Limit Dextrinase from Malted Barley: Extraction, Purification, and Characterization[1]

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Cereal Chem. 71(6):610-617

ABSTRACT

Limit dextrinase was prepared from malted barley with a high degree of purity and a yield of 17% of the total limit dextrinase. Enzyme activity was not affected by dithiothreitol, indicating that limit dextrinase is not a sulfhydryl enzyme. However, enzyme levels were enhanced significantly in the presence of proteins such as bovine serum albumen (BSA); the pH optimum of the enzyme was also affected by BSA. The enzyme was inhibited strongly by low levels (100 μg/ml) of β-cyclodextrin. Low levels of enzyme activity were present in barley, but enzyme levels increased rapidly during germination in a manner similar to that of α-amylase. A significant proportion of malt limit dextrinase was present in malt extracts in a soluble but inactive form. Conditions were established for the complete extraction of active limit dextrinase from malt and from barley germinated for varying periods of time (one to eight days).

Complete degradation of starch to fermentable carbohydrates during the mashing stage of brewing requires the action of three hydrolytic enzymes from malt (Manners 1985). α-Amylase rapidly hydrolyzes starch in a random fashion to a mixture of linear and branched dextrins. Linear dextrins, in turn, are hydrolyzed further by β-amylase to maltose, but the branched dextrins are incompletely hydrolyzed because neither α- nor β-amylase is able to hydrolyze the α-(1→6) bonds originating from the amylopectin component of starch. A third enzyme, limit dextrinase, is required to cleave these bonds and so render the branched dextrins susceptible to further hydrolysis by β-amylase. The central role played by limit dextrinase in starch hydrolysis was recognized several years ago (Enevoldsen and Schmidt 1973, Enevoldsen 1975). Several studies were conducted to isolate and purify the enzyme and to characterize its action on amylopectin and amylopectin-degradation products (Manners et al 1970, Manners and Yellowlees 1973, Manners and Hardie 1977). Interest in the enzyme declined when it was reported that 15–20% of malt starch was incompletely hydrolyzed during mashing and could be detected in the finished beer as highly branched dextrins (Enevoldsen and Bathgate 1969, Enevoldsen 1978). These results suggested that limit dextrinase had minimal influence on starch degradation during malting and brewing.

Limit dextrinase hydrolyzes α-limit dextrins much faster than it hydrolyzes amylopectin (Manners et al 1970). Therefore, it would not be effective during mashing until the starch has been hydrolyzed extensively by α- and β-amylases. The enzyme is heat labile (Lee and Pyler 1984) and so may be largely inactivated during mashing, before it has an opportunity to hydrolyze a significant amount of starch. In addition, the results of some studies have indicated that limit dextrinase is synthesized slowly during germination, thus, enzyme levels present in normal malts would be quite low (Lee and Pyler 1984). More recent studies have indicated, however, that it is difficult to extract all of the limit dextrinase from malt or germinated barley in an active form (Longstaff and Bryce 1991, McCleary 1992, Kristensen et al 1993). The enzyme may have been incompletely extracted in previous studies, so the enzyme levels reported may have been too low, especially during early stages of germination.

There has been an upsurge in interest in malt limit dextrinase recently (Sissons et al 1992a,b; Kristensen et al 1993; Longstaff and Bryce 1993; Sissons et al 1993). This has probably been sparked by advances made in the molecular biology of malt hydrolytic enzymes that now offer the possibility of improving the heat stability of the enzyme and increasing its rate of synthesis during germination. The goals of the current study were to develop optimum conditions for extraction of active enzyme from germinated barley and malt, to determine the rate of synthesis of the enzyme in germinating barley, and to develop an efficient method for purifying the enzyme so that its chemical, biochemical, and physical properties could be reevaluated.

MATERIALS AND METHODS

Development of Limit Dextrinase and α-Amylase During Germination

Barley kernels (cv. Harrington) were soaked for 20 min in sodium hypochlorite (1.5%) and rinsed thoroughly with deionized, sterile water. Kernels (50 per dish) were placed, crease down, in sterile petri dishes containing two pieces of Whatman No. 1 filter paper. Sterile water (4 ml) was added to each dish, and samples were germinated at 18°C and 90% rh in a growth cabinet (model FR-381C, Blue M Electric Co., Blue Island, IL). Filter papers were kept damp by the addition of sterile water as required.

Samples (50–150 kernels) were removed from the growth cabinet at daily intervals for eight days; they were freeze-dried and cleaned to remove shoots, rootlets, and discolored seeds. Kernel weights were determined, and samples were ground in a Udy cyclone mill (0.5-mm screen).

Limit dextrinase activity was assayed by extracting ground grist (0.4 g) with 8.0 ml of sodium acetate buffer (0.1*M*, pH 5.5) containing 25 m*M* dithiothreitol (DTT) (Sigma, 99% purity) in a 40°C water bath. Samples were extracted for 1, 5, 10, 16, 24, or 36 hr. Extracts were centrifuged (20,000 × *g*, 15 min, 20°C). Appropriate aliquots of the supernatants were made up to 1.0 ml with extract buffer containing 25 m*M* DTT. Samples were warmed for 5 min at 40°C and then assayed with Limit DextriZyme tablets (MegaZyme Australia, Warriewood, NSW) as directed by the manufacturer. Results are given for the average of four replicates (quadruplicate extractions, one analysis per extract).

α-Amylase activity was assayed by a modified Briggs method (Briggs 1961) using β-limit dextrin substrate prepared from waxy maize starch (MacGregor et al 1971). Ground samples were extracted by tumbling grist (0.2 g) with 5.0 ml of sodium acetate buffer (0.2*M*, pH 5.5, 1 m*M* $CaCl_2$) acetate containing 1 m*M* $CaCl_2$) for 16 hr at 4°C. Extracts were centrifuged (20,000 × *g*, 15 min, 5°C). Aliquots of diluted supernatants were assayed for 10 min at 35°C. Results are given for the average of six replicates (duplicate extractions, three analyses per extract).

Effect of DTT Concentration on Extraction and Activation of Limit Dextrinase from Green Malt

Green (unkilned) malt of Harrington barley was prepared in equipment previously described (Bettner et al 1962). Barley was

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germinated for eight days to maximize enzyme levels and then freeze-dried, cleaned, and ground in a Udy cyclone mill (0.5-mm screen). The ground grist was then extracted for 5 hr at 40°C with sodium acetate buffer (0.1*M*, pH 5.5) containing 0, 1, 5, 10, 25, or 50 m*M* DTT (1.2 g of grist per 24.0 ml of buffer). Samples were resuspended once every hour.

Extracts were centrifuged (20,000 × *g*, 15 min, 20°C), and appropriate aliquots of each supernatant were made up to 1.0 ml with buffer containing the same concentration of DTT (0, 1, 5, 10, 25, or 50 m*M*) used for extraction of the grist. Samples were warmed for 5 min at 40°C and assayed with Limit DextriZyme tablets. Three aliquots were assayed for each extract.

DTT was added to 10-ml portions of the remaining supernatants to increase the DTT level of all supernatants to 50 m*M*. Supernatants were returned to the 40°C bath for 16 hr, recentrifuged, and reassayed for limit dextrinase activity.

Purification of Limit Dextrinase

Extraction. Green malt of Harrington barley was ground in a Wiley mill (1.0-mm screen), and 500 g of grist was extracted with 1.5 L of sodium acetate buffer (0.1*M*, pH 5.5, 25 m*M* DTT) by shaking gently in a 40°C water bath for 16 hr. The slurry was centrifuged (9,000 × *g*, 15 min, 20°C), and the supernatant was collected. The pellet was reextracted (1 hr) with 1 L of the same buffer at 40°C and recentrifuged. The supernatants were combined.

Protein in the supernatant was precipitated overnight at 4°C with ammonium sulfate (80% saturation). After centrifugation (9,000 × *g*, 15 min, 4°C), the clear supernatant was discarded. Phosphate-citrate buffer (0.02*M*, pH 6.0) containing 1 m*M* monothioglycerol (Sigma, 98% purity) was added to the protein pellet until all protein was solubilized (400 ml of buffer). This solution was then dialyzed at 4°C against the same phosphate-citrate buffer (containing 1 m*M* monothioglycerol) to remove all traces of ammonium sulfate (SpectraPor 4 dialysis membrane, three changes of dialysis buffer, 18 L each). The dialyzed extract was centrifuged (8,000 × *g*, 15 min, 10°C) and filtered through glass wool.

Ion-exchange chromatography. A diethylaminoethyl (DEAE) ion-exchange column (4.8 × 45 cm, Whatman DE52) was equilibrated with phosphate-citrate buffer (0.02*M*, pH 6.0, 1 m*M* monothioglycerol). The dialyzed extract was loaded on to the column at 100 ml/hr, and the column was washed at 100 ml/hr with the same phosphate-citrate buffer until the A_{280} of the eluent returned to baseline. The column was then eluted at 100 ml/hr with a 4-L linear salt gradient (0.0–0.5*M* NaCl) formed with 2 L of equilibration buffer and 2 L of equilibration buffer containing 0.5*M* NaCl.

Column fractions (18 ml) were assayed for limit dextrinase activity essentially as described by Lee and Pyler (1982), using a soluble pullulan substrate. α-Amylase activity was measured by the modified Briggs method (Briggs 1961) as described above.

Fractions containing limit dextrinase were pooled, concentrated to ~85 ml in an Amicon ultrafiltration cell (PM10 membrane), and dialyzed for 48 hr at 4°C against several changes (each 18 L) of sodium acetate buffer (0.02*M*, pH 5.5, 1 m*M* monothioglycerol).

Affinity chromatography. A β-cyclodextrin affinity column (0.9 × 50 cm, Sepharose CL6B covalently linked to β-cyclodextrin) was prepared as described by Silvanovich and Hill (1976) and equilibrated with sodium acetate buffer (0.02*M*, pH 4.0, 1 m*M* monothioglycerol). The dialyzed pool from DEAE chromatography was adjusted to pH 4.0 with acetic acid, centrifuged (10,000 × *g*, 10 min, 4°C), and loaded immediately onto the affinity column at 30 ml/hr. The column was washed with sodium acetate buffer (0.02*M*, pH 5.5, 1 m*M* monothioglycerol), and eluted with a linear β-cyclodextrin gradient formed with 200 ml of sodium acetate buffer (0.02*M*, pH 5.5, 1 m*M* monothioglycerol) and 200 ml of the same buffer containing 0.5 mg/ml β-cyclodextrin. Fractions (4 ml) were collected into tubes containing 5 ml of acetate buffer (0.2*M*, pH 5.5, 1 m*M* monothioglycerol) to ensure that the limit dextrinase eluted from the column would not remain in a denaturing environment (i.e., at pH 4.0) for any longer than was necessary and to dilute the β-cyclodextrin concentration.

Column fractions were assayed for limit dextrinase and α-amylase activity as described for DEAE chromatography. Fractions containing limit dextrinase were pooled, concentrated to 50 ml in an Amicon ultrafiltration cell (PM10 membrane), and dialyzed overnight at 4°C against 18 L of acetate buffer (0.02*M*, pH 5.5, 1 m*M* monothioglycerol) to remove residual β-cyclodextrin. The pool was concentrated to 2.0 ml in an Amicon ultrafiltration cell in preparation for gel-permeation chromatography.

Gel-permeation chromatography. A Sephacryl S-200 gel filtration column (2.5 × 100 cm, Pharmacia) was equilibrated with sodium acetate buffer (0.02*M*, pH 5.5, 1 m*M* monothioglycerol). The postaffinity pool of enzyme (2 ml) was applied to the bottom of the column and eluted with equilibration buffer using an upward flow rate of 25 ml/hr. Fractions (5 ml) were assayed for limit dextrinase activity, and active fractions were pooled and concentrated in an Amicon ultrafiltration cell (PM 10 membrane).

Specific activity and protein determination. To determine the specific activity of limit dextrinase at each stage of purification, appropriate aliquots of each enzyme pool were made up to 1.0 ml with sodium acetate buffer (0.1*M*, pH 5.5, 25 m*M* DTT). Samples were warmed in a 40°C water bath and assayed with Limit DextriZyme tablets as directed by the manufacturer.

The protein content of each enzyme pool was determined by the method of Lowry et al (1951). Sample absorbance was read at 680 nm. Interference of the method by thiol reagents was removed by diluting all samples to reduce the thiol concentration (DTT and monothioglycerol) to 1 m*M*. Aliquots of these solutions were mixed with 1.0 ml of N′-ethylmaleimide (0.05*M*) as described by Hughes et al (1981). Standards were prepared containing BSA and either DTT (1 m*M*) or monothioglycerol (1 m*M*), depending on the thiol reagent in the sample.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis. SDS-PAGE was performed by the method of Laemmli (1970), using a 6% stacking gel and 10% running gel. The gel was cast with a BioRad Mini-Protean II cell and run at 200V constant voltage for 1 hr. Gels were stained for protein with a modified silver stain (Blum et al 1987). Broad range SDS-PAGE molecular weight standards (BioRad) were used to estimate molecular weights.

Isoelectric focusing and activity overlay gels. IEF of purified limit dextrinase was performed at 4°C using an LKB 2217 Ultrophor electrofocusing unit and a 4-mm thick gel ($\%T = 6.5$, $\%C = 2.4$) containing pH 4–8 Bio-Lyte ampholytes (BioRad). Samples were applied at the cathode end and focused for 2 hr at 10 W constant power. Gels were stained for protein with a modified silver stain (Blum et al 1987).

Limit dextrinase activity was detected on focused, unstained gels with an overlay substrate gel containing amylopectin β-limit dextrin (MacGregor and Dushnicky 1989, MacGregor et al 1994).

Effect of BSA Concentration on Limit Dextrinase Activity

A series of six sodium acetate buffers (0.1*M*, pH 5.5) containing 0, 0.05, 0.1, 0.25, 0.5, and 1.0 mg/ml BSA (Sigma RIA grade) was prepared. For each BSA concentration, 35 μl of purified limit dextrinase was added to 465 μl of buffer. After a 30-min preincubation at room temperature, 0.5 ml of 50 m*M* DTT (in the corresponding buffer and BSA) was added to each enzyme solution. Samples were assayed for limit dextrinase as described above. Triplicate analyses were performed for each BSA concentration.

Effect of DTT Concentration on Limit Dextrinase Activity

A series of six sodium acetate buffers (0.1*M*, pH 5.5) containing 0, 1, 5, 10, 25, and 50 m*M* DTT was prepared. For each DTT concentration, 35 μl of purified limit dextrinase was added to 965 μl of buffer and DTT, left for 30 min at room temperature, and assayed for limit dextrinase. Triplicate analyses were performed for each DTT concentration. A second buffer and DTT series was prepared with acetate buffer containing 0.5 mg/ml BSA. Assays were repeated as described above for buffer and DTT mixtures.

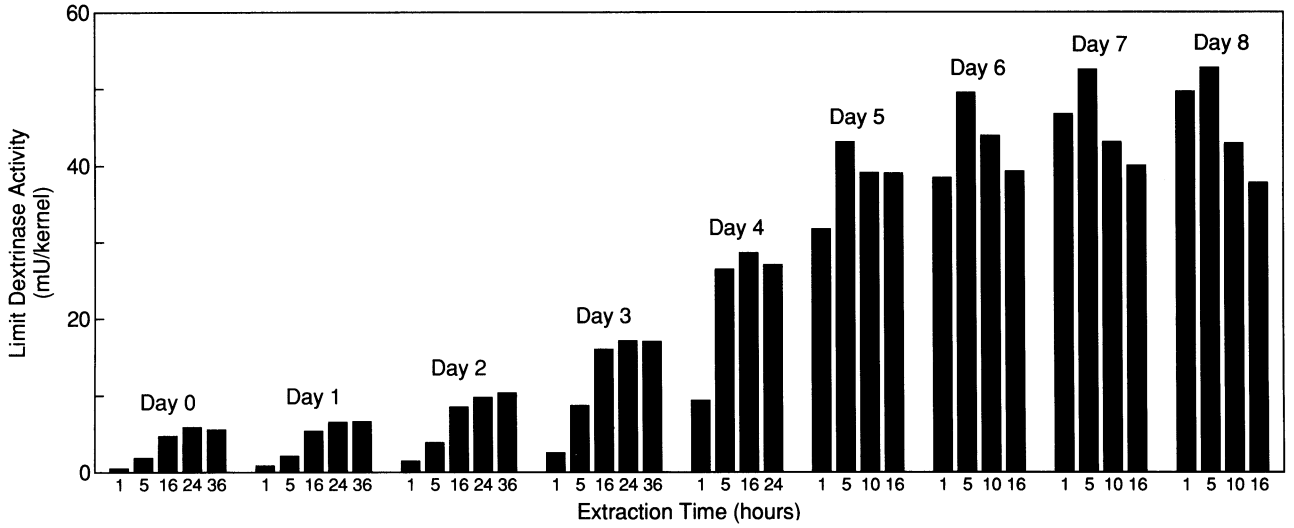

Fig. 1. Effect of time on the extraction of limit dextrinase from barley germinated for 0 to 8 days. Maximum standard deviation within replicates ranged from ±0.1 mU per kernel (Day 0) to ±1.0 mU per kernel (Day 8).

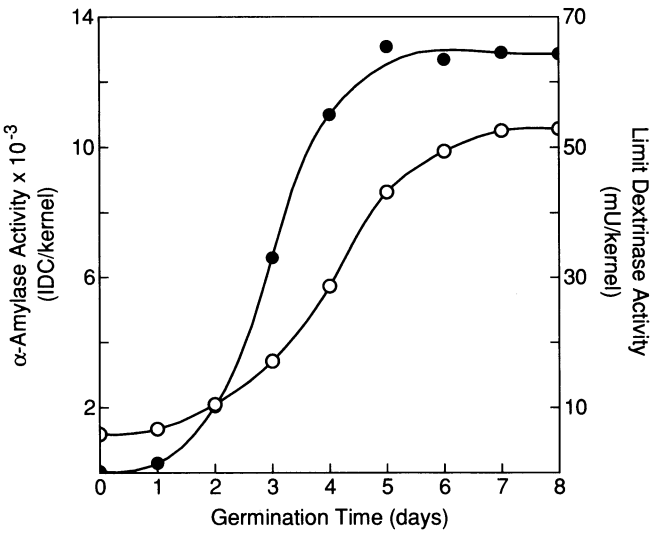

Fig. 2. Changes in limit dextrinase and α-amylase activities during germination. Limit dextrinase values are the maximum obtained at each stage of germination (Day 0 to Day 8). ○ = limit dextrinase activity; ● = α-amylase activity.

Effect of pH on Limit Dextrinase Activity (±0.5 mg/ml BSA, ±25 m*M* DTT)

Four experiments were conducted to determine whether BSA or DTT influenced enzyme activity over the pH 4.9–6.5 range using 0.2*M* sodium acetate buffer: 1) activity in buffer alone; 2) activity in buffer + 25 m*M* DTT; 3) activity in buffer + 0.5 mg/ml BSA; 4) activity in buffer + 25 m*M* DTT + 0.5 mg/ml BSA. In experiments 1 and 2, 50 μl of purified limit dextrinase was added to 950 μl of buffer (±DTT), left for 30 min at room temperature, and assayed for activity. In experiments 3 and 4, 30 μl of purified limit dextrinase was added to 970 μl of buffer (±DTT) and assayed in a similar fashion.

Limit dextrinase activity was also assayed over a wider pH range using a series of McIlvaine's buffers at pH 4.0–8.0 (0.1*M* citric acid/0.2*M* Na_2HPO_4). Limit dextrinase (30 μl) was added to 970 μl of McIlvaine's buffer containing both 0.5 mg/ml BSA and 25 m*M* DTT. The mixture was preincubated for 15 min at room temperature and assayed as described above.

Effect of Cyclodextrins on Limit Dextrinase Activity (with 25 m*M* DTT)

The effect of α-, β-, and γ-cyclodextrins (Sigma) on limit dextrinase activity was compared at two concentrations (90 μg/ml and 180 μg/ml) of cyclodextrin. Cyclodextrins were dissolved in sodium acetate buffer (0.1*M*, pH 5.5) containing 0.5 mg/ml BSA. Thirty-five microliters of purified limit dextrinase was added to 765 μl of each buffer/BSA/cyclodextrin mixture and left for 30 min at room temperature. After the 30-min preincubation, 200 μl of 125 m*M* DTT (in the same buffer/BSA/cyclodextrin mixture) was added. Samples were warmed for 5 min at 40°C and assayed with Limit DextriZyme tablets as previously described. Analyses were performed in triplicate.

The inhibitory effect of β-cyclodextrin on limit dextrinase activity in the presence of BSA was examined in greater detail by preincubating limit dextrinase enzyme with various levels of β-cyclodextrin (0, 10, 25, 30, 50, 60, 90, 100, 120, 150, and 180 μg/ml cyclodextrin in buffer containing 0.5 mg/ml of BSA) and assaying as described above. The effect of β-cyclodextrin on limit dextrinase activity without BSA was also examined by preincubating 35 μl of limit dextrinase with 765 μl of acetate buffer containing 0, 30, 60, 90, 120, 150, or 180 μg/ml of cyclodextrin (no BSA) for 30 min at room temperature, adding 200 μl of 125 m*M* DTT (in acetate buffer, no BSA), and assaying as described above.

RESULTS AND DISCUSSION

Changes in Limit Dextrinase Activity During Germination

Limit dextrinase activity increased rapidly after two to three days and reached maximum levels within seven to eight days of germination (Fig. 1). As germination progressed, shorter extraction times were required to yield maximum enzyme levels. For barley germinated for three days, for example, maximum limit dextrinase activity required 24–36 hr of extraction at 40°C when 25 m*M* DTT was included in the extract buffer. Barley germinated for eight days required just over 1 hr of extraction to reach maximum activity under the same experimental conditions. Beyond five days of germination, grists extracted for longer than 5 hr showed decreased limit dextrinase activity, probably because of denaturation of this heat-labile protein when maintained in solution for prolonged periods at 40°C.

These results show clearly that optimum extraction time for limit dextrinase depends on the extent of germination of the barley sample. The requirement for relatively long extraction times (5–16 hr) confirms results of previous studies (Longstaff and Bryce 1991, McCleary 1992, Kristensen et al 1993).

The small but significant level of limit dextrinase activity detected in the ungerminated grain was probably due to residual activity of enzyme synthesized during kernel development (Laurière et al 1985, Sissons et al 1993). Other research workers have also reported detectable levels of limit dextrinase in ungermi-

nated barley (Manners and Yellowlees 1973, Lenoir et al 1984, Laurière et al 1985, Sissons et al 1993). Enzyme levels in barley are below those found in ungerminated oats and rice (McCleary 1992). Extraction time is important because very little enzyme was detected after 1 hr compared to that found after a 24-hr extraction period.

Maximum enzyme levels obtained at each stage of germination were plotted against germination time (Fig. 2). For comparison purposes, α-amylase levels were also included. α-Amylase activity was very low in the ungerminated barley, indicating that the barley was unlikely to have been sprout-damaged. This supports the contention that the limit dextrinase present in the barley was residual activity from earlier stages of kernel development and was not a result of preharvest sprouting.

α-Amylase activity increased rapidly during germination and reached maximum levels after five days. However, the increase in limit dextrinase activity appeared to be slower; maximum levels of activity were not attained until day 7. Nevertheless, limit dextrinase activity did increase significantly between days 2 and 5, which is in agreement with other results reported by Longstaff and Bryce (1991) and Kristensen et al (1993). Lee and Pyler (1984) reported that the increase in limit dextrinase activity during germination was much slower than it was for other hydrolytic enzymes, such as α-amylase. It should be noted, however, that in this latter study the enzyme was assayed under different conditions, and optimum extraction of the enzyme may not have been achieved. The rate of enzyme synthesis is also dependent on the cultivar used (Longstaff and Bryce 1991, McCleary 1992).

Effect of DTT Concentration on Extraction and Activation of Limit Dextrinase from Green Malt

The limit dextrinase activities shown in Figure 2 represent the maximum, or total, activity found in germinating barley. Kristensen et al (1993) described three distinct forms of limit dextrinase: 1) free or fully active; 2) latent limit dextrinase in crude extracts that must be activated or released by the aid of reducing agents; and 3) bound limit dextrinase remaining in the pellet after extraction.

From the data shown in Figure 1, it is not possible to determine whether the low levels of limit dextrinase activity obtained after short extraction times (e.g., 1 hr) were due to incomplete solubilization of enzyme from the grist, or whether the enzyme was solubilized quickly, but existed in the extract supernatant in a latent and inactive form.

To examine this phenomenon of free and latent limit dextrinase, green malt of Harrington barley was first extracted for 5 hr at 40° C with different amounts of DTT in the extraction buffer. Limit dextrinase activity in the crude extract increased as the concentration of DTT in the extraction buffer increased (Fig. 3). A similar trend was observed by Longstaff and Bryce (1991) for malt and by Yamada (1981) for extraction of rice limit dextrinase. However, when the supernatants were made up to 50 m*M* DTT and maintained at 40° C for a further 16 hr, there was a significant increase in limit dextrinase activity in all the supernatants, particularly for those prepared with 0–10 m*M* DTT (Fig. 3). Since the pellet was discarded before the supernatants were returned to the water bath for the further 16 hr of activation with 50 m*M* DTT, the increase in activity could not have been caused by further solubilization of enzyme from the grist. The increase in activity must have been due to activation of latent enzyme already in the supernatant, or possibly, to an increase in efficiency of active enzyme due to conformational changes in the enzyme.

Results in Figure 3 indicate that the maximum amount of limit dextrinase was extracted from malt grist within 5 hr, with as little as 5 m*M* DTT. Lower concentrations of DTT did not appear

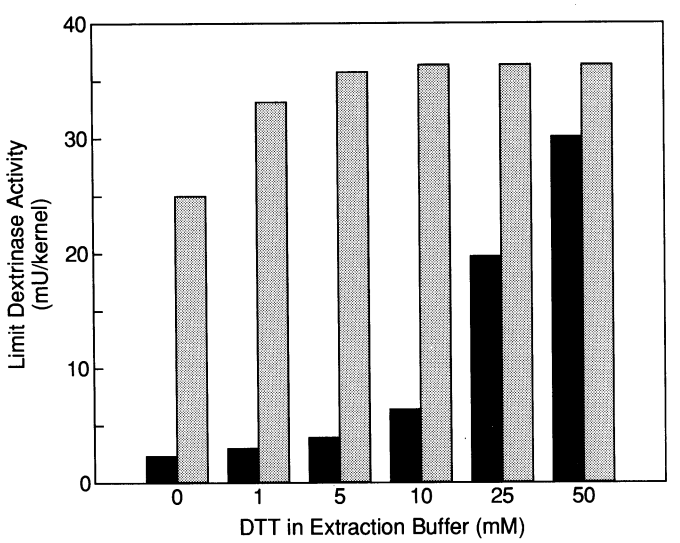

Fig. 3. Effect of dithiothreitol (DTT) concentration on the extraction and activation of limit dextrinase from green malt. ■ = activity after a 5-hr extraction; ▨ = activity after activation of the supernatants for 16 hr with 50 m*M* DTT. Maximum standard deviation within replicates was ±0.4 mU per kernel.

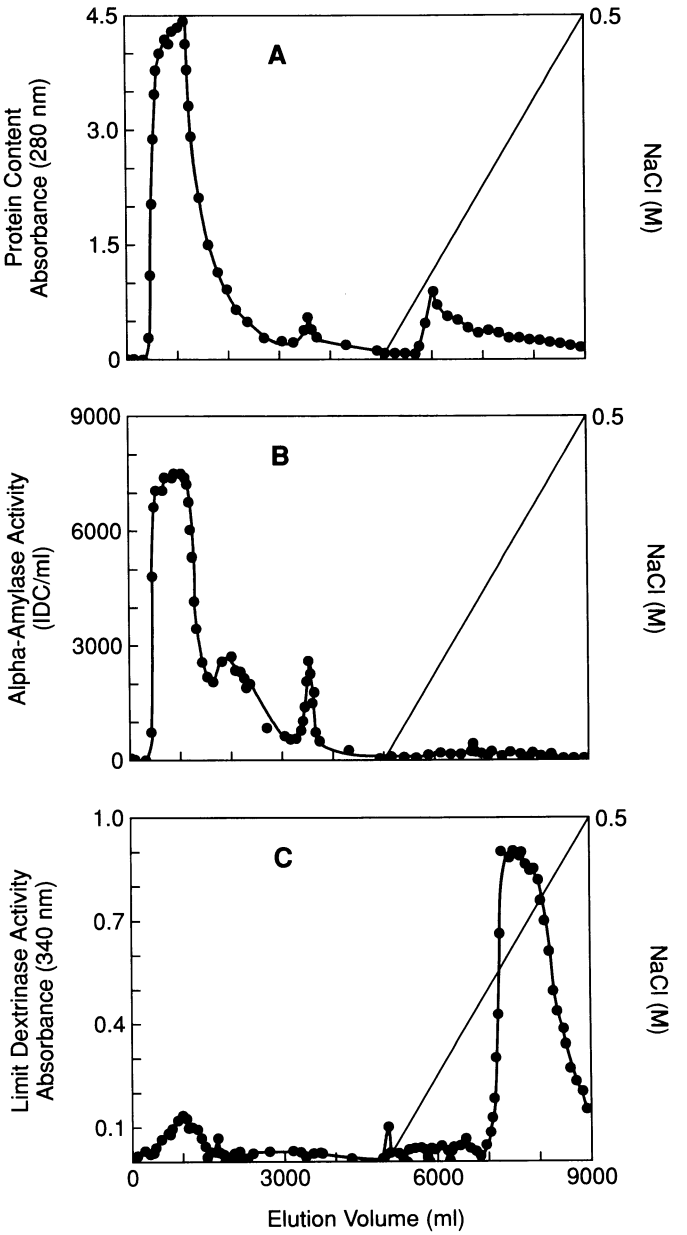

Fig. 4. Diethylaminoethyl (DEAE) ion-exchange chromatography. **A,** protein content (A_{280}); **B,** α-amylase activity; **C,** limit dextrinase activity. Solid line is salt gradient.

to extract all limit dextrinase from the grist within the 5-hr period. Activated extracts prepared with buffer alone (no DTT) contained only 70% of maximum activity, indicating that 30% of the total activity remained bound in the discarded pellet.

Two theories have been proposed to explain how latent limit dextrinase might be activated or released by reducing agents in the supernatant of crude malt extracts. Longstaff and Bryce (1993) proposed that reducing agents such as DTT activate sulfhydryl proteases, which, in turn, activate limit dextrinase by proteolytic modification of the inactive enzyme. Macri et al (1993) reported that barley contains an endogenous inhibitor of limit dextrinase that forms an inactive but soluble complex with limit dextrinase, and that this complex is somehow disrupted by the addition of reducing agents. It is quite possible that further research will show that the role of reducing agents in the activation of limit dextrinase is a combination of both proteolytic modification by sulfhydryl proteases and a disruption of the limit dextrinase inhibitor complex. Levels of limit dextrinase inhibitor in Harrington barley appear to decrease as germination proceeds (A. W. MacGregor, *unpublished data*), and the proportion of total limit dextrinase extracted from the grist as free or active enzyme increases as germination proceeds (Longstaff and Bryce 1991, 1993; Kristensen et al 1993). It is possible that sulfhydryl proteases that are synthesized during germination (Mikola 1983, Wrobel and Jones 1993) are responsible for hydrolysis of the inhibitor itself, either in the germinating kernel or during extraction of malt. Proteolytic degradation of the inhibitor might explain the trend observed in Figure 1. As sulfhydryl protease activity increases during germination, increasing amounts of the inhibitor would be destroyed, thus shorter extraction times would be required to achieve maximal limit dextrinase activity.

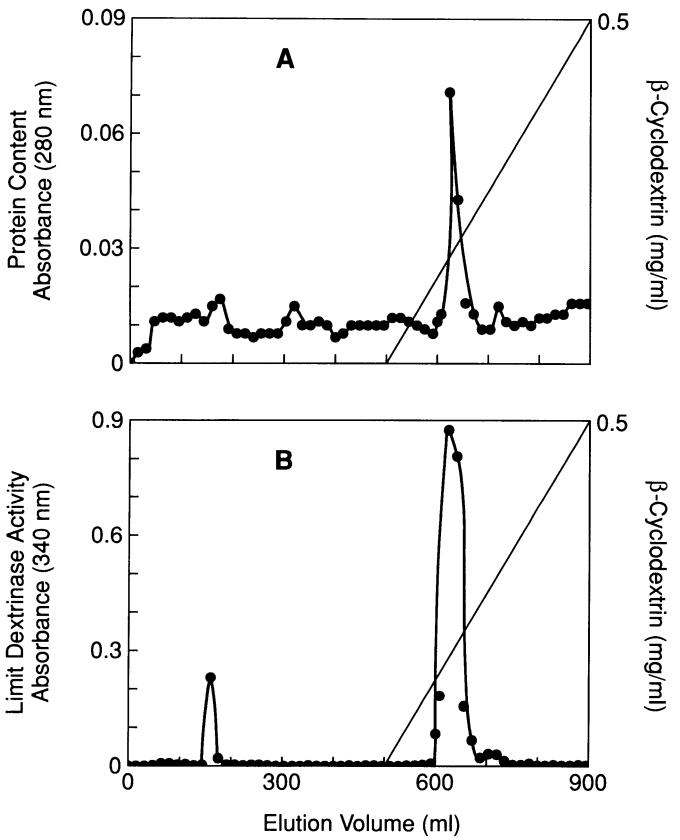

Fig. 5. Affinity chromatography on a column of β-cyclodextrin. **A,** protein content (A_{280}); **B,** limit dextrinase activity. Solid line is β-cyclodextrin gradient.

Purification of Limit Dextrinase

Preliminary experiments showed that green malt of Harrington barley required 10–16 hr of extraction at 40°C with 25 m*M* DTT to achieve maximum limit dextrinase activity in the grist extract. These same conditions were chosen for the bulk extraction of the enzyme used during purification. Because preliminary experiments had shown that such activation steps were not required, limit dextrinase assays performed during the purification procedure were not subjected to a further activation step.

DEAE chromatography was used in the first step of the purification to remove as large a portion of the α-amylase activity as possible from the extract. This was achieved in part by equilibrating and loading the column at pH 6.0, so that the large amounts of α-amylase 2 (pI 6.0–6.4) in the extract would be eluted in the load and wash fractions (Fig. 4).

Binding of limit dextrinase to the β-cyclodextrin column was improved in two ways. First, the affinity column was equilibrated to pH 4.0, and the pH of the DEAE pool was adjusted to pH 4.0 just before loading the column. Preliminary experiments showed that the percentage of limit dextrinase binding to the affinity column increased as the starting pH of the column decreased. This improved binding resulted in 70% of the limit dextrinase that was applied to the column being recovered in the postaffinity pool (Fig. 5, Table I). This degree of binding is similar to that reported by Maeda et al (1978) and is higher than that reported by other researchers, who typically used a higher pH (5.5 or 6.0) for β-cyclodextrin columns (Lecommandeur et al 1984, Sissons et al 1992a, Kristensen et al 1993). It should be noted that limit dextrinase activity is lost quickly at pH 4.0, and it was necessary to bring the pH of the eluted enzyme back up to 5.5 as quickly as possible (e.g., by collecting column effluent in tubes containing a small volume of strong pH 5.5 buffer).

Binding of limit dextrinase to the affinity column was also improved by first removing other β-cyclodextrin-binding enzymes, such as α-amylase, from the preparation during DEAE chromatography. α-Amylase binds to β-cyclodextrin columns much tighter than does limit dextrinase (Lecommandeur et al 1988) and would be eluted after limit dextrinase in the β-cyclodextrin gradient. Removal of α-amylase during DEAE chromatography should allow free access of limit dextrinase to the binding sites on the β-cyclodextrin column.

Limit dextrinase was eluted from the column by a low concentration of β-cyclodextrin (Fig. 5). A large increase in the specific activity of the enzyme was achieved by this step (Table I), confirming the effectiveness of an appropriate affinity chromatography method for protein purification.

Gel-permeation chromatography on Sephacryl S-200 was used as the final step in the purification of the enzyme to remove β-cyclodextrin, as well as a small amount of a low molecular weight protein contaminant, from the preparation.

Electrophoretic techniques showed that the post-gel filtration enzyme preparation was highly purified. A single band of protein

TABLE I
Purification of Limit Dextrinase

Purification Stage	Volume (ml)	Total Activity (mU)	Total Protein (mg)	Specific Activity (mU/mg)	% Recovery	Purification (Fold)
Extract	1,720	412,000	50,200	8.21	100	1
Ammonium sulfate precipitate	575	230,000	7,740	29.7	55.8	3.6
After ion-exchange chromatography	93	161,000	144	1,120	39.1	136
After affinity chromatography	61	116,000	13.1	8,860	28.2	1,080
After gel-permeation chromatography	44	70,400	8.32	8,460	17.1	1,030

(~105 kDa) was revealed by SDS-PAGE (Fig. 6). IEF resolved a single band of protein at approximately pI 4.6 (Fig. 7). This molecular weight and pI are similar to those previously reported for limit dextrinase isolated from various grains (Serre and Laurière 1989), although other investigators have reported finding multiple bands of limit dextrinase (i.e., isoenzymes) in malt, differing only slightly in pI (Lenoir et al 1984, Lecommandeur et al 1988, Sissons et al 1992a, Kristensen et al 1993). There was no evidence of α-amylase activity on β-limit dextrin overlay gels (i.e., there were no clear areas in the gel due to hydrolysis of the dextrin substrate by α-amylase) even after prolonged incubation (20 hr). There was one dark blue band on the overlay gel (indicative of limit dextrinase hydrolysis) that corresponded to the position of the focused protein band on the IEF gel (Fig. 7).

Characterization of Purified Limit Dextrinase

Effect of BSA. Addition of as little as 0.05 mg/ml BSA to assay buffers increased the activity of purified limit dextrinase threefold (Fig. 8). Enzyme activity increased only slightly as the BSA concentration increased above this level. This stabilization or activation effect of BSA on limit dextrinase was not specific to BSA. Proteins in crude barley extracts showed the same ability to boost the activity of purified limit dextrinase, providing that endogenous limit dextrinase inhibitor was first removed from the extracts (A. W. MacGregor, *unpublished data*).

During purification of limit dextrinase, there was a large decrease in the specific activity of the enzyme when the protein concentration of the preparation decreased to a critical level (i.e., after affinity chromatography). For this reason, 0.5 mg/ml BSA was added routinely to assay buffers to mask the effect that variable protein levels in the samples might have on limit dextrinase activity.

Effect of DTT. The addition of 1 m*M* DTT to purified limit dextrinase raised the enzyme activity slightly (Fig. 9), but little further increase in activity was obtained when the DTT concentration was increased to 25 m*M*. In the presence of BSA, even low levels of DTT had no effect on limit dextrinase activity (Fig. 9). This confirms the findings of Longstaff and Bryce (1993) that limit dextrinase itself is not a sulfhydryl enzyme requiring reducing agents for full activity. It should be stressed that reducing agents such as DTT must be added to buffers during the purification procedure to obtain high yields of pure enzyme. Whether reducing agents are required during purification for activation of sulfhydryl proteases, stabilizing the limit dextrinase, or whether they play a role in blocking the endogenous limit dextrinase inhibitor from complexing the enzyme is still unknown. These results confirm that the effect of DTT on limit dextrinase levels in malt extracts (Fig. 3) is not caused by simple activation of the enzyme by sulfhydryl groups.

pH Optimum. The effect of pH on the activity of purified limit dextrinase was dependent to a large degree on whether BSA was present in the assay buffer (Fig. 10). With 0.5 mg/ml of BSA

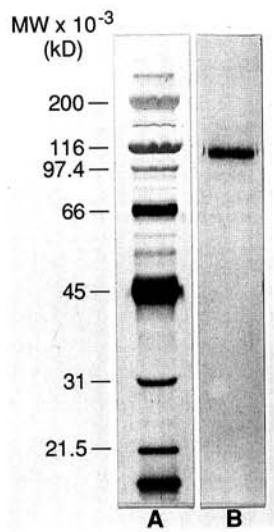

Fig. 6. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of purified limit dextrinase. **A,** broad range molecular weight markers; **B,** limit dextrinase.

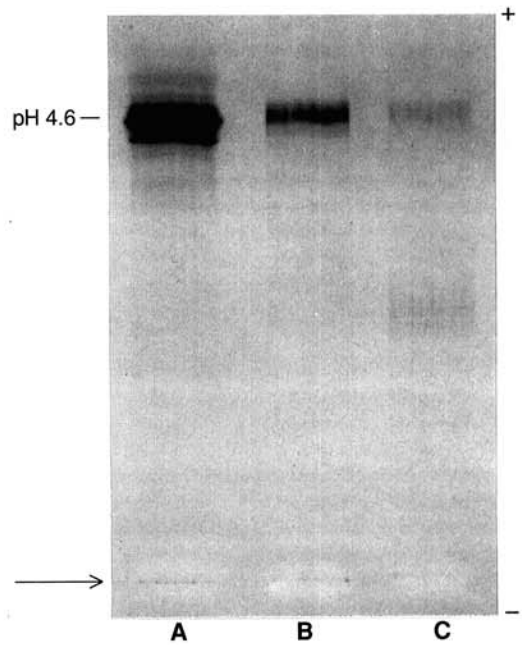

Fig. 7. Isoelectric focusing of purified limit dextrinase using a pH 4–8 gradient. **A,** soybean trypsin inhibitor, pI 4.6 (protein stain); **B,** limit dextrinase (protein stain); **C,** limit dextrinase (activity overlay gel using amylopectin β-limit dextrin); + = pH 4.0; − = pH 8.0; → = point of application of samples.

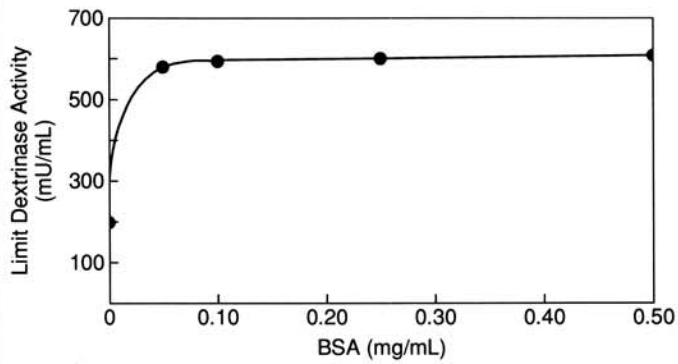

Fig. 8. Effect of bovine serum albumen (BSA) on the activity of purified limit dextrinase. All assay buffer contained 25 m*M* dithiothreitol.

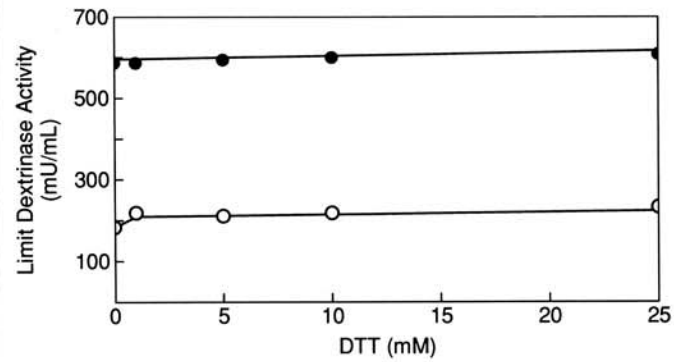

Fig. 9. Effect of dithiothreitol (DTT) on the activity of purified limit dextrinase. ○ = no bovine serum albumen (BSA); ● = with 0.5 mg/ml BSA.

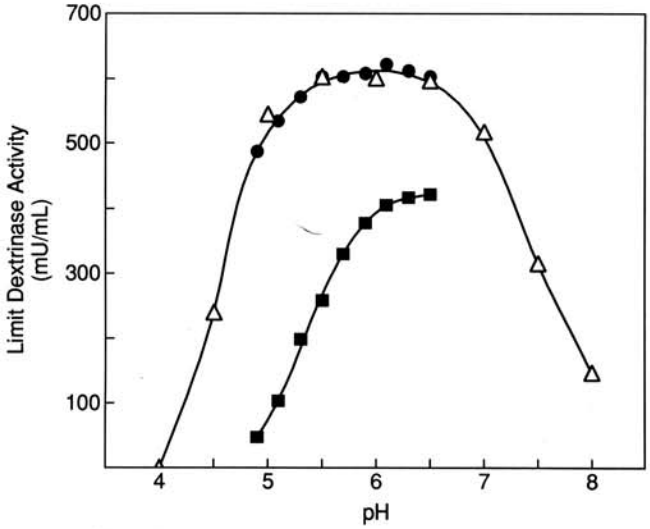

Fig. 10. Effect of pH on the activity of purified limit dextrinase with and without bovine serum albumen (BSA). △ = Phosphate-citrate buffer with 0.5 mg/ml BSA; ● = acetate buffer with 0.5 mg/ml BSA; ■ = acetate buffer, no BSA. All assay buffers contained 25 m*M* dithiothreitol.

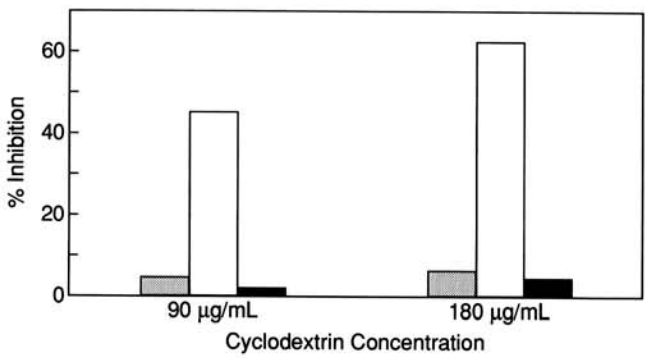

Fig. 11. Inhibition of purified limit dextrinase by cyclodextrins. ▨ = α-cyclodextrin; □ = β-cyclodextrin; ■ = γ-cyclodextrin. All assay buffers contained bovine serum albumen (0.5 mg/ml). Maximum standard deviation within replicates was ±1.2% inhibition.

and 25 m*M* DTT, limit dextrinase showed maximum activity at pH 5.5–6.5 in both acetate and phosphate-citrate buffers. Without BSA (i.e., with 25 m*M* DTT alone), enzyme activity was reduced at lower pH values, and maximum activity occurred well above pH 5.5.

All data shown in Figure 10 are given for assays conducted with 25 m*M* DTT. When parallel experiments were conducted without DTT (i.e., buffer + 0.5 mg/ml BSA or buffer alone), limit dextrinase activities were decreased only slightly (data not shown). However, the general shape of the curves and pH optima were very similar to results obtained in corresponding experiments conducted with DTT, and still depended, in large part, on the presence or absence of BSA in the assay buffer.

Published values for the pH optimum of limit dextrinase from various cereals are generally in the range of pH 5.3–5.5 (Manners and Yellowlees 1971, Maeda et al 1978, Serre and Laurière 1989, Sissons et al 1992a), but buffer systems, incubation times, and substrates differed from those used in the present study.

Effect of Cyclodextrins on Limit Dextrinase

Cyclodextrins have been shown to inhibit limit dextrinases from several sources, such as sweet corn (Marshall 1973), rice (Iwaki and Fuwa 1981), barley (Maeda et al 1978, 1979) and sugar beet leaves (Li et al 1992). The effect of all three cyclodextrins (α-, β-, and γ-cyclodextrins) on malt limit dextrinase was determined at two cyclodextrin concentrations in the presence of BSA (0.5 mg/ml). The results are shown in Figure 11. β-Cyclodextrin was

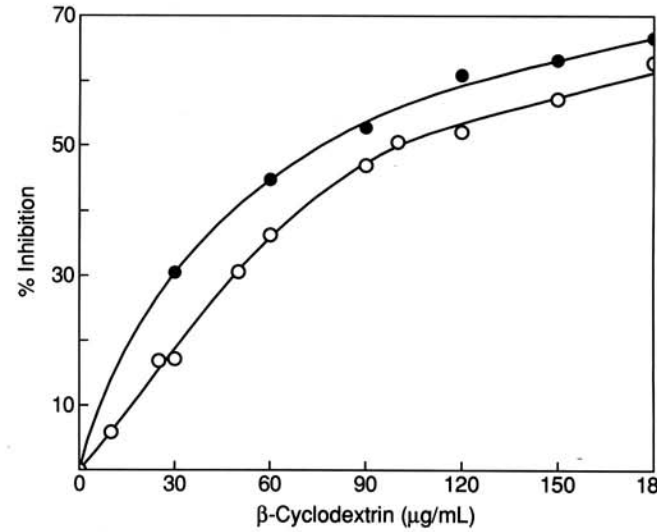

Fig. 12. Inhibition of purified limit dextrinase by β-cyclodextrin. ● = No bovine serum albumen (BSA); ○ = with 0.5 mg/ml BSA.

the most potent inhibitor, and the extent of inhibition was similar to that reported by Maeda et al (1979). A more indepth study of inhibition by β-cyclodextrin was conducted in the presence or absence of BSA (Fig. 12). Percent inhibition was not linearly related to β-cyclodextrin concentration, and significantly more inhibition occured without BSA at any given level of β-cyclodextrin. Under the conditions of this experiment, 50% of the enzyme activity was inhibited by 75 µg/ml of β-cyclodextrin without BSA, but 100 µg/ml of β-cyclodextrin was required to achieve 50% inhibition when BSA was included in the assay.

SUMMARY

Limit dextrinase was purified from unkilned barley malt by extraction at 40°C with 25 m*M* DTT, ammonium sulfate precipitation, and chromatography on ion-exchange (DEAE, pH 6.0), affinity (β-cyclodextrin Sepharose with pH 4.0 for loading and pH 5.5 for elution), and gel-filtration (Sephacryl S-200, pH 5.5) columns. The enzyme had high specific activity, was homogeneous, and gave single bands of protein when examined by SDS-PAGE (~105 kDa) and IEF (~pI 4.6). Activity of the purified enzyme increased threefold when BSA was added to the assay buffer. DTT had little effect on activity of the purified enzyme, indicating that limit dextrinase itself is not a sulfhydryl enzyme. The enzyme had an pH optimum of 5.5–6.5 in the presence of 0.5 mg/ml BSA and 25 m*M* DTT and was inhibited by low levels of β-cyclodextrin.

The amount of limit dextrinase extracted from malted barley depended on several factors including time of extraction, amount of DTT in the extraction buffer, and degree of germination of the sample. For samples germinated for one to eight days and extracted at 40°C with 25 m*M* DTT, the extraction time required to achieve maximum activity decreased as germination time increased. Ungerminated barley contained a small but significant level of limit dextrinase activity that increased rapidly after two days of germination and reached a maximum after five to six days of germination. For green malt extracted at 40°C for 5 hr with different levels of DTT, a high percentage of the total enzyme in the grist was solubilized with as little as 5 m*M* DTT. However, a major portion of this solubilized enzyme was inactive. Full enzyme activity required further activation of the extract supernatant by incubation at 40°C with reducing agents.

ACKNOWLEDGMENT

We thank M. Rudd for technical assistance.

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[Received May 17, 1994. Accepted August 15, 1994.]