STUDIES ON THE ENDO-BETA-GLUCANASE SYSTEM OF BARLEY MALT[1]

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ABSTRACT

Two endo-beta-glucanases were separated from barley green malt. The two enzymes were distinguished by their heat sensitivities, solubilities in ammonium sulfate, and pH optima.

Both enzymes rapidly decreased the viscosity of barley glucan solutions. Neither enzyme was capable of decreasing the viscosity of carboxymethyl cellulose solutions; thus they were distinguished from malt cellulase.

The primary factor in the general softening or physical modification of barley during germination is the breakdown of cell-wall materials. The principal carbohydrate constituents of barley cell walls are cellulose and hemicellulose.

Typically, barley hemicelluloses are mixed hexose-pentose polymers containing glucose, arabinose, and xylose, with perhaps traces of other sugars and sugar derivatives. Glucose polymers isolated from barley hemicelluloses were reported by Aspinall and Telfer (1) to consist of unbranched chains containing approximately equal numbers of beta-1,3- and beta-1,4-linkages. More recent work by Smith's group on glucose polymers isolated from oat and barley hemicelluloses indicates that the -1,3- and -1,4-linkages are not evenly distributed. There are present sequences of -1,3-, and sequences of -1,4-linkages which are separated from one another by much larger molecular segments containing alternating -1,3- and -1,4-linkages (4).

The investigations described in this paper are concerned with the barley malt enzymes which attack the inner linkages of the glucose polymers present in barley hemicellulose. Study of the heat stability of the barley malt endo-beta-glucanase[3] system supported earlier suggestions that there was more than one enzyme responsible for its viscosity-reducing activity (2,5). Lack of a hemicellulose substrate containing only beta-1,3- or beta-1,4-polyglucosidic linkages has been a serious handicap in attempts to determine whether more than one specific polyglucosidase in fact is involved. This difficulty is readily apparent when it is considered that substrates available from barley gums contain both 1,3- and 1,4-beta-polyglucosidic linkages and prob-

[1] Manuscript received November 27, 1959.

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[3] Previously this system was designated endo-beta-polyglucosidase (5). The nomenclature has been changed to conform to that of the substrate, glucan.

ably 1,6 linkages. Hence, viscosity measurements do not distinguish whether the enzyme acting is specific for beta-1,3-linkages, beta-1,4-linkages, for both, or for some other linkage.

The work presented here describes experiments in which heat-stability of the enzymes which hydrolyze barley gums is used to distinguish the types of activity. The heat-stability criterion was used by applying the principle that the heat inactivation of an individual enzyme usually follows first-order kinetics.

Materials and Methods

Enzymes. Crude malt extracts were prepared by mashing finely ground malt for 2 hours in a 0.5% solution of sodium chloride at room temperature. The insoluble material was eliminated by filtration or centrifugation (5). Separation of the activities is described in the text.

Substrate. A 1.72% solution of Kindred barley gum prepared as described previously was used as the general substrate (5). Other substrates used in the investigations are described in the text.

Activity Determination. Enzyme activity was determined by measuring the change in viscosity of the substrate at pH 4.7 and 30°C. in an Ostwald No. 200 viscometer. Endo-beta-glucanase activities were expressed as the change in the reciprocal specific viscosity per 30 minutes per g. of dry malt (5).

Results

Differential Heat-Inactivation of Malt Endo-Beta-Glucanases. A green malt extract was heated at 50°C. for 10, 20, 30, and 60 minutes. The log of the activity remaining after each time interval is plotted in Fig. 1. There was a rapid decrease in activity for the first 20 minutes, after which the rate of inactivation decreased and, as evidenced by the straight-line function, appeared to follow first-order kinetics during the latter portion of the heating. Kinetics of this sort suggest that the green malt extract contained two or more endo-beta-glucanases, one of which was more heat-stable than the others. If this is true, then the heat applied during malt kilning should destroy relatively more of the less stable enzyme.

Figure 2 shows the effects of heating at 40°C. on the endo-beta-glucanase activity of extracts prepared from green malt and from the corresponding kilned malt. The amounts of the more heat-stable activity (A_S) present in the malt extracts can be estimated by extrapolating the straight-line portion of the log curves to zero heating time. The value obtained represents the amount of A_S which was pres-

ent before the heat inactivation was begun. Subtraction of this figure from the initial activity of the extract provides an estimate of the amount of less heat-stable activity (A_L) which was present in the original extract. These values were calculated from the curves in Fig. 2 and are presented in the table below. The results show that

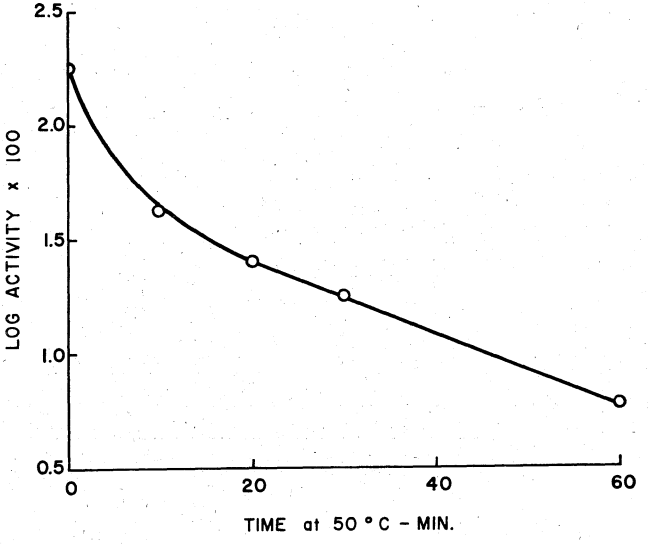

Fig. 1. Heat-inactivation of green malt extract at 50°C.

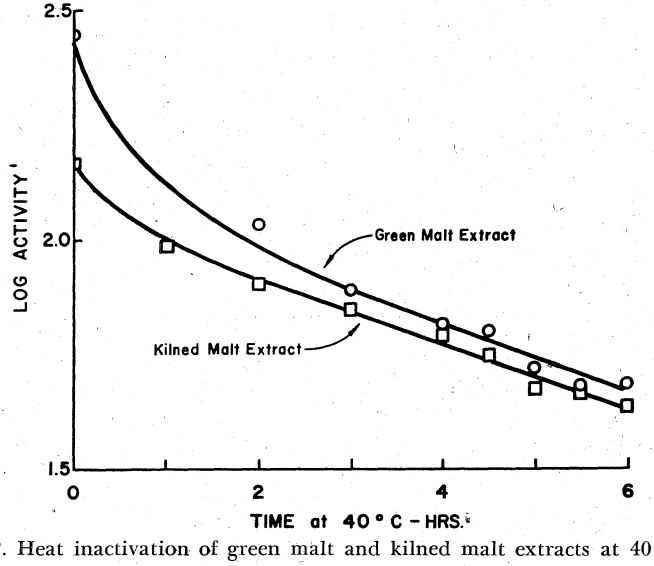

Fig. 2. Heat inactivation of green malt and kilned malt extracts at 40°C.

kilning resulted in a much greater loss of A_L than of A_S. About 90% of A_L was lost during kilning, as compared to about half of A_S.

	Activity	
	Green malt	*Kilned malt*
A_L	11.0	1.3
A_S	9.2	4.8

The data in Figs. 1 and 2 and the table above show that there are two or more endo-beta-glucanases present in barley green malt and in addition, that the activities can be distinguished by their behavior during heat-inactivation. With this tool for identifying the activities, separation of malt endo-beta-glucanases was undertaken.

Separation of Barley Malt Endo-Beta-Glucanases. The classic method of ammonium sulfate fractionation was selected for the separation. In general, the enzymes were precipitated at various levels of ammonium sulfate, followed by dialysis to remove the ammonium sulfate.

The results of a preliminary fractionation experiment are shown in the table below. Nearly one-third of the activity was precipitated between 20 and 40% saturated ammonium sulfate, and two-thirds of the activity between 40 and 60% saturated ammonium sulfate.

Ammonium Sulfate	*Activity of Precipitate*
% saturated	
20	0.1
40	4.9
60	10.4
80	1.2
95	0.0
Original extract	21.1
Original extract dialyzed	17.8

In order to facilitate fractionation and location of the activities it was desirable to omit the rather time-consuming dialysis step. The maximum possible carryover of ammonium sulfate with the enzyme precipitates was 0.27 g. per 100 ml. of reaction solution. Quantities of ammonium sulfate as high as 0.5 g. per 100 ml. of reaction solution were tested and did not affect the endo-beta-glucanase activity.

On the other hand, the results in Fig. 3 show that a small amount of ammonium sulfate did affect the kinetics of the enzyme inactivation. Dilution of the malt extract with an ammonium sulfate solution before heat-inactivation appeared to afford some protection to A_S. The smaller slope of the log curve reflects a lower rate of enzyme inactivation. Dilution of the malt extract with water before heat-in-

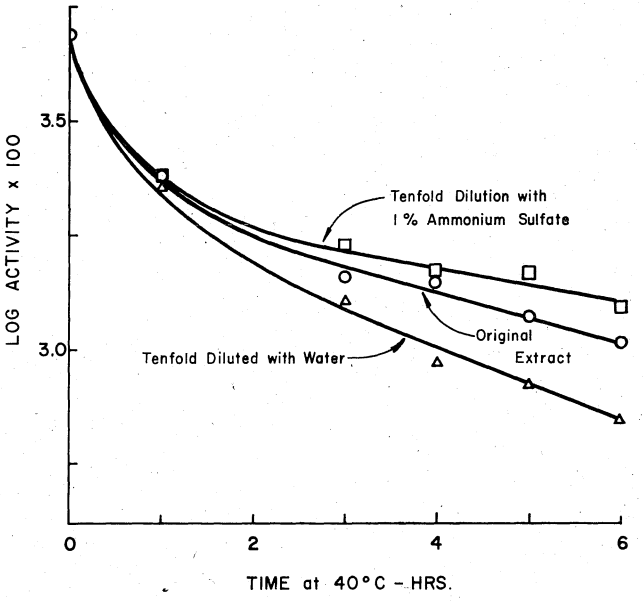

Fig. 3. Effect of ammonium sulfate on the heat sensitivity of malt endo-beta-glucanases.

activation resulted in a more rapid inactivation of A_S, as evidenced by the increased negative slope of the log curve. Even so, all samples provided the same estimation of the amounts of A_L and A_S present, showing the feasibility of directly assaying the ammonium sulfate precipitates.

Enzyme Preparation. The fractionation of a 0.5% sodium chloride extract of barley green malt is presented as a flow diagram in Fig. 4. The insoluble and precipitated materials were removed from the solubles by centrifugation.

Distribution of the endo-beta-glucanase activities in the first five fractions is shown in the table below. Heat-inactivation curves showed that fraction II was 85% A_L and 15% A_S, fraction III was 60% A_L and 40% A_S, and fraction IV was 30% A_L and 70% A_S. Refractionation improved the separation of the activities but resulted in marked

Fraction	*Activity*
	% of total recovered
I	4
II	27
III	36
IV	29
V	4

losses of enzyme. Fraction VII was 95% A_L and fraction X was 85% A_S.

Enzyme Purification. Portions of fractions VII and X were again fractionated. The precipitates which formed from VII at 40% saturated ammonium sulfate, and from X at 60% saturated ammonium sulfate, were collected and dialyzed. For dialysis the precipitates were dissolved in small volumes of water and dialyzed at 0 to 4°C. against 30-volume aliquots of distilled water. The water was replaced at 2.5, 5, and 7.5 hours. The final dialysis proceeded for 16 hours. Containers were subjected to gentle agitation during dialysis.

Very heavy precipitates which formed in both samples during dialysis were removed by centrifuging. The precipitates were resuspended in 0.5% sodium chloride solutions and tested for endo-beta-glucanase activity. Approximately one-tenth of the total activity remaining after dialysis was present in the precipitates; the remaining nine-tenths of the recovered activity was present in the supernatants. The dialysis

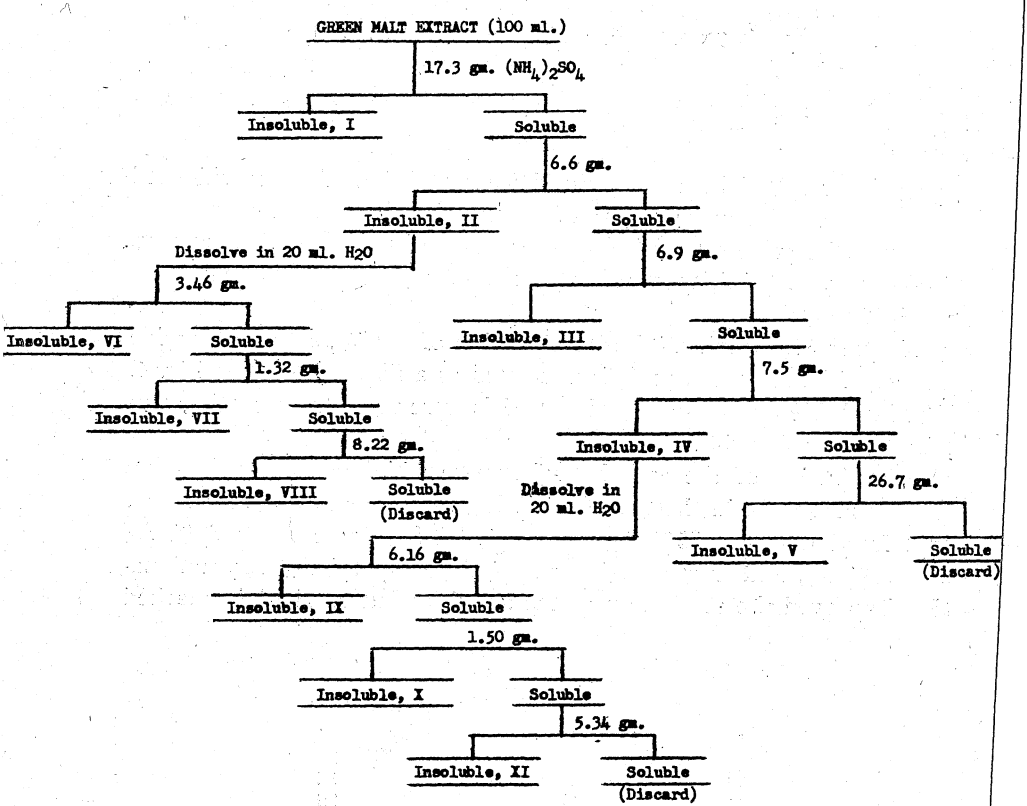

Fig. 4. Flow diagram of the enzyme fractionation.

step resulted in losses of nearly 30% of the initial activities.

The purity of the endo-beta-glucanase present in each supernatant was tested by heat-inactivation. The results are presented in Fig. 5. A_L was 95% free of A_S, and A_S was 90% free of A_L. In addition, the results in Fig. 5 show that A_L was half inactivated by heating for 40 minutes at 40°C. The more heat-stable A_S loses half of its activity

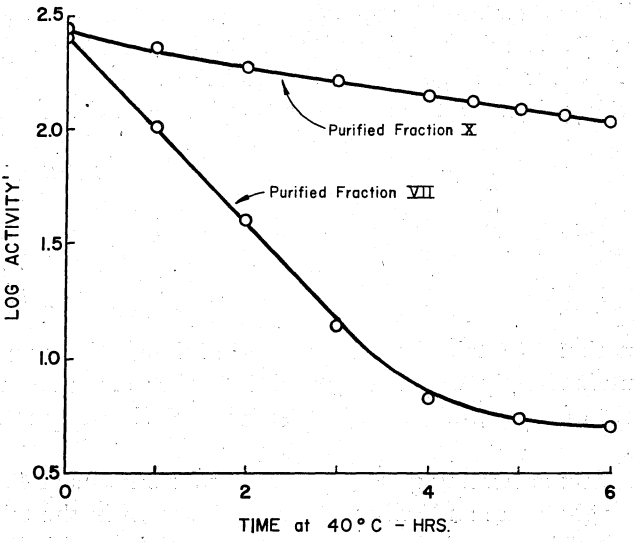

Fig. 5. Heat inactivation of purified endo-beta-glucanases at 40°C.

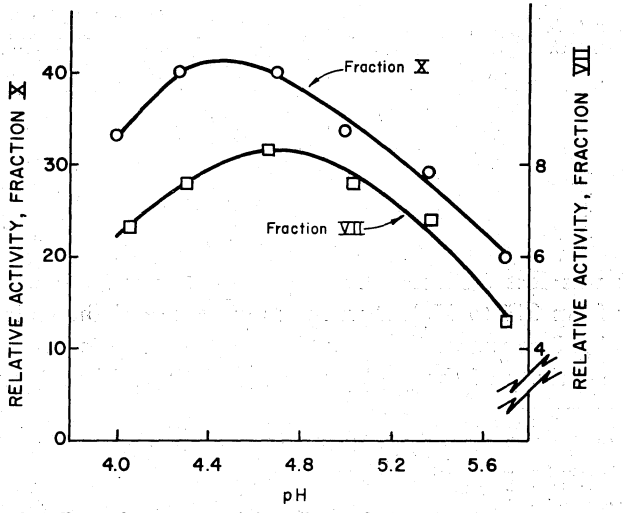

Fig. 6. Effect of pH on activity of purified endo-beta-glucanases.

after being heated for 5 hours and 15 minutes at 40°C.

Effect of pH on Activity. The endo-beta-glucanase activities[4] of fractions VII and X were measured over the pH range of 4.0 to 5.7. pH values reported are those found immediately following the reaction. Replicate experiments, illustrated by the data in Fig. 6, showed that fraction VII exhibited the greatest activity between pH 4.6 and 4.8, and fraction X between pH 4.3 and 4.7.

Studies on Substrate Specificity. Green malt extracts contain cellulase activity[5]. The distribution of malt cellulase in certain ammonium sulfate fractions is shown in the table below. Apparently the malt cellulase is quite soluble in less than 40% saturated ammonium sulfate. Fraction I contained no detectable cellulase activity and fraction VII contained only very little cellulase. Other experiments showed that most of the cellulase present in fraction X precipitates during dialysis.

	Relative Cellulase Activity
Original extract	19.2
Fraction I	0.0
Fraction VII	1.4
Fraction X	5.7

The effect of heating green malt extract for 5 hours at 40°C. on its endo-beta-glucanase and cellulase activity is shown in the table below. During the 5-hour period nearly all the A_L activity and half of the A_S activity was lost (reflected by a decrease of the endo-beta-glucanase activity from 915 to 178). During the same period the cellulase activity remained constant. Thus neither A_L nor A_S contributed to the cellulase activity of the malt extract. That is, neither A_L nor A_S was

Extract Held at 40°C.	*Relative Activity*	
	Endo-beta-glucanase	*Cellulase*
hours		
0	915	18.6
1	318	19.3
2	267	19.4
3	213	19.2
4	191	20.4
5	178	18.4

[4] The results in Fig. 6 and the next two tables are expressed as relative activities. Different amounts of the two enzymes were lost during fractionation and purification; hence, expression of activity on a dry malt basis would be misleading. The activities were compared by adjusting the enzyme solutions to a standard volume and using standard aliquots for the assays.

[5] As reflected by the ability to decrease the viscosity of a carboxymethyl cellulose solution. The activity is expressed as 100 times the change in reciprocal specific viscosity of a carboxymethyl cellulose solution per 30 minutes.

able to attack beta-1,4-linkages distant from the ends of carboxymethyl cellulose molecules.

The action of barley malt extract on a "rye pentose gum" substrate[6] is shown in Fig. 7. The viscosity dropped rapidly from 30 c.p.

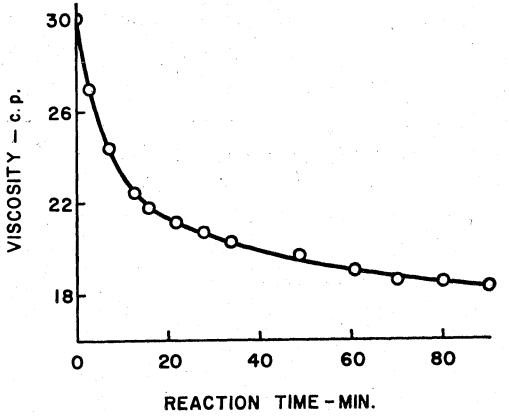

Fig. 7. Effect of barley malt extract on the viscosity of rye pentose gum.

to about 20 c.p., followed by a very slow decrease in viscosity as the reaction continued. The high-molecular-weight carbohydrate[7] which remained after 2 hours' hydrolysis contained 39% arabinose, 54% xylose, 3% glucose, and 4% fructose and/or mannose. It appears that the barley malt extract lacked certain endo-enzymes required to extensively degrade the "rye pentose gum." The same extract rapidly degrades barley glucan to small fragments (as reflected by solution viscosities approaching one).

Discussion

The experiments described herein demonstrated the presence of at least two endo-beta-glucanases in barley malt. One enzyme was considerably more heat-sensitive than the other. Nearly 90% of the more heat-sensitive enzyme was lost during kilning, as opposed to approximately half of the less heat-sensitive enzyme. Thus both enzymes likely are active during germination, while the less heat-sensitive enzyme may play the dominant role during the mashing step of the brewing process.

Preliminary fractionation of the barley malt extracts with more refined procedures has suggested the possible presence of one or more

[6] The "rye pentose gum" contained 18% glucose, 6% fructose and/or mannose, 30% arabinose, and 46% xylose on a weight basis; analyzed per Luchsinger, English, and Kneen (6).

[7] Molecular weights greater than 5,000 to 10,000 on the basis of dialysis experiments (3).

additional endo-beta-glucanases. The recent discovery that barley glucan contains -1,6- in addition to -1,3- and 1,4-linkages (4) likewise may be evidence that the two enzymes described herein do not constitute the entire beta-glucanase system.

The barley malt extracts were unable to extensively hydrolyze rye pentose gums showing a lack of certain endo-pentosanases. Whether a similar behavior would prevail with barley pentose gums has not been determined.

Additional experiments with substrate showed that neither A_L nor A_S contributes to the cellulase activity of barley malt as it is reflected by the decrease in viscosity of carboxymethyl cellulose (CMC). Thus, neither system showed significant activity toward beta-1,4-linkages distant from the ends of the CMC molecules. One might speculate from these results that the two systems also are unable to attack beta-1,4-linkages in barley beta-glucan.

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