 min, a large downward shift in the molecular weight of the β-limit dextrin was observed. This is shown in Fig. 1 as an increase in elution time. Very little intermediate and low molecular weight material, ie, less than 6.0 × 10⁴ daltons, is formed during this transformation. The next stage in the reaction is the gradual formation of intermediate molecular weight products in the range of 1.0–6.0 × 10⁴ daltons. Simultaneously, the high molecular weight component is almost completely broken down. This transformation occurs in Fig. 1 in reaction times between 33 and 113 min. Finally, the intermediate molecular weight products

break down gradually, with a progressive downward shift in molecular weight. At the same time, low molecular weight dextrins of less than 1.0 × 10⁴ daltons progressively increase.

The very rapid initial molecular weight shift that occurred in the β-limit dextrin at early reaction times was of particular interest
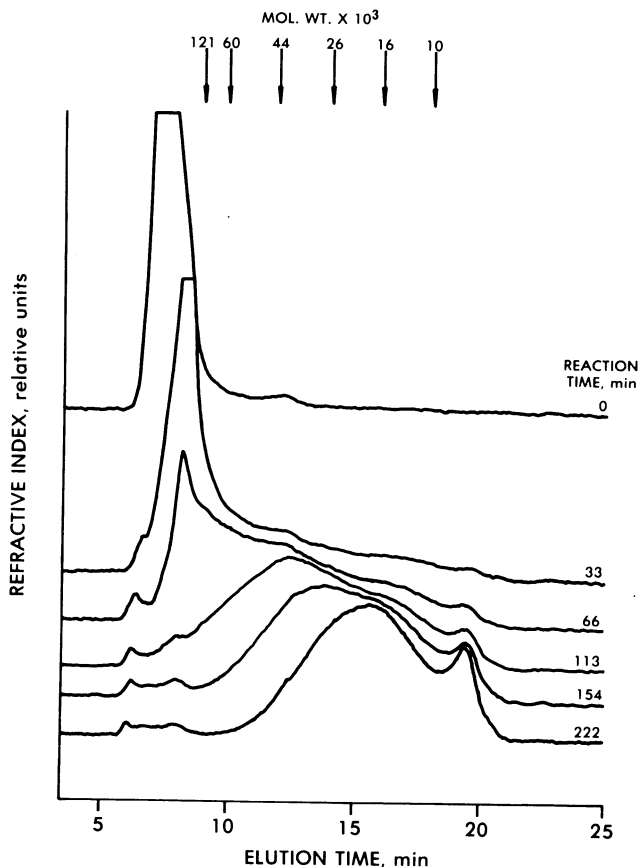

Fig. 1. Molecular weight distribution of products formed at increasing reaction times due to malted hard red spring wheat α-amylase hydrolysis of β-limit dextrin.
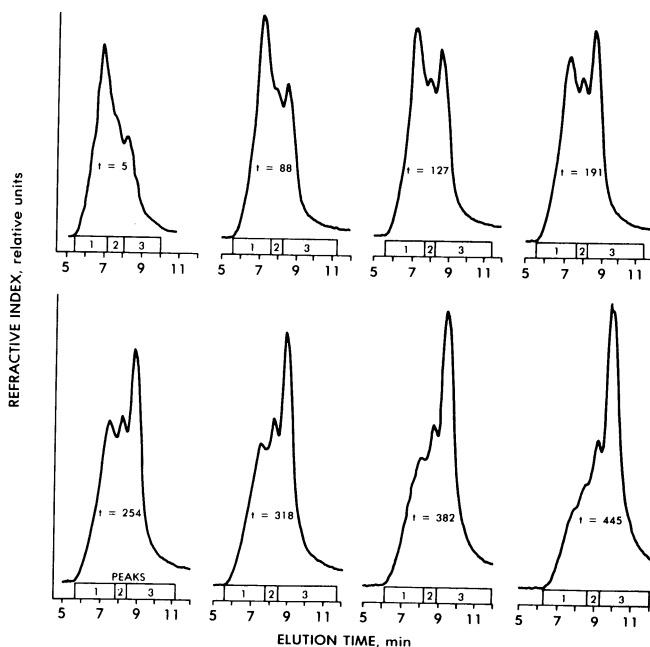

Fig. 2. Changes in high molecular weight products formed by malted hard red spring wheat α-amylase hydrolysis of β-limit dextrin with increasing hydrolysis time.

because dramatic decreases in turbidity (cloudy to clear) of the β-limit dextrin solution occurred simultaneously. As such, these changes form the basis for the nephelometric method of determining α-amylase in cereals (Campbell 1980a, 1980b). To better understand this phase of the reaction, a decreased amount of enzyme (approximately one twentieth) was used, such that the reaction proceeded at a much slower rate. The resulting chromatographic profile is shown in Fig. 2. Three discrete dextrin peaks can be noted in the early eluting high molecular weight part of the chromatogram. At very early reaction times, the bulk of the β-limit dextrin appears predominantly as one main peak with the highest molecular weight (peak 1). This material was degraded with time to the species present in peaks 2 and 3. Peak 2 species gradually increased in concentration until a maximum was reached at t = 191 min. At the same time, peak 3 species exhibited a steady increase in concentration during the course of the reaction. This suggests that peak 3 species were being formed slowly by the degradation of peak

2 species, as well as by the rapid degradation of peak 1 species. The decrease in turbidity of the original β-limit dextrin solution paralleled the transition of peak 1 species to peak 3 species. At the stage when only peak 3 species were present, the solution had become completely clear. Negligible low molecular weight sugars were produced during this transition. This finding explains why the turbidometric method for determining α-amylase is more sensitive than methods based upon the formation of reducing sugars.

The molecular weight program of the Spectrophysics SP 4000 computing integrator calculates the relative areas of the chromatographic components within defined elution time intervals, which in the present study were set at 1 min. These time intervals corresponded to specific molecular weight ranges, and areas from more than one molecular range were sometimes combined as shown in Table I. From this, the percentage of dextrins in any particular molecular weight segment can be calculated. The percentage dextrins present in any molecular weight range will be somewhat approximate because of the overlap between groups.

Percent distributions of the reaction products differing in molecular size were plotted against reaction time, as shown in Fig. 3. The molecular weight distribution of the unreacted substrate is indicated by the zero reaction time. The overall changes shown in Fig. 1 are more clearly defined. Thus, the highest molecular weight species (high mol wt 1) decreased rapidly in the early part of the reaction, whereas the other high molecular weight species (high mol wt 2 and 3) increased.

These latter components then began to decrease, whereas all of the remaining molecular weight ranges increased. A small amount of high molecular weight dextrins remained even after lengthy incubation. These dextrins are presumably resistant to α-amylase attack.

TABLE I
Distribution of Molecular Weights at Various Elution Times from HRS Wheat, Durum Wheat, and Fungal α-Amylase Hydrolysis of β-Limit Dextrin

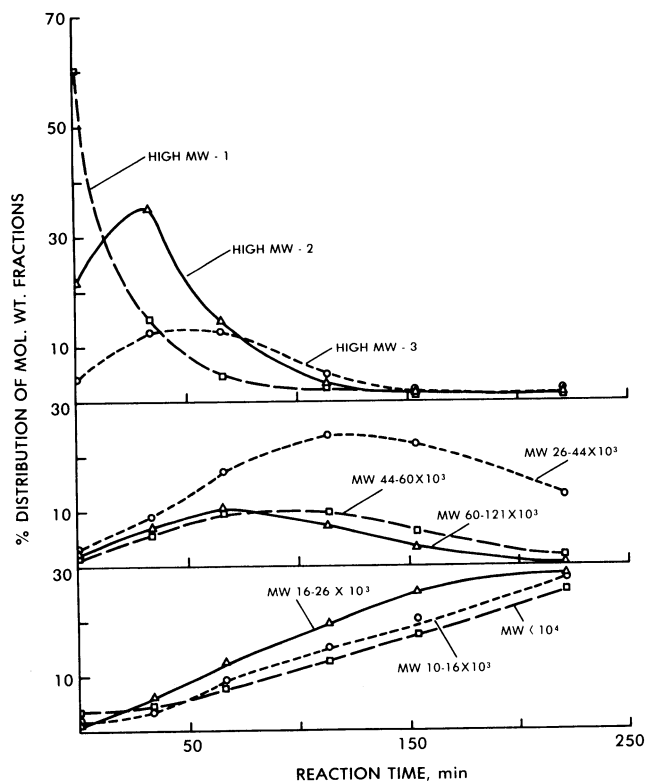| Elution Time (min)	Molecular Weight (×10³ daltons)		
	HRS Wheat α-Amylase	Durum Wheat α-Amylase	Fungal α-Amylase
7–8	High mol wt 1	High mol wt 1	High mol wt 1
8–9	High mol wt 2	High mol wt 2	High mol wt 1
9–10	High mol wt 3	High mol wt 3	High mol wt 2
10–11	60–121	61–163	High mol wt 3 (71–205)
11–12	44–60	43–61	53–71
12–14	26–44	26–43	31–53
14–16	16–26	16–26	19–31
16–18	10–16	9.5–16	11.5–19
18–23	Low mol wt <10	Low mol wt <9.5	Low mol wt <11.5

Fig. 3. Percent distribution of products by weight in different molecular weight fractions from malted hard red spring wheat α-amylase hydrolysis of β-limit dextrin with increasing reaction time. High mol wt (MW) 1, 2, and 3 refer to dextrins excluded from the column as described in Table I.
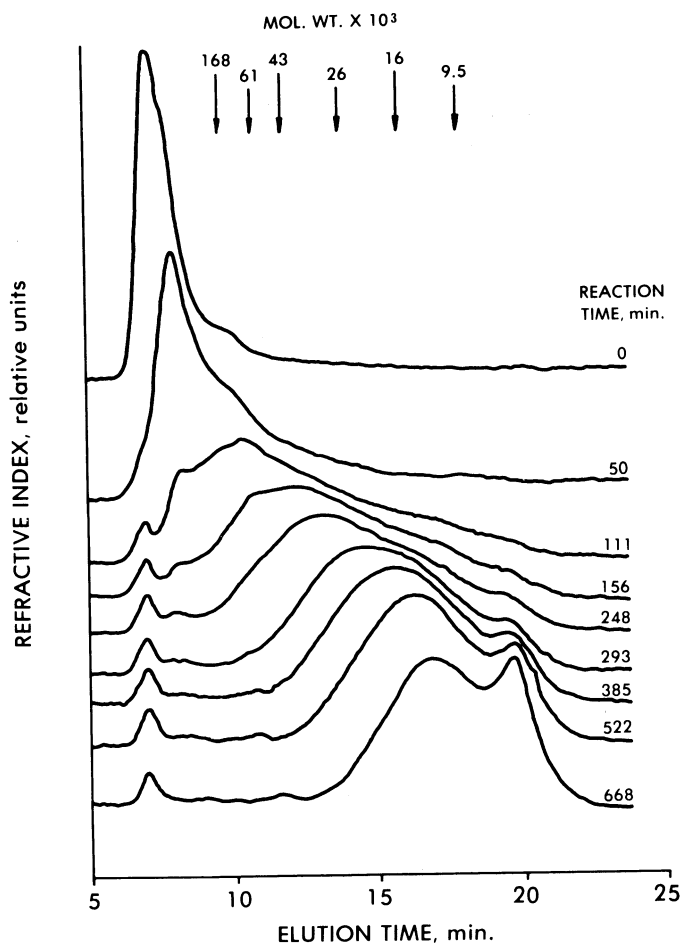

Fig. 4. Molecular weight distribution of products formed at increasing reaction times due to malted durum α-amylase hydrolysis of β-limit dextrin.

Of the intermediate molecular weight products, those in the $2.6-12.1 \times 10^4$ dalton range increase with increasing reaction time followed by subsequent decreases. Those products less than 2.6×10^4 daltons, however, progressively increased in concentration during the reaction interval studied.

The catalytic breakdown of β-limit dextrin by malted durum wheat α-amylase was examined in the same manner as described for malted HRS wheat α-amylase. The chromatographic profiles are shown in Fig. 4, and the percent distributions of dextrins in the different molecular weight ranges are shown in Fig. 5. Comparison of Fig. 4 with Fig. 1 and Fig. 5 with Fig. 3 indicates that malted durum wheat α-amylase hydrolysis is almost identical to that of malted HRS wheat α-amylase. This suggests that very similar reaction mechanisms operate for the two sources of α-amylase in breaking down β-limit dextrin.

Fungal α-amylase hydrolysis of β-limit dextrin with increasing reaction time is shown in Fig. 6, and the percent distribution of dextrins present in the different molecular weight ranges with increasing reaction time is shown in Fig. 7. The mode of hydrolysis was noticeably different from that of the malted wheat α-amylases. As with the plant α-amylases, the initial reaction was characterized by a rapid breakdown of β-limit dextrin to high molecular weight dextrin species not excluded from the column and by little formation of small molecular weight oligosaccharides and reducing sugars. This phase of the reaction was accompanied by a rapid decrease in cloudiness of the β-limit dextrin extract to a clear solution. Subsequent amylase hydrolysis, however, favored the increased formation of smaller molecular weight dextrins of less than 3.1×10^4 daltons in preference to intermediate-size dextrins in the ranges $3.1-7.1 \times 10^4$ daltons. Thus, with malted wheat α-amylase hydrolysis, products in the molecular weight range of $2.6-4.3 \times 10^4$ daltons account for approximately 25% of the total products formed at one point in the reaction. With fungal α-amylase hydrolysis, on the other hand, products in the $3.1-5.3 \times 10^4$ dalton range maximally comprised about 15% of the total reaction products. A larger amount of β-limit dextrin eluting at 7 min, apparently resistant to fungal α-amylase hydrolysis, was present compared to the plant α-amylase hydrolysis.

CONCLUSIONS

High-performance aqueous gel permeation chromatography is ideally suited to measuring dynamic progress in the hydrolytic breakdown of β-limit dextrin by α-amylase. Information about the molecular weight distribution of any hydrolysate can be obtained in 20 min with this method. This then could have wide applicability in the sugar, brewing, paper, glue, and textile industries, which require the rapid assessment of dextrin blends. Removal of acid or high salt concentrations is essential before analysis, however, because of an adverse effect on the column or chromatographic profile.

Initial α-amylase hydrolysis of β-limit dextrin results in rapid breakdown to molecular species having molecular weights in excess of 1.5×10^5 daltons with insubstantial amounts of low molecular weight dextrins or simple sugars. This phase of the reaction is of great interest because it is responsible for the decrease in turbidity of β-limit dextrin solutions, which is the basis for nephelometric assays. Although such changes could be visualized using the Spherogel-TSK 3000 SW HPLC column, precise information on the molecular weight changes could not be obtained because of the exclusion limit restriction of the column. An HPLC column with a dextrin exclusion limit of 6×10^5 daltons is available commercially (Spherogel-TSK 4000 SW, Altex Scientific, Berkeley, CA), and this column might provide more information in future experiments.

The breakdown of β-limit dextrin as hydrolyzed by malted HRS and malted durum wheat α-amylases appeared almost identical. This is not surprising, because the two types of α-amylases have similar isoenzymes (Warchalewski and Tkachuk 1978), and cereal α-amylases have similar action patterns (Greenwood and Milne 1968). Aside from molecular weight product differences, other conclusions concerning the nature of the products cannot be made because of the complexity of β-limit dextrin, the fine structure of which has yet to be established (Manners and Matheson 1981). The observed behavior certainly would conform to a cluster-type
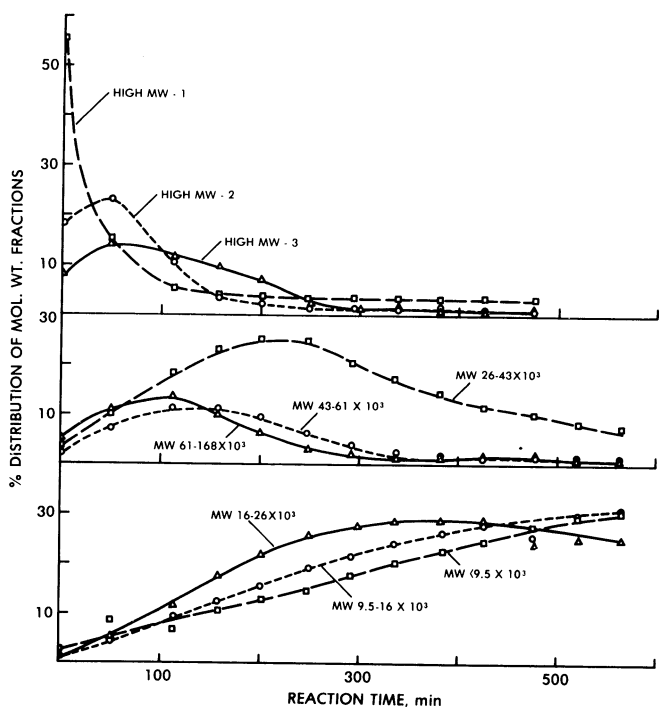

Fig. 5. Percent distribution of products by weight in different molecular weight fractions from malted durum wheat α-amylase hydrolysis of β-limit dextrin with increasing reaction time. High mol wt 1, 2, and 3 refer to dextrins excluded from the column as described in Table I.
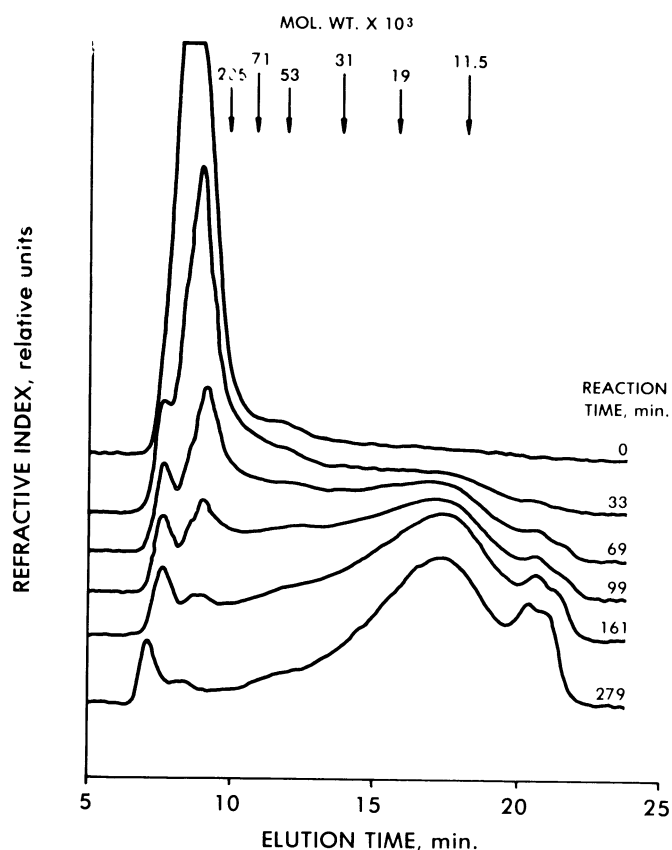

Fig. 6. Molecular weight distribution of products formed at increasing reaction times due to fungal α-amylase hydrolysis of β-limit dextrin.
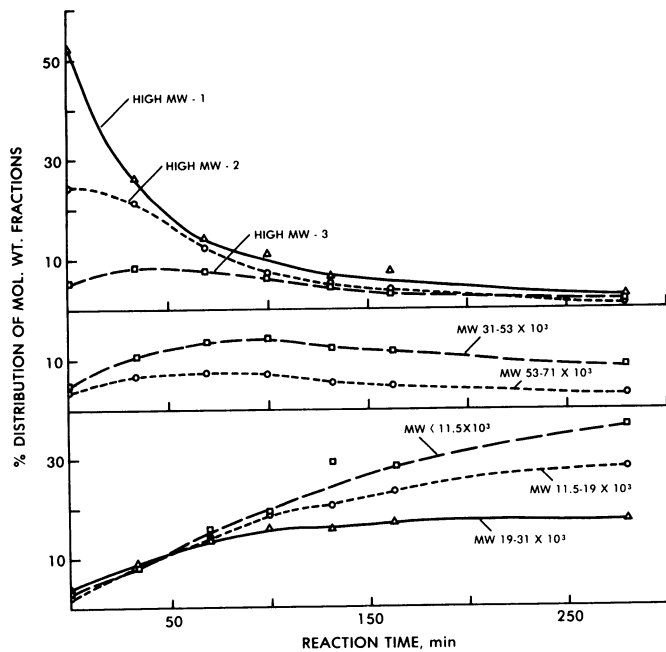
Fig. 7. Percent distribution of products by weight in different molecular weight fractions from fungal α-amylase hydrolysis of β-limit dextrin with increasing reaction time. High mol wt (MW) 1, 2, and 3 refer to dextrins excluded from the column as described in Table I.

structure for amylopectin (French 1972, Nikumi 1975, Robin et al 1974, Tamaguchi et al 1979). The initial rapid decrease in molecular weight could be accounted for by the cleavage of bonds linking the clusters. This would be followed by a slower degradation of the individual clusters, assuming that they were more densely packed.

Fungal α-amylase hydrolysis was different from that of the malted wheat α-amylase, in that the breakdown of high to low molecular weight dextrins was not as gradual. Such differences would be expected, as many previous studies have emphasized the large differences in properties between cereal and fungal α-amylases (Greenwood and Milne 1968). The pH chosen for this study was 5.5, which is optimal for malted wheat α-amylase, and the concentration of enzyme was chosen to best portray the dynamic changes occurring upon hydrolysis. However, pH, temperature, and concentration of enzyme may influence the mode of attack. Future research within the limitations imposed by HPLC gel permeation columns presently available is planned to study the influence of these factors.

LITERATURE CITED

CAMPBELL, J. A. 1980a. Measurement of alpha-amylase in grains. Cereal Foods World 25:46.

CAMPBELL, J. A. 1980b. A new method for detection of sprout damaged wheat using a nephelometric determination of alpha-amylase activity. Cereal Res. Commun. 8:107.

FRENCH, D. 1972. Fine structure of starch and its relation to the organization of starch granules. J. Jpn. Soc. Starch Sci. 19:8.

FUKANO, K., KOMIYA, K., SASAKI, H., and HASHIMOTO, T. 1978. Evaluation of new supports for high-pressure aqueous gel permeation chromatography: TSK-Gel SW type columns. J. Chromatogr. 166:47.

GREENWOOD, C. T., and MILNE, E. A. 1968. Studies on starch degrading enzymes. Part VIII. A comparison of α-amylases from different sources: Their properties and action patterns. Staerke 20:139.

INTERNATIONAL ASSOCIATION FOR CEREAL CHEMISTRY STANDARD METHODS. ICC Standard 107, 1968; ICC Standard 108, 1968. The Association, Vienna, Austria.

KATO, Y., KOMIYA, K., SASAKI, H., and HASHIMOTO, T. 1980. Separation range and separation efficiency in high-speed gel filtration on TSK-Gel SW columns. J. Chromatogr. 190:297.

KRUGER, J. E., and TKACHUK, R. 1969. Wheat alpha-amylases. I. Isolation. Cereal Chem. 46:219.

KRUGER, J. E., and MARCHYLO, B. 1972. The use of reduced β-limit dextrin as substrate in an automated amylase assay. Cereal Chem. 49:453.

KRUGER, J. E., and TIPPLES, K. H. 1981. Modified procedure for use of the Perkin-Elmer model 191 Grain Amylase Analyzer in determining low levels of α-amylase in wheats and flours. Cereal Chem. 58:271.

MANNERS, D. J., and MATHESON, N. K. 1981. The fine structure of amylopectin. Carbohydr. Res. 90:99.

MARCHYLO, B., and KRUGER, J. E. 1978. A sensitive automated method for the determination of α-amylase in wheat flour. Cereal Chem. 55:188.

NIKUNI, Z. 1975. Historical Review of our studies on starch granules. J. Jpn. Soc. Starch Sci. 22:78.

O'CONNELL, B. T., RUBENTHALER, G. L., and MURBACH, N. L. 1980. Evaluation of a nephelometric method for determining cereal alpha-amylase. Cereal Chem. 57:411.

OSBORNE, B. G., DOUGLAS, S., FEARN, T., MOORHOUSE, C., and HICKLEY, M. J. 1981. Collaborative evaluation of a rapid nephelometric method for the measurement of alpha-amylase in flour. Cereal Chem. 58:474.

ROBIN, J. P., MERCIER, C., CHARBONNIERE, R., and GUILBOT, A. 1979. Lintnerized starches. Gel filtration and enzymatic studies of insoluble residues from prolonged acid treatment of potato starch. Cereal Chem. 51:389.

WARCHALEWSKI, J. R., and TKACHUK, R. 1978. Durum wheat α-amylases: Isolation and purification. Cereal Chem. 55:146.

YAMAGUCHI, M., KAINUMA, K., and FRENCH, D. 1979. Electronic microscopic observations of waxy maize starch. J. Ultrastruct. Res. 69:249.