AN INVESTIGATION OF THE α-AMYLASE FROM SELF-LIQUEFYING BARLEY STARCH[1]

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ABSTRACT

Starch prepared from Washonupana, a short awn hulless waxy barley developed at Montana State University, has an associated enzyme that will liquefy the starch. With Washonupana starch the Brabender curves attain a maximum value and then the viscosity drops rapidly, but $HgCl_2$ negates the effect of the enzyme and results in a normal Brabender viscosity curve for a waxy barley starch. Addition of $HgCl_2$ results in increased viscosity obtained on cooling for several other barley starches; apparently these starches also have traces of associated enzyme. The enzyme associated with Washonupana starch behaves like an α-amylase. The enzyme releases dye from a Cibachron Blue-Amylose complex, is enhanced at lower pH levels by the addition of calcium ions, and has an optimum pH level of 5.3. The enzyme is fairly thermo-stable, maintaining activity at temperatures of 50°–60° C. The products of the autolysis were studied by high pressure liquid chromatography. The principal sugars following digestion at 50° C were glucose, maltose, and maltotriose, but digestion at 60° C yielded higher oligosaccharides, with maltose and G_6 being the predominant fractions.

A short awn hulless waxy Compana barley, known as Washonupana, has been developed at Montana State University. Starch prepared from this barley has an associated α-amylase that will liquefy and digest the host starch (1). This study is concerned with this enzyme, the conditions under which the enzyme is active, and the reaction products formed.

MATERIALS AND METHODS

The Washonupana barley used in this study was grown near Mesa, Arizona, in 1974 and near Kalispell, Conrad, and Huntley, Montana, in 1975. The barleys were harvested under dry conditions, except for the samples grown at Kalispell and Conrad. There were no signs of sprouting in any of the samples except those grown in Kalispell which included a few sprouted seeds. However, the samples grown at Conrad had been weather-damaged. Waxy Oderbrucker, Short Awn Hulless (SAH) Compana and Long Awn Covered (LAC) Compana barleys were grown in Montana.

The corn starch used was unmodified corn starch from CPC International obtained through the courtesy of Jiun Keng. The waxy corn starch was Amioca Waxy Maize Starch obtained from American Maize-Products Co., Roby, IN.

Preparation of Starches

The starches were separated by a wet-milling technique. Barley was steeped for 24 hr at 45° C in 0.2% sodium metabisulfite, then thoroughly washed. The grain was mixed with 2 vol of tap water at 37° C and milled in a 1-gallon Waring

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Blendor for several minutes at low speed. The slurry was then screened on a 115-mesh screen. The residue was replaced in the blender and homogenized with 2 vol of water. The slurry was again screened. This procedure was repeated until the residue appeared to be essentially free of starch, which required 5–6 cycles.

The material passing through the 115-mesh screen was screened on a 270-mesh screen. The suspension that passed through the screen was centrifuged in a solid bowl centrifuge, and the dark layer on top of the bowl was removed with a spatula. The starch remaining was air dried.

After drying, the starch was resuspended in warm water and again screened through a 270-mesh screen. This drying step denatured some of the contaminating protein which was then removed by the screen. The starch slurry was again centrifuged in a solid bowl centrifuge and air dried at room temperature.

Although many barley and wheat starches have been separated at Montana State University over a period of 15 years using this technique, only the Washonupana barley variety has shown appreciable enzyme activity associated with the separated starch as determined by the Brabender Amylograph.

Brabender Cooking Curves

Brabender pasting curves were determined at the 8% starch level with and without the addition of 200 mg mercuric chloride dissolved in the 420 g water used to suspend the starch sample (1).

Enzyme Activity

Enzyme activity was estimated by two methods. α-Amylase was determined by measuring the dye released from a Cibachron Blue-Amylose complex using an adaptation of the procedure reported by Klein *et al.*, (2) and investigated further by Marshall (3). Exo-enzymes such as β-amylase and glucoamylase do not cause dye release from this complex. The color formed by released dye is proportional to the α-amylase activity. A 100 mg sample of starch was mixed with 200 mg Cibachron Blue-Amylose (CB-A) and equilibrated at 50°C in a water bath shaker. To this was added 4.5 ml 40m*M* acetate buffer, pH 5.3 containing 12m*M* calcium acetate, also equilibrated at 50°C. After 30 min shaking 2.4 ml 0.5*M* tris, pH 10.3 was added to stop the reaction. The slurry was centrifuged, and the supernatant solution was passed through a Millipore filter. The absorbance was read at 625 nm and the amount of dye released was determined by reference to a standard curve prepared using an aqueous solution of Cibachron Blue dye. α-Amylase activity of ground barley was determined in the same way, but blank samples were prepared consisting of ground barley, CB-A substrate, and buffer containing tris by shaking for 30 min and used to correct for extraneous color leached from the grain. The procedure was carried out at various temperatures to determine the optimum temperature for digestion.

The enzyme activity was also measured by the decrease in iodine stain in digests of Lintner starch. The effects of the addition of calcium ions and of changing the pH were studied using this method. Buffers were prepared by the procedures of Gomori (4) and included glycine-HCl (below pH 3.5), acetate (pH 3.8–5.5), and tris-maleate (pH 5.6–8.5). The pH values of buffers were determined accurately using a Corning Model No. 12 pH meter.

The digests were made in buffers with and without calcium acetate added.

Samples of starch from Washonupana barley grown at Mesa (100 mg) were weighed into test tubes, and 5 ml of the digest solution (2 ml buffer, 2 ml 2% Lintner starch and 1 ml either 60 m*M* $Ca(OAc)_2$ or glass-distilled water) was added. The suspensions were mixed well and topped with toluene. The tubes were incubated for 24 hr at 37° C. At the end of this period, 0.5 ml aliquots were added to 0.5 ml 0.1*N* HCl and color was developed by the addition of 5.0 ml iodine reagent (0.05 mg I_2 and 0.5 mg KI/ml). The solutions were diluted to 20 ml and read at 660 nm after standing 10 min. Absorbance of a Lintner starch solution in the absence of the Washonupana starch was also determined. The calculations were:

$$\%\ \text{Color Loss} = \frac{\text{Abs}_{\text{Lintner}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{Lintner}}} \times 100$$

Products of Autolytic Digestion

To study the products of autolytic digestion at two different temperatures, 5.0 g samples of starches were stirred at 50° or at 60°C for 24 hr in 50 ml 50m*M* acetate buffer, pH 5.3 containing 12m*M* calcium acetate. The digests were centrifuged, and the supernatant solutions were heated in a boiling water bath for 20 min. Following centrifugation, the extracts were used for the determination of total carbohydrate by the phenol-sulfuric acid method (5), reducing substances by 3,5-dinitrosalicylic acid (6), and glucose by glucose oxidase (7). Aliquots of

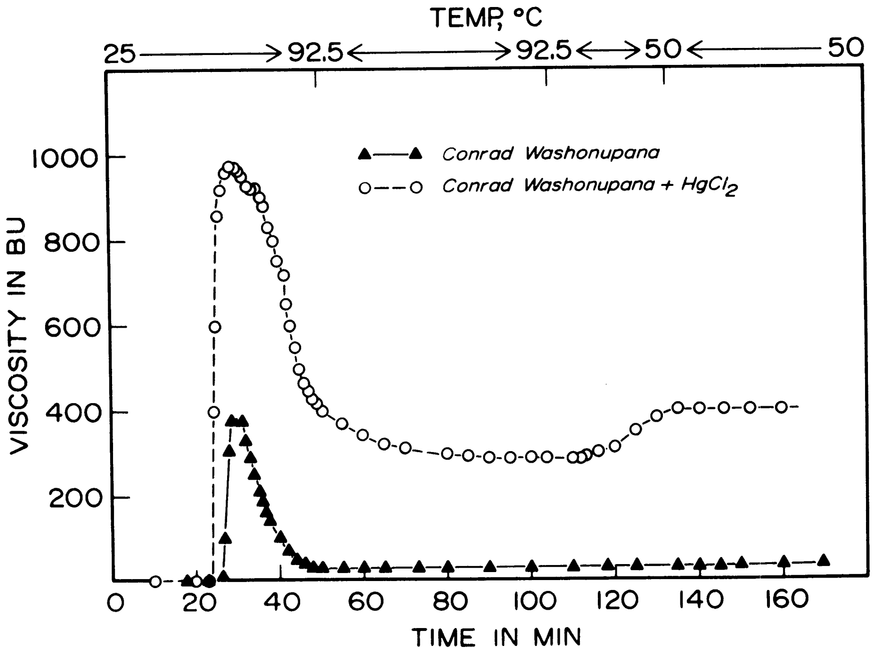

Fig. 1. Brabender amylograms of Washonupana starch with and without the addition of an inhibitor.

the solutions were deionized with Amberlite MB-3, concentrated to 0.25 vol and analyzed by high pressure liquid chromatography (HPLC) to further determine the products of autolytic digestion.

A μ-BONDAPAK/Carbohydrate column (4 mm ID × 30 cm) from Waters Associates was used. The eluting solvent was double-distilled deionized water and acetonitrile of a glass-distilled or better grade (35:65v/v). A linear flow program using program No. 6 on a Waters Associates Model 660 solvent programmer was employed. The samples (25–50 μl) were separated chromatographically using a 25-min program with an initial flow rate of 1.0 ml/min and final flow rate of 2.5 ml/min, except for the samples of Mesa 60° C and Conrad 60° C digests which were run using a 34-min program having an initial flow rate of 1.0 ml/min and a final flow rate of 3.0 ml/min. In all cases the pump was allowed to run isocratically at the final flow rate until a flat recorder response was reached. Column effluents were monitored using a Waters Associates Model R401 Differential Refractometer.

Pullulanase from Boehringer Mannheim Corporation was diluted to 1 mg/ml. An aliquot of 0.1 ml was added to 10 ml of the supernatant solutions from the autolytic digestions performed at 60° C. These were digested 48 hr at 30° C, and then heated in a boiling water bath 30 min. Pullulanase digests were deionized, concentrated to 0.25 vol and analyzed by HPLC by the procedure described above.

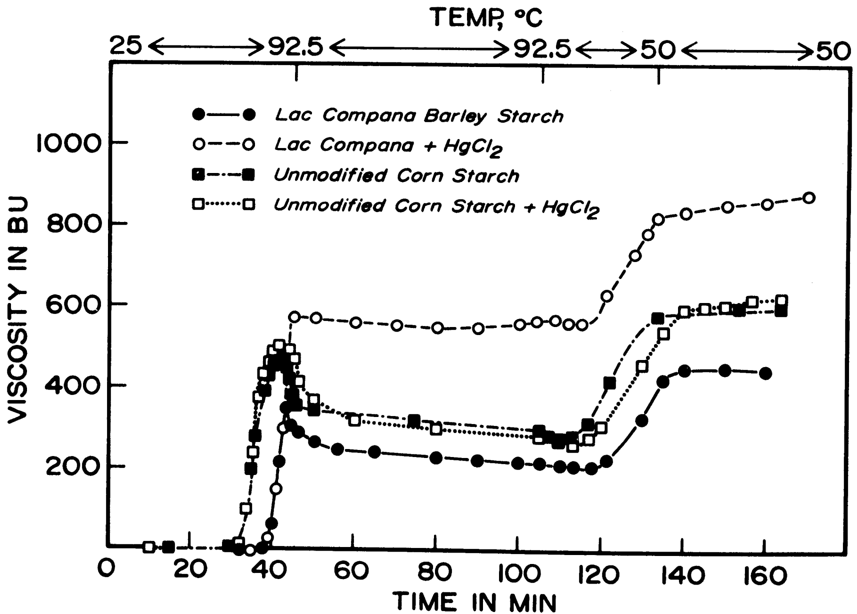

Fig. 2. Brabender amylograms of Long Awn Covered Compana barley starch and unmodified corn starch with and without the addition of an inhibitor.

RESULTS AND DISCUSSION

The Brabender curves of Washonupana starch with and without inhibitor ($HgCl_2$) differ drastically, as shown in Fig. 1.

Fig. 2 illustrates the effect of an inhibitor on the Brabender curve of Long Awn Covered Compana barley starch. Long Awn Covered Compana is the parent

TABLE I
α-Amylase Activity of Barleys and Barley Starches Determined by the CB-A Method[a]

Variety	Cibachron Blue released/min/mg (ng)	
	Barley	Barley Starch
Long Awn Covered Compana	40	0.8
Conrad Washonupana	104	48
Huntley Dryland Washonupana	21	46
Mesa Washonupana	...	78
Kalispell Washonupana[b]	>8800	78

[a]CB-A = Cibachron Blue-Amylose.
[b]Evidence of sprouting in sample.

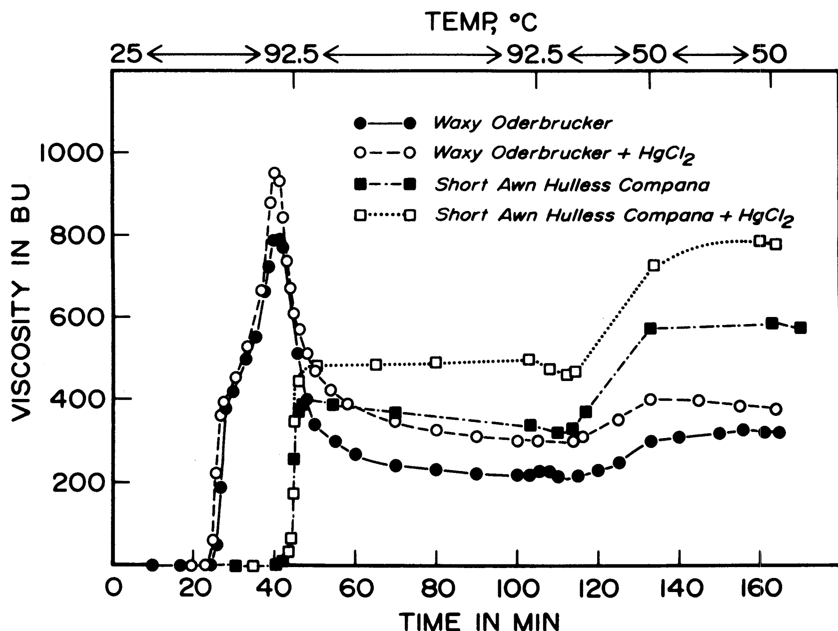

Fig. 3. Brabender amylograms of Waxy Oderbrucker and Short Awn Hulless Compana barley starches with and without the addition of an inhibitor.

stock of Washonupana. The Brabender curves of unmodified corn starch with and without $HgCl_2$ are shown for comparison.

Barley starches in general seem to have an associated α-amylase. The curves of Fig. 3 were obtained from shelf samples of starches prepared within the past few

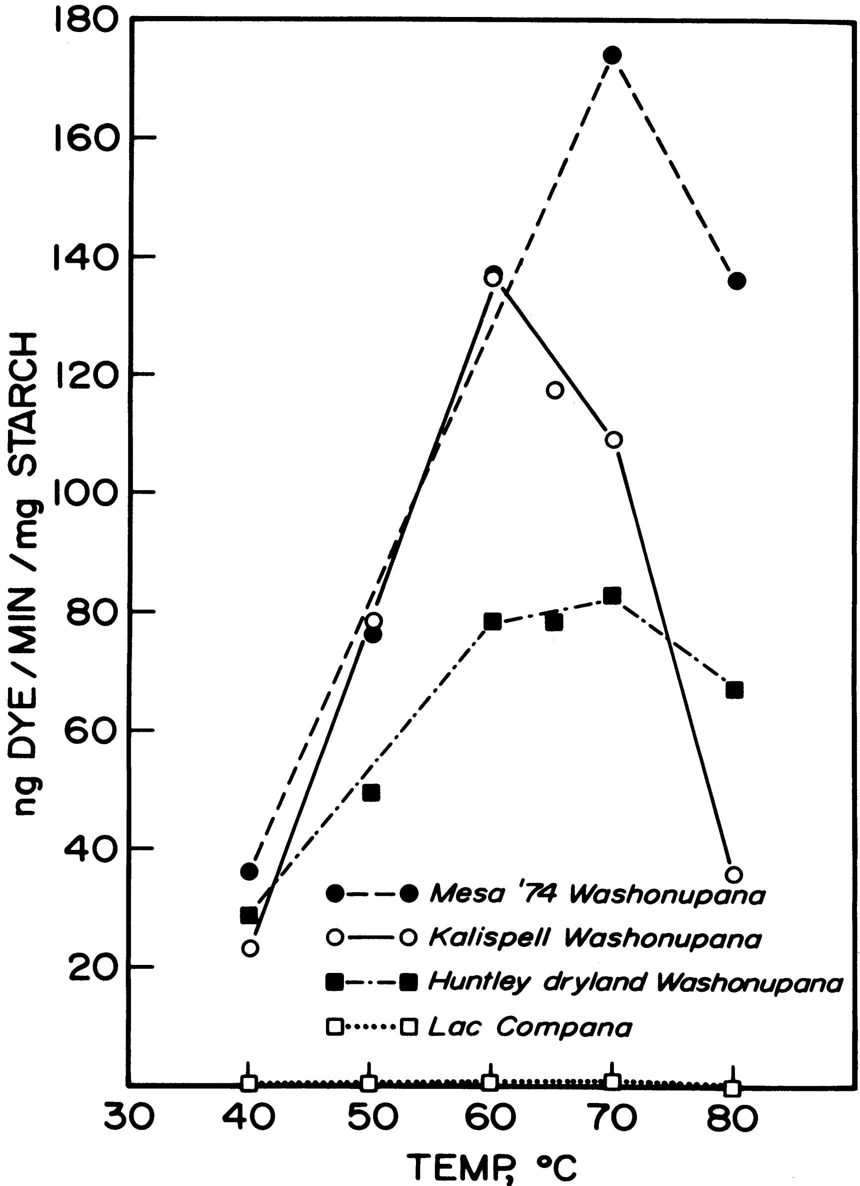

Fig. 4. Effect of temperature on the release of dye from Cibachron Blue-Amylose by Washonupana barley starches.

years. In each case the presence of the inhibitor, $HgCl_2$, changed the curve appreciably. The waxy gene of Washonupana is derived from Waxy Oderbrucker.

The α-amylase activities of Washonupana barley grown at different locations and the activities of starches from these barleys were determined by the Cibachron Blue-Amylose method and compared to the activity of LAC Compana barley. The results are shown in Table I.

Although there are appreciable differences in the α-amylase activities of the barley, only the Washonupana sample grown at Kalispell has an activity different from the group by more than an order of magnitude. The sample grown

TABLE II
Products of Autolytic Digestion of Washonupana Starches

Location	Carbohydrate (mg/ml)		Reducing Substances (mg maltose/ml)		Glucose (mg/ml)	
	50°C	60°C	50°C	60°C	50°C	60°C
Conrad	6.0	29.6	4.3	13	.04	.04
Huntley dryland	4.2	6.9	5.5	5.0	.40	.14
Mesa	7.3	8.6	5.4	5.4	1.8	1.1
Kalispell	4.9	10.3	4.0	5.8	.07	.13

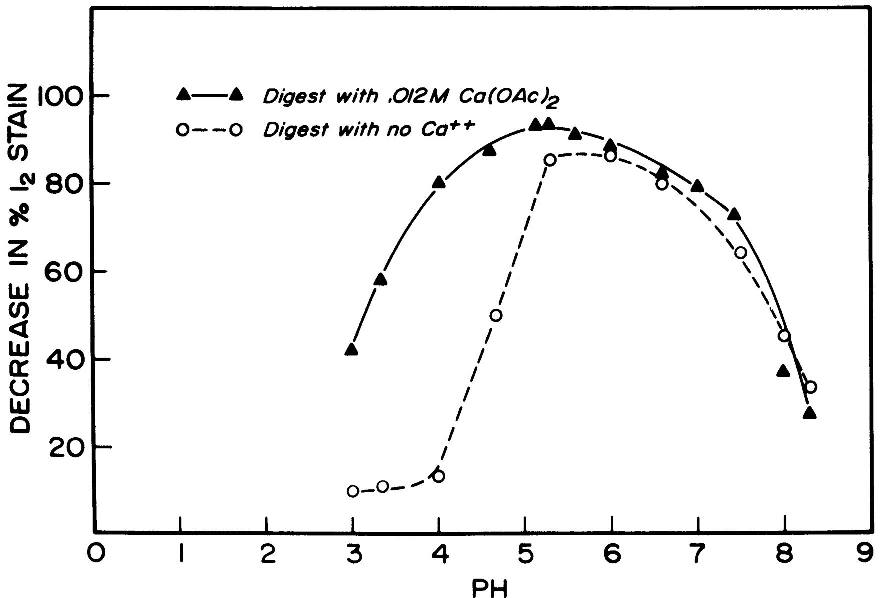

Fig. 5. Effect of pH on digest of Lintner starch by Mesa Washonupana barley starch with and without the presence of 12 m*M* $Ca(OAc)_2$.

at Kalispell contained a few sprouted seeds and has approximately 100 times the activity of the most active of the other samples. However, starch prepared from this sample is less than twice as active as the starches prepared from the other Washonupana barleys grown the same year. The LAC Compana barley shows approximately the same α-amylase activity as the Washonupanas (with the exception of the sample grown at Kalispell) but starch prepared from LAC Compana has negligible α-amylase activity. The samples grown at Conrad were weather-damaged, however, which may have caused increased α-amylase activity (8). In the case of the sample grown at Huntley, adsorption of enzyme on the starch is apparently essentially quantitative. Possibly two species of α-amylase are present: one that is peculiar to the Washonupana variety and has a strong affinity for the starch, and the other that is the usual α-amylase of germinating seeds. Work is currently in progress to isolate the Washonupana α-amylase for further study.

The effect of temperature on the starch digestion was studied by the CB-A method and is shown in Fig. 4.

The optimum temperature for autolytic digestion of starch from Washonupana barley grown at Kalispell is about 55° C while for starches from barley grown at Mesa and at Huntley it is about 70° C.

The effects of the presence of calcium ions and of changing the pH are shown in Fig. 5.

This determination reflects the optimum reaction conditions of the starch/enzyme system, rather than necessarily the optimum conditions for the starch/α-amylase since the Lintner starch is digested also by β-amylase and by other carbohydrases that may be present.

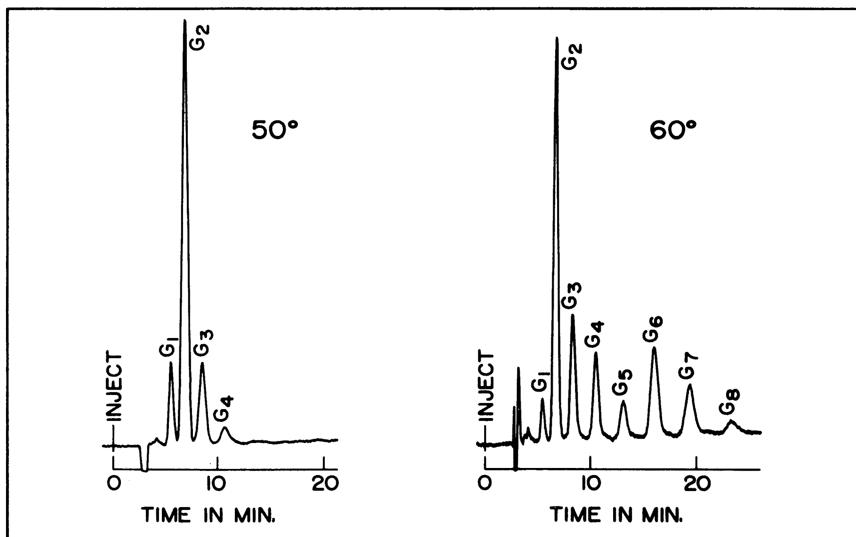

Fig. 6. High pressure liquid chromatography separation of oligosaccharides formed from Huntley Washonupana starch by autolysis at 50° and 60° C.

Table II shows the products obtained by autolysis of the starches at 50° and at 60° C.

At 50° C the total carbohydrate found in the supernatant solution was higher than at 60°C. Generally the reducing value was also higher in the digestions carried out at 50° C, although in some cases the differences were slight. "Blank" determinations were made with starches grown at Kalispell and Huntley by allowing autolytic digestion in the same buffer with the addition of 1 mg/ml mercuric chloride. Negligible amounts of reducing substances were found in both digests. However, the digest of the starch from barley grown at Kalispell had 0.4 mg/ml carbohydrate solubilized at 50°C and 0.6 mg/ml solubilized at 60°C while the digest of the starch from barley grown at Huntley had 1.3 mg/ml and 1.5 mg/ml carbohydrate solubilized at 50° and 60° C, respectively.

Separations obtained by high pressure liquid chromatography are shown in Figs. 6, 7, and 8.

The relative amounts of oligosaccharides formed by the autolytic digestion of starches prepared from barleys grown in different locations are shown in Table III. At 50° C the principal sugars from a 24 hr digestion of Huntley Washonupana starch are glucose, maltose, and maltotriose while at 60° C the glucose yield drops sharply, as does the maltose, and considerable amounts of G_6 and G_7 appear. This pattern of increased amounts of higher oligosaccharides from the digestion at the higher temperature is followed for all the starches from the various locations although quantities differ. Autolytic digests of starches from barley samples grown at Mesa and Huntley yield appreciable amounts of glucose at 50° C but starches from barley samples grown at the other two locations do not.

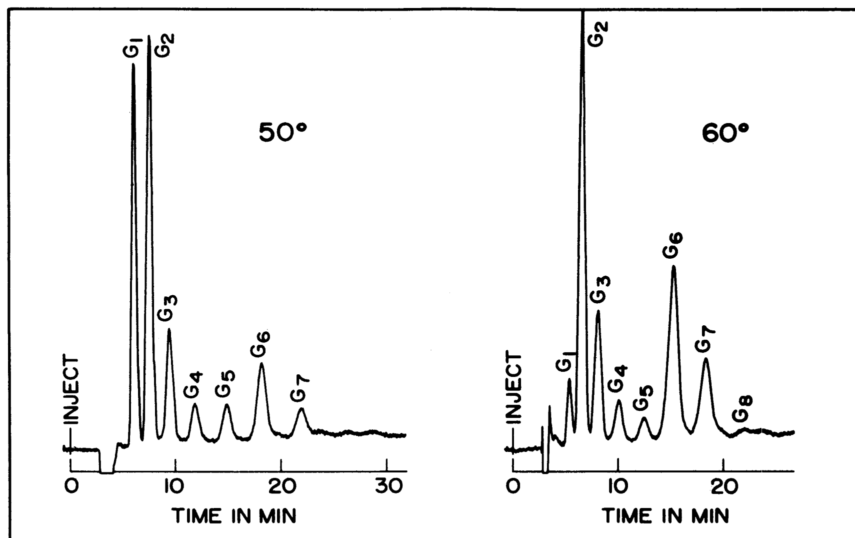

Fig. 7. High pressure liquid chromatography separation of oligosaccharides formed from Mesa Washonupana starch by autolysis at 50° and 60° C.

TABLE III
Oligosaccharides from Autolytic Digestion of Washonupana Barley Starches

Number of Glucose Units	Peak Area (%) Mesa 50° C	Mesa 60° C	Conrad 50° C	Conrad 60° C	Huntley 50° C	Huntley 60° C	Kalispell 50° C	Kalispell 60° C
G_1	26	4	2	1	10	3	2	3
G_2	34	31	69	15	67	36	67	23
G_3	12	13	16	9	15	13	14	8
G_4	5	5	8	7	4	11	5	7
G_5	5	4	2	5	3	6	2	5
G_6	11	25	2	24		14	2	21
G_7	5	13	2	19		10	3	26
Unidentified[a]	1	...		...		...	1	...
G_8	1	1		10		5	trace	3
Unidentified[a]	1	1		...		...	3	1
G_9		2		5		2		2
G_{10}		2		4				2
G_{11}				1				
G_{12}				1				

[a]Apparently branched polymers whereas all others are linear.

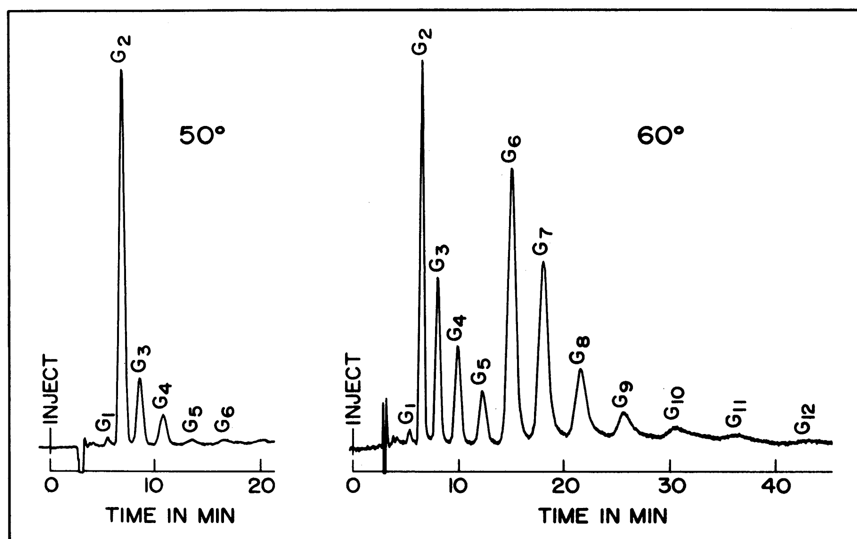

Fig. 8. High pressure liquid chromatography separation of oligosaccharides formed from Conrad Washonupana starch by autolysis at 50° and 60° C.

At 60° C the enzyme system does not cleave the starch into units as small as at 50° C. There seems to be a preference for leaving chains of six and seven glucose units at the higher temperature. While the primary enzyme involved here is α-amylase, traces of β-amylase are also present with the starch. There may also be other carbohydrases present. The β-amylase and other enzymes may be partially inactivated at the higher temperature, accounting for the less complete digestion.

When the digests were treated with pullulanase and again subjected to HPLC the patterns of peaks were essentially unchanged for all the samples, except that the "unidentified" small peaks between G_7 and G_8 and between G_8 and G_9 disappeared. This indicates these small peaks were the only branched polymers that were separated by HPLC. The pullulanase digest of the solution prepared from the autolytic digestion of the starch of the barley sample grown at Mesa developed a precipitate of retrograded starch and the other pullulanase digests became slightly cloudy. The precipitate and cloudiness disappeared when the digests were heated but reappeared and were removed in the procedure of concentrating and deionizing the solution. The HPLC separations of the debranched digests showed increased amounts of higher maltodextrins of G_{10}, G_{11}, and G_{12} and at the end of the runs small peaks that might correspond to G_{13} and G_{14} were found.

The question of whether the α-amylase of Washonupana barley has some special characteristic that makes it more easily adsorbed on the starch or whether the starch of Washonupana is more able to adsorb α-amylase has not been answered. Further work is contemplated to try to answer this question and to determine conditions whereby maximum liquefaction of the starch is obtained.

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