

Large-Scale Purification and Characterization of Barley Limit Dextrinase, a Member of the α -Amylase Structural Family

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ABSTRACT

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Homogeneous barley limit dextrinase (LD) was isolated on a large scale in a yield of 9 mg/kg of 10-day germinated green malt. This represents a 9,400-fold purification and 29% recovery of the activity in a flour extract in 0.2M NaOAc (pH 5.0) containing 5 mM ascorbic acid. The purification protocol consists of precipitation from the extract at 20–70% saturated ammonium sulfate (AMS), followed by diethylaminoethyl (DEAE) 650S Fractogel anion-exchange chromatography, and affinity chromatography on β -cyclodextrin-Sepharose in the presence of 2M AMS. LD was eluted by 7 mM β -cyclodextrin and contains a single polypeptide chain of 105 kDa (SDS-PAGE) and pI 4.3. Sequence analysis of tryptic fragments,

prepared from 2-vinylpyridinylated LD and purified by RP-HPLC, identified short motifs recognized in β -strand 2, 3, and 5 characteristic of a catalytic (β/α)₈-barrel domain of the α -amylase family of amylolytic enzymes. Barley LD has \approx 50 and 85% sequence identity to bacterial pullulanases and rice starch debranching enzyme, respectively. By using ¹H-NMR spectroscopy, LD hydrolyzes specifically α -1,6-glycosidic linkages in pullulan and a branched oligodextrin, 6²-O- α -maltotriosyl-maltotriose, with retention of the α -anomeric configuration. β -Cyclodextrin competitively inhibits the LD activity with K_i of 40 μ M, while K_i is 1.9 mM and 2.4 mM for α -cyclodextrin and γ -cyclodextrin, respectively.

The enzyme-catalyzed mobilization of storage starch granules in the endosperm of germinating cereal seeds involves an initial solubilization, mainly by α -amylase, which is followed by hydrolysis of the resulting dextrans to oligosaccharides and glucose by α -amylase in conjunction with β -amylase, limit dextrinase (LD), and α -glucosidase (MacGregor 1987). Of these activities, only LD (pullulanase, R-enzyme, EC 3.2.1.41) acts specifically on the α -1,6-glycosidic linkages, and it shows decreasing activity toward β -limit dextrin, α -limit dextrans, pullulan, and amylopectin (MacGregor 1987, Maeda et al 1979).

LD is synthesized in germinating barley seeds in response to the phytohormone gibberellic acid (Hardie 1975, Lee and Pyler 1984). Small amounts of LD, however, appear during grain filling and remain in the mature seeds (Manners and Yellowlees 1973, Lenoir et al 1984, Sissons et al 1993). In contrast, LD in pea (Vlodavsky et al 1971) and rice (Dunn and Manners 1975) accumulates in an inactive form in the developing seed and is activated at germination. Papain or thiol compounds can trigger rice LD activation in vitro (Yamada 1981), reminiscent of the release by thiols of the bound LD activity from barley malt (Longstaff and Bryce 1991, McCleary 1992). In vivo, the fraction of bound LD in malt is reduced from \approx 85% at day 3 to \approx 35% at day 7 of germination (Kristensen et al 1993). This mobilization presumably stems from the action of germination-induced thiol endoproteases (Longstaff and Bryce 1993) or natural reductants such as thioredoxins (Kobrehel et al 1992) and thionins (Johnson et al 1987). Recently, endogenous proteinaceous LD inhibitors were identified and proposed to play a role in the regulation of LD activity in germinating barley (Macri et al 1993).

There are subtle specificity differences among the various debranching enzymes. Thus, the smallest substrate for barley LD is isopanose, 6-O- α -maltosyl-glucose (Maeda et al 1979), but 6²-O- α -maltosyl-maltose and 6³-O- α -maltosyl-maltotriose for *Klebsiella aerogenes* pullulanase and *Pseudomonas amyloclavata* isoamylase, respectively (Kainuma et al 1978). LD and the closely related enzyme pullulanase both catalyze hydrolysis of α -1,6-linkages in pullulan

to produce maltotriose. The related enzymes, isopullulanase and neopullulanase act on α -1,4-linkages of the maltotriose repeat unit in pullulan to yield isopanose (Sakano et al 1971) and panose (Kuriki et al 1988), respectively. α -Amylase-pullulanase (Melasniemi 1988), amylopullulanase (Saha et al 1988), and TVA α -amylase (Sakano et al 1982, Tonuzaka et al 1995), which possess dual α -1,4 and α -1,6 specificity and thus catalyze hydrolysis of both bond-types in pullulan, have intermediary specificity.

Sequence comparison shows that the various starch debranching and pullulan-hydrolyzing enzymes are large multidomain members of the α -amylase (β/α)₈-barrel family of amylolytic enzymes (Jespersen et al 1991, 1993; Svensson 1994). However, a starch granule binding domain, present in some members of the superfamily (Jespersen et al 1991, Svensson et al 1989), has not been recognized in any of the debranching enzymes. On an evolutionary tree constructed from a total of 37 residues, which includes the four best conserved β -strands and the short C-terminal extensions in the (β/α)₈-barrel, the strictly α -1,6 specific debranching enzymes form a cluster next to the plant starch-branching enzymes, whereas the dual-bond type specific enzymes are close to α -glucosidases and bacterial branching enzymes (Jespersen et al 1993). This distinct discrimination among enzymes involved in the metabolism of α -1,6-linkages is further supported by sequence motifs present in two different types of putative structural domains located N-terminally to the (β/α)₈-barrel in pullulanase, isoamylase, and branching enzyme, respectively, neopullulanase and α -amylase-pullulanase (Jespersen et al 1991). By sequence analysis of peptide fragments, we identified the motif from the first group of enzymes in barley LD (V. Planchot, I. Svendsen, M. Kristensen and B. Svensson, unpublished data). Comparison with the recent cDNA sequence encoding starch debranching enzyme from rice developing endosperm (Nakamura et al 1996) suggests high sequence similarity to barley LD and inclusion with the first group of strictly α -1,6-specific enzymes.

In the present work, an efficient large-scale purification procedure for LD from barley malt has been established. Using pullulan and 6²-O- α -maltotriosyl-maltotriose as substrates, the enzyme catalyzes hydrolysis of branched oligosaccharides and pullulan with retention of the anomeric configuration and acts on only α -1,6-bonds. Finally, sequence comparison reveals a strong similarity between barley LD and α -1,6 specific bacterial hydrolases, as well as a high homology with rice starch debranching enzyme.

MATERIALS AND METHODS

Plant Material

Seeds of malting barley (*Hordeum vulgare* L.) cultivars Natasha or Alexis (Carlsberg Maltings) were treated six days in the malt

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house on production scale. Germination was continued for another four days in trays at 16°C in darkness. The green malt was air-dried in a kiln at room temperature to a water content of ≈8% (w/w).

Enzyme Activity Assay

LD activity was determined in 200 μL of 0.2M NaOAc (pH 5.0) containing 5 mM CaCl₂ and 0.1% BSA by addition of 100 μL of substrate, 2% (w/v) Red-Pullulan (Megazyme) in 0.5M KCl. After 20 min at 40°C, the reaction was stopped by addition of 600 μL of 85% (v/v) ethanol (Serre and Lauriere 1990, McCleary 1992). The mixture was left at room temperature (10 min), centrifuged (10,000 rpm, 8 min), and the absorbance of the supernatant was measured at 510 nm. One unit (U) of LD is defined as the amount that gives $(A_{510}^S - A_{510}^0) / \text{min} = 1$. The LD concentration was either calculated from amino acid analysis or measured spectrophotometrically at 280 nm using $A_{0.1\%} = 1.2$, determined by aid of amino acid analysis.

Small-Scale Extraction

Flour of air-dried malt (0.5 g) was extracted by vigorous shaking at regular intervals during 2 hr in 5 mL of 0.2M NaOAc (pH 5.0) or 0.1M Tris-HCl (pH 7.0) containing 2–5 mM ascorbic acid and 0.1M monothioglycerol in various combinations. The samples were left overnight at room temperature, centrifuged (4,000 rpm, 20 min), and the LD activity in the supernatant was measured.

Large-Scale Extraction and Purification

Flour of air-dried malt (14 kg of Natasha) was extracted in 120 L of 0.2M NaOAc (pH 5.0) containing 5 mM ascorbic acid with slow stirring for 2–4 hr at room temperature and left to settle overnight at 4°C. The supernatant was concentrated to 12 L by ultrafiltration (DDS-RO GR81P membrane, 20 kDa cut-off; Danisco, Copenhagen) and ammonium sulfate (AMS) was added to 20% saturation. The pellet was discarded after centrifugation, and the supernatant was saturated to 70% in AMS. The precipitate was collected in six portions by centrifugation and stored at –18°C. Two portions were redissolved in 1 L of 0.5M CaCl₂ (pH 5.0) and 50 mM NaOAc, desalted by diafiltration (10 kDa cut-off, Pellicon, Millipore), and applied to DEAE-Fractogel 650S (2.5 × 50 cm) in 20 mM NaOAc (pH 5.0). The column was washed with the same buffer and eluted using a linear gradient of 0–1M NaCl (2 × 900 mL). LD containing fractions were pooled. AMS was added to 1.5–2M and applied to β-cyclodextrin (βCD) Sepharose (5 × 30 cm) in 2M AMS with 50 mM NaOAc (pH 5.0) at a flow rate of 600 mL/hr. After a wash with this buffer, followed by 50 mM NaOAc (pH 5.0), until $A_{280} < 0.05$. LD was eluted with 7 mM in the latter buffer (500 mL). Fractions containing LD activity were pooled, applied to DEAE-Fractogel

650S (1.6 × 30 cm) in 20 mM NaOAc (pH 5.5) at a flow rate of 75 mL/hr. Following a wash with this buffer, LD was eluted by a linear gradient of 0–0.5M NaCl (2 × 500 mL). Fractions containing activity were pooled and stored at –18°C.

LD used for immunization was purified from an AMS precipitate prepared as above from an extract of malt (30 kg in 250 L) in 20 mM Tris-HCl (pH 7.0), 2 mM ascorbic acid, and 1 mM CaCl₂. One sixth of the precipitate was redissolved in 1 L of 0.5M CaCl₂ with 50 mM NaOAc (pH 5.5) and applied to βCD-Sepharose (5 × 30 cm), preequilibrated in 50 mM NaOAc, 5 mM CaCl₂, 0.2M NaCl (pH 5.5). After washing with 20–30 volumes of this buffer at a flow rate of 130 mL/hr followed by 2 volumes of 20 mM NaOAc, 1 mM CaCl₂ (pH 5.5), LD1 was eluted by 35 μM and LD2 was eluted by 7 mM βCD (2 × 400 mL). The LD1 and LD2 pools were concentrated as above, applied to DEAE-Fractogel 650S (1.6 × 30 cm) in 20 mM NaOAc, 1 mM CaCl₂ (pH 5.5), and eluted by a linear gradient of 0–0.5M NaCl (2 × 200 mL). Fractions containing activity were pooled, concentrated, chromatographed on Fractogel HW55S (1.6 × 95 cm) in the same buffer at a flow rate of 16 mL/hr, and the activity was pooled. After dialysis against water, LD1 was lyophilized and used for rabbit immunization (150 μg, followed by five boostings of 150 μg) (Dako A/S, Denmark).

Enzymic Properties

Pullulan (Sigma), DP17 amylose EX-1 (Hayashibara Chemical Laboratories, Okayama), and 6'-O-α-maltotriosyl-maltotriose (from a house collection) were used to determine the specificity of LD. Substrate (1 mg) in 0.5 mL of deuterium oxide, ²H₂O, and 0.2M C²H₃CO₂²H adjusted to pH 5.0 with NaO²H, was added to LD (0.35 μg/mL; 20 U/mg in 1 μL of 20 mM NaOAc (pH 5.0) to yield a final LD concentration of 12 nM. ¹H-NMR spectra were recorded on a Bruker AM500 instrument at 40°C (27°C for pullulan degradation) with appropriate time intervals up to 70 min.

The influence of DTT, CaCl₂, EDTA, and the inhibitors αCD, βCD, and γCD as well as acarbose on LD activity was examined in 0.2M NaOAc (pH 5.0) 0.1% BSA using the assay with Red-Pullulan as substrate and 5 mU of LD (20 U/mg). $K_{i,app} = (V_x/V_{max} - V_x)I_x$ was calculated, V_{max} and V_x being activities at 0 and other I_x concentrations of inhibitor in the ranges 0.01–5 mM βCD, 0.1–10 mM αCD or γCD, and 0.25–10 mM acarbose.

Raw Starch Binding Test

LD was added (final concentration 10 nM) to a suspension of raw barley starch (10 g/L) (gift of the late B. S. Enevoldsen) in 20 mM NaOAc, 1 mM CaCl₂, 0.05% BSA (pH 5.0) (1 mL) at 4°C and whirl-mixed at 5-min intervals. After 30 min, the starch was spun

TABLE I
Purification of Limit Dextrinase (LD) from Flour of Air-Dried Barley Green Malt (cv. Natasha)

Fractions ^a	Activity (U)	Protein ^b (mg)	Specific Activity (U/mg)	Yield (%)	Purification (fold)
A					
Extract	3,620	1.1 × 10 ⁶	0.003	(100)	(1)
20–70% saturated AMS precipitate	2,150	50 × 10 ³	0.04	100 (59)	1 (13)
DEAE-Fractogel 650S	1,930	2720	0.7	90 (53)	18 (230)
βCD-Sepharose in 2M AMS	1,500	40	37.5	70 (41)	938 (12,500)
DEAE-Fractogel 650S	1,050	38	28.2	49 (29)	705 (9,400)
B					
20–70% saturated AMS precipitate	2,360	1.2 × 10 ⁶	0.02	100	1
βCD-Sepharose					
Peak 1	nd ^c	28			
Peak 2	nd	188			
Pass-through	2,030	9.2 × 10 ⁴	0.02	86	
DEAE-Fractogel 650S					
LD1	91	4.8	18.9	3.6	945
LD2	13	4.0	3.2	0.5	160

^a Fraction A: 4.6 kg of extract in 0.2M NaOAc (pH 5.0) containing 5 mM ascorbic acid. Fraction B: 5 kg of extract in 0.1M Tris Cl (pH 7.0) containing 2 mM ascorbic acid and 5 mM CaCl₂. AMS = ammonium sulfate; DEAE = diethylaminoethyl.

^b Protein estimated by absorbance at 280 nm.

^c Not determined because βCD is present.

down (4,000 rpm, 10 min) and the LD activity in the supernatant was measured against Red-Pullulan.

Electrophoresis and Immunoblotting

SDS-PAGE and isoelectric focusing (IEF) were performed (Phast-System) using 7.5% and pH 3–9 PhastGels, respectively, and Pharmacia marker protein kits. Protein was visualized by staining with PhastGel Blue R or Silver Kit (Pharmacia). For immunoblotting, protein after SDS-PAGE was transferred by diffusion onto moist BA85 nitrocellulose (Schleicher and Schull) for 20 min at 70°C using the PhastSystem. The filters were subsequently blocked by 1% BSA, and incubated with LD1 antiserum (1:1,000 dilution) The primary antibody was detected using the ProtoBlot (Promega) alkaline phosphatase kit.

Amino Acid and N-Terminal Sequence Analyses

Amino acid analysis was performed on acid hydrolysates of 10–50 µg of LD (6M HCl; 110°C; 24, 48, and 72 hr) using an LKB model Alpha Plus amino acid analyzer. LD was reduced and 2-vinylpyridinylated, digested with trypsin, and the fragments obtained were purified by RP-HPLC essentially as described earlier (Svensson et al 1983) using a C₁₈ column (Vydac 218TP54) eluted by an acetonitrile gradient (2–90%) in TFA (0.1%). The N-terminal sequencing was performed using an Applied Biosystems 470A Protein Sequencer equipped with an on-line PTH-analyzer.

The partial LD sequence was aligned using MACAW software (Schuler et al 1991) to the related sequences (Jespersen et al 1993) of: Taka-amylase A (Matsuura et al 1984), pullulanase from *Klebsiella aerogenes* (Katsuragi et al 1987), *Bacillus acidopullulyticus* (Kelly et al 1994), *Thermus* sp. AMD-33 (Sashihara et al 1993), and *B. stearothermophilus* (Kuriki et al 1990), α -amylase-pullulanase from *Thermoanaerobacter ethanolicus* E101 (formerly *Clostridium thermohydrosulfuricum*) (Melasniemi et al 1990), and *T. saccharolyticum* (EMBL:L07762), neopullulanase from *B. stearothermophilus* (Kuriki and Imanaka 1989) and *Bacillus* sp. neopullulanase (Igarashi et al 1992), a neopullulanase-type α -amylase II from *Thermoactinomyces vulgaris* R-47 (Tonozuka et al 1993), *Pseudomonas amyloclavata* isoamylase (Amemura et al 1988), maize *sugary1* gene (James et al 1995), and rice starch debranching enzyme (Nakamura et al 1996).

RESULTS

Purification of Barley LD

Modest variation was found in the amount of LD activity extracted under different conditions from flour of air-dried malt of barley cultivars Natasha and Alexis. In the small-scale experiments, the presence of ascorbic acid or monothioglycerol or both enhanced the yield by up to 15%. Moreover, the yield obtained at pH 5.0 was superior to that obtained at pH 7.0 by \approx 15%. In large-scale extractions, monothioglycerol was omitted to prevent activation of malt thiol endoproteases that degrade LD at later stages of the purification (V. Planchot and M. Kristensen, unpublished data). The scaling-up by a factor of 5×10^4 from 5 mL to 250 L decreased the yield of LD activity in the extract by two- to threefold.

Two different protocols used for large-scale purification resulted in homogeneous, highly active LD, albeit in very different yields. Thus, extraction at pH 5.0, followed by AMS precipitation, anion-exchange chromatography, and affinity chromatography in the presence of 2M AMS, resulted in 940-fold purification of LD and a recovery of 70% of the activity present in the redissolved AMS precipitate (Table I). The initial extract contained active LD corresponding to \approx 800 U/kg air-dried malt, 29% of which was recovered as finally purified homogenous LD. After the β CD-Sepharose affinity chromatography, removal of β CD by a second anion-exchange chromatography step slightly decreased the specific activity of LD from 37.5 U/mg to 28.2 U/mg (Table I). In immunoblots (Fig. 1), antiserum raised against LD1 recognized both this highly purified

LD and LD in malt extracts. The antibodies were of good sensitivity, the detection limit being 10 pg of LD. It therefore would be a useful tool in monitoring the occurrence of LD in developing seeds or under germination. Moreover, this polyclonal antibody cross-reacted with proteins of the same apparent molecular weight as barley LD in sorghum, rice, wheat, rye, and pearl millet. Minor bands of lower molecular weight apparent in the extract from green malt most likely represent LD after minor proteolytic degradation, since such bands would arise also when a solution of highly purified LD was left at room temperature overnight or for prolonged periods at 4°C, or when highly purified LD was treated with proteases (V. Planchot, M. Kristensen, and B. Svensson unpublished data).

The LD activity isolated after extraction at pH 7.0 and AMS precipitation corresponded to 470 U/kg of malt (Table I). Although LD was completely inhibited by 1 mM β CD, \approx 90% of this activity was not adsorbed on β CD-Sepharose, and consistently $<$ 1% of the LD activity in the pass-through was retained on β CD-Sepharose by reapplication. A fraction of LD (LD1) was eluted by 35 μ M β CD which was subsequently purified (945-fold) to homogeneity by anion-exchange chromatography with 3.6% recovery of LD in the AMS precipitate. A smaller amount of LD (LD2) was eluted by 7 mM β CD corresponding to 150-fold purification and recovery of 0.5% of activity (Table I).

Structural Properties

The molecular mass of LD was estimated to be 105 kDa by SDS-PAGE (Fig. 2A) in agreement with earlier reports (Maeda et al 1978). In IEF LD (Fig. 2B), LD1 and LD2 (data not shown) migrate as a major and two minor components of slightly different levels at \approx pI 4.3. The N-terminal sequences determined for LD and LD1 were identical: ATQAFMPDAR¹⁰AYWVTSDLIA²⁰GNVGE. In \approx 50% of the molecules, the sequence started at AFM and thus lacked the first three residues. LD (Table II), LD1, and LD2 (not shown) have essentially identical amino acid composition. The sequences of three selected peptides of LD were identified to comprise β -strands 2, 3, and 5 of the catalytic (β/α)₈-barrel domain characteristic of members of the α -amylase family (Jespersen et al 1993) and have been compared to the corresponding stretches from bacterial pullulanases, isoamylase, neopullulanases, amylopullulanases, the maize *sugary1* gene, and starch debranching enzyme from rice (Fig. 3). The calculated identity of the LD sequences were 46, 58, and 53% to the corresponding three regions from *Klebsiella* pullulanase and overall 85% to the rice debranching enzyme. Barley LD most closely resembles enzymes that are strictly specific for α -1,6-linkages compared to those of dual specificity for both α -1,4 and α -1,6 bonds.

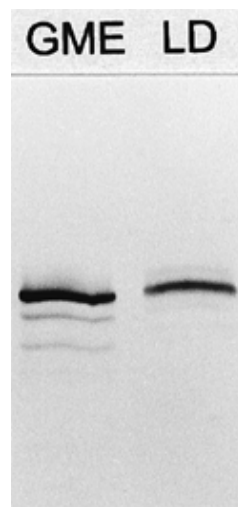


Fig. 1. Immunoblot of barley: 10 µg of green malt extract (GME) and 1 ng of highly purified limit dextrinase (LD).

Enzymatic Properties

The activity of the barley malt LD increased by 20% in the presence of 100 mM DTT. While thiol groups thus played a small role in the activity of barley LD, no effect of Ca²⁺ (1–100 mM) was observed. EDTA (10 mM) caused only a 15% decrease in activity.

βCD inhibits LD with $K_{i,app}$ of 40 ± 12 μM, while αCD and γCD have significantly lower affinity with $K_{i,app}$ of 1.9 ± 0.6 μM and 2.4 ± 0.9 μM, respectively. All three cyclodextrins were found to be competitive inhibitors. The pseudotetrasaccharide acarbose, which is a potent inhibitor of α-1,4 specific amylolytic enzymes such as α-amylase and glucoamylase, reduced the activity of LD by only 30% at a high concentration of 10 mM.

A functional raw starch binding site has not been demonstrated for barley LD, since neither the LD nor the LD1 preparation were adsorbed onto barley starch granules at pH 5.0 and 4°C, as shown by complete recovery of the activity toward Red-Pullulan in supernatants after incubation with barley starch granules.

The action of LD toward 6²-O-α-maltotriosyl-maltotriose was followed using ¹H-NMR spectroscopy. The α-1,6-bond of the branched oligosaccharide, of which the anomeric proton was assigned the resonance δ = 4.95 ppm, was confirmed to be hydrolyzed to yield

maltotriose with the characteristic resonances δ = 5.21 ppm and δ = 5.37 ppm (Fig. 4), assigned the α-anomeric protons from the reducing end and the α-1,4-linkage, respectively (Fig. 4). Weak signals near these chemical shift values (Fig. 4) stem from small amounts of singly branched hepta- and octasaccharides present in the substrate. A rate constant of 90/sec was calculated from the progress of hydrolysis of 6²-O-α-maltotriosyl-maltotriose catalyzed by LD and monitored as product formation using ¹H-NMR.

Furthermore, ¹H-NMR spectroscopy demonstrates that all α-1,6-linkages in pullulan (δ = 5.10 ppm) disappear by LD-catalyzed degradation concomitant with production of pure maltotriose with characteristic chemical shifts (δ = 5.21 ppm and δ = 5.37 ppm) (Fig. 5). The increase in reducing groups of α-anomeric configuration, corresponding to δ = 5.2 ppm (Figs. 4 and 5) confirms that LD acts in a manner similar to that of other members of the α-amylase family (Svensson and Sogaard 1993) and catalyzes hydrolysis with retention of the anomeric configuration, thus acting by a double-displacement mechanism in glycoside bond hydrolysis. Finally, the barley LD (2.4 nM) did not hydrolyze DP17 amylose (0.2%) during 24 hr of incubation at room temperature as followed by both the ¹H-NMR and reducing sugar analysis. The LD prepared is thus

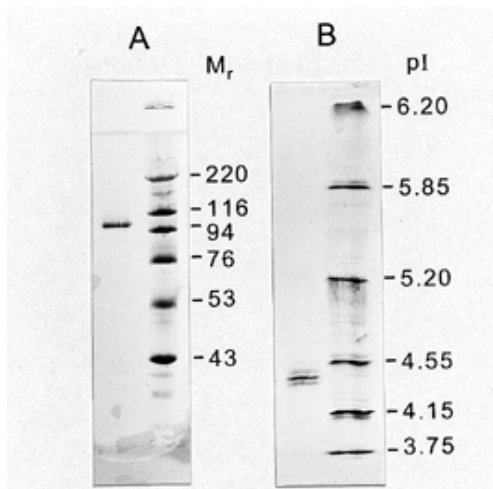


Fig. 2. SDS-PAGE (A) and isoelectric focusing (IEF) (B) of limit dextrinase (2 μg) from barley green malt. Molecular weights and isoelectric points of the protein markers are indicated.

TABLE II
Amino Acid Composition of Barley Limit Dextrinase

Amino Acid	%
Aspartic acid/Asparagine	12.5
Threonine	4.8
Serine	8.1
Glutamic acid/Glutamine	9.1
Proline	6.2
Glycine	8.6
Alanine	8.6
Cysteine	0.6
Valine	7.7
Methionine	2.5
Isoleucine	4.5
Leucine	8.2
Tyrosine	3.1
Phenylalanine	4.5
Histidine	2.5
Lysine	3.5
Tryptophan	nd ^a
Arginine	4.5

^a Not determined.

	β-strand E2		β-strand E3		β-Strand E5
TAKAMY	56 iggmGftaiwitPvtaqlpqdcay	79..114	lmvDvVaNHmgdydgagssvdysvf	137..227	vycigEvlldgdpayt 241
	* * *		* * *		*
LDHORV	lsdaGlthvhllPsfhfagvddik	vvmDvVyNHldssgpcgissvidk	iydygEgwdfalvar ...
SDEORZ	lsaaGlthvhllPsfhfafsvddnk	422..499	vvmDvVyNHldssgpgfgvssvldk	522..608	iylygEgwdfgevaq 622
PULKLA	lsasGvthiellPvfdlatvnefs	489..599	vimDvVyNHtNaagptdrtsvldk	622..700	iyffgEgwdsnqsdR 714
PULBST	lkelGvthvellPfnfdagvdenp	230..278	vimDvVyNHvyirdqssfekivpg	301..376	ilvfgEgwldptpls 390
PULTHE	vkelGvthvqlmPfmfdagvderd	287..336	vvmDaVvNHvydregspkklvpg	359..434	ilvygEgwldptlfh 448
PULBAC	lkelGinavqlqPieefnsidetq	432..479	vnmDvVyNHtfnvgvsdfdkivpg	502..577	ivlygEpwtggtsgl 591
ISOPSA	laslGvtaveflPvqetqndandv	262..314	vymDvVyNHtaeggtwtssdptta	337..437	nrilrEftvrpaagg 451
SU1ZEA	lkelGvncielmPchefneleyfs	240..292	vimDvVfNHtaegnekpilsfrg	315..405	vkliAEawdagglyq 419
NPUBST	lvdlGitgiyltPifrspshhkyd	208..239	vmlDaVfNHcgiefapfqdvwnk	262..352	vyilgEiwhdampwl 366
NPUBAC	lkelGingiytlPifkarnhkyd	207..239	vmlDaVfNHsgfyefqfvdvlehq	262..351	vyilgEvhwhdampwl 365
NPUTVA	leelGvtalyftPifaspshhkyd	204..236	iildAvfNHagdqffafrdvllqk	259..349	alivgEiwhdasgwL 363
APUTHE	lkslGisviylnPifqspshhryd	454..485	vildGvFNHtsdssiyfdrygkyl	508..621	apmvaEnwndasldl 635
APUTHS	lkgLGvsviylnPifespshhkyd	487..518	iildGvFNHtsdssiyfnrygkyp	541..652	apmiaEnwgdasldl 663
	hhhh βββββ		βββββ		βββββ

Fig. 3. Alignment of the partial amino acid sequence of barley limit dextrinase (LD), Taka-amylase A, and bacterial debranching enzymes. h and β indicate residues from secondary structure elements in Taka-amylase A; asterisks mark fully conserved residues. LD tryptic peptides are homologs of β-strand 2, β-strand 3, and β-strand 5 of the catalytic (β/α)₈-barrel domain in known amylolytic enzymes. Sequences: LD from barley, LDHORV; rice starch debranching enzyme, SDEORZ; pullulanase from *Klebsiella aerogenes*, PULKLA; *B. stearothersophilus*, PULBST; *Thermus* sp. AMD-33, PULTHE; *B. acidopulluliticus*, PULBAC; isoamylase from *Pseudomonas amyloclavata*, IAPSA; neopullulanase from *B. stearothersophilus*, NPUBST; *Bacillus* sp., NPUBAC; neopullulanase-type α-amylase from *Thermoactinomyces vulgaris*, NPUTVA; α-amylase-pullulanase from *Thermoanaerobacter ethanolicus* E101, APUTHE; *Thermoanaerobacter saccharolyticum*, APUTHS.

free from detectable contamination by α -glucosidase, α -amylase, and β -amylase and is classified as a type of pullulanase that is especially efficient in hydrolysis of the α -1,6-linkage in α -limit dextrins.

DISCUSSION

Homogeneous barley LD with a high specific activity (28.2 U/mg) toward Red-Pullulan is obtained by $\approx 10^3$ -fold purification in 70% yield after AMS precipitation of an extract of malt flour. This corresponds to 29% of the activity measured in the crude extract, which may, however, have been underestimated because of the presence of endogenous LD specific inhibitors (Macri et al 1993). We conclude that four factors contribute to the high yield of LD: 1) prolonged germination (10 days); 2) extraction of the flour at pH 5.0; 3) the presence of ascorbic acid during extraction; and 4) the use of concentrated AMS in the affinity chromatography on β CD-Sepharose. Others reported that 2M AMS enhanced the adsorption of plant β -amylases on α CD-Sepharose (Totsuka and Fukazawa 1993).

Barley LD was previously purified $\approx 10^3$ -fold in 20% yield after AMS precipitation of a malt extract (1.9% yield of the crude extract) (Maeda et al 1978). Recently, 17% yield was obtained by performing the β CD-Sepharose affinity chromatography at pH 4.0, although the lack of stability of LD in acidic solution required rapid pH adjustment toward neutrality (MacGregor et al 1994). Whereas β CD-Sepharose efficiently and specifically adsorbs α -amylase from wheat and barley malt (Silvanovich and Hill 1976), and retains $\approx 50\%$ of the α -amylase in extracts of sorghum and maize (Mundy 1982, Warner et al 1991), barley LD was earlier only obtained in low yields using either β CD- (Lecommandeur et al 1988) or α CD-Sepharose (Maeda et al 1979, Sissons et al 1992a), despite substantial purification of LD. Also, in the present work, 86% of the LD activity in the extract was not retained on β CD-Sepharose at moderate salt concentrations, perhaps due to complexation of LD with the endogenous LD-inhibitor. Thus, an extract of dehusked barley is reported to inactivate 80% of the LD in a malt extract (Macri et al 1993). Perhaps the efficient retention of LD on β CD-Sepharose in 2M AMS is due to dissociation of the LD/LD-inhibitor complex.

The nature of the β CD-binding site and the forces that stabilize the β CD-barley LD complex have not been identified. β CD, however, is a competitive inhibitor of LD as of several amyolytic enzymes and thus binds at the active site. Hydrophobic forces may be important because high concentrations of AMS enhanced the affinity of LD for β CD-Sepharose. Crystal structures of different amylases reveal that binding of cyclodextrins can be reminiscent of formation of inclusion complexes. Thus, the side chain of Leu383 in soybean β -amylase interacts with the cavity of α CD near C-3

of four of the glucose residues (Mikami et al 1993). Furthermore, three α CD molecules are bound to pig pancreatic α -amylase, one has included an asparagine side-chain; a tryptophan side-chain is directed toward the center of the cavity in another molecule; three glucose residues of the third α CD interact with different protein side chains (Larson et al 1994). It seems less likely that β CD in LD binds to a separate raw starch binding site or domain, as in the cases of barley α -amylase (Gibson and Svensson 1987, Sogaard et al 1993) and glucoamylase (Svensson et al 1989; Jespersen et al 1991, 1993), respectively, because β CD acts as competitive inhibitor and a homologue of a starch-binding domain is not recognized in the LD sequence (data not shown). The different affinity of LD1 and LD2 for β CD-Sepharose may indicate some variation in the structure of the binding site, which may originate from differences in sequence or posttranslational modification in accordance with a slight difference in the pI values of LD1 and LD2 (data not shown). The higher affinity of LD (Maeda et al 1979, Ludwig et al 1984, MacGregor et al 1994) for the inhibitor β CD, reported by others, may reflect the same point. Pullulanase from *Klebsiella pneumoniae* also has a lower K_i (higher affinity) than barley LD for α CD, β CD, and γ CD (Iwamoto et al 1993).

Several reports demonstrate the beneficial influence of reducing conditions in extraction and activity of plant debranching enzymes (Ludwig et al 1984, Doehlert and Knutson 1991, Li et al 1992). For barley LD, a comparable increase in yield was obtained by continuation of the germination for 10 days (Kristensen et al 1993). Sulfhydryl compounds stimulate the conversion in vitro of the bound form to free barley LD, perhaps because they activate malt thiol endoproteases (Wrobel and Jones 1992, Longstaff and Bryce 1993) or release LD inhibitors (Macri et al 1993). But thiol compounds barely influenced the activity of barley LD, whereas 5–10 mM DTT increased the activity of debranching enzymes from sugar beet and spinach leaves two- to fourfold (Ludwig et al 1984, Li et al 1992), and maize debranching enzyme requires 5 mM DTT for activity (Doehlert and Knutson 1991). Furthermore, many amyolytic enzymes require Ca^{2+} for activity and stability, but since EDTA (10 mM) caused only 15% decrease in activity for barley LD, as also found earlier with sorghum LD (Hardie et al 1976), Ca^{2+} (if required) must be firmly bound. Cross-reactivity of barley LD antibodies with a single protein band of 105 kDa demonstrated the close similarity between the barley LD and proteins in extracts of sorghum as well as rice, corn, wheat, rye, and pearl millet (Kristensen et al 1995). Despite its role in degradation of endospermal starch, barley LD was not adsorbed onto starch granules at pH 5.0 and, although raw starch adsorption of amyolytic enzymes is generally favored at pH 3.0–4.0, experiments were not performed in this pH range because LD is rapidly inactivated at pH < 4.5.

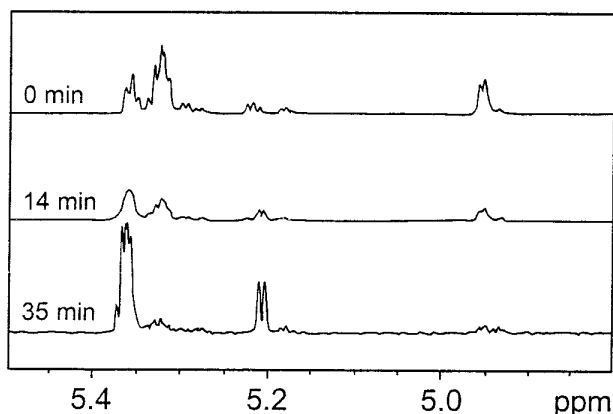


Fig 4. Limit dextrinase catalyzed hydrolysis at 40°C of 6²- α -O-maltotriose monitored by ¹H-NMR spectroscopy (from top to bottom) before and after 14 and 35 min of hydrolysis.

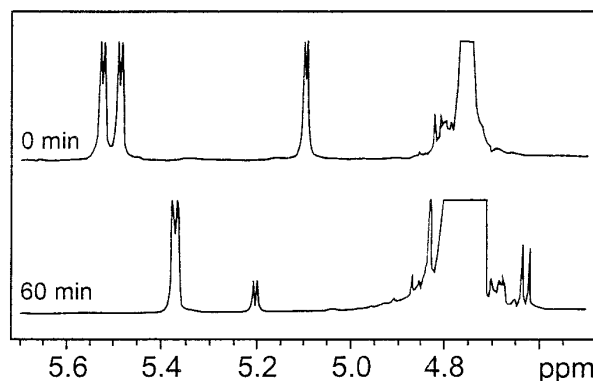


Fig 5. Limit dextrinase catalyzed hydrolysis at 27°C of pullulan monitored by ¹H-NMR spectroscopy before (top) and after 60 min (bottom) of incubation.

LD shows very little polymorphism compared to barley α -amylase and β -amylase (MacGregor 1987). Even though others reported as many as eight LD forms of different pI values in the range pH 4.2–5.0 (Lenoir et al 1984, Sissons et al 1992a), the present preparations of LD have pI 4.3. While wild barley (*H. spontaneum*) contained an extra LD form of higher pI value, six LD forms were invariably seen from 135 barley lines (*H. vulgare*) of wide genetic background (Sissons et al 1992b).

The partial sequence provided evidence that barley LD belongs to the α -amylase family of multidomain amylolytic enzymes with a characteristic $(\beta/\alpha)_8$ -barrel catalytic domain (Jespersen et al 1991, 1993; Svensson 1994). The three short sequences comprise β -strands with catalytic site and typical structural motifs. G and P in GithvlllP (Fig. 3) are thus conserved at β -strand 2, in the vast majority of the family members, representing 15 different specificities (Janecek 1995). The invariant D in DvVvNH of β -strand 3 is involved in maintaining the geometry of the catalytic site, and H, conserved in most family members (Svensson 1994), was demonstrated by mutational analysis in barley α -amylase to be critical for transition state stabilization (Søgaard et al 1993). The invariant E in Egwd at β -strand 5 is identified to act as general acid catalyst (Svensson 1994). The binding motif corresponding to Taka-amylase K²⁰⁹-H²¹⁰ which is GI, GY, SV, QY, or NE in α -1,6-bond specific enzymes pullulanase, isoamylase, amylase-pullulanase, neopullulanase, and TVA α -amylases (Svensson 1994) was GH in rice debranching enzyme (Nakamura et al 1996) and also GH in barley LD (not shown).

Barley LD hydrolyzes the α -1,6-linkages in pullulan and is thus classified with the pullulanases (EC 3.2.1.41). This is in accordance with the sequence similarity between barley LD and bacterial pullulanases and the action on branched oligosaccharides of both malt LD and different plant debranching enzymes (Hardie 1975, Dunn and Manners 1975, Maeda et al 1979, Yellowlees 1980). The barley LD hydrolyzes the α -1,6-linkage in a typical α -limit dextrin at a rate of 90/sec which is comparable to 53.2/sec reported earlier for the pullulanase from *Klebsiella pneumoniae* (Iwamoto et al 1993).

The knowledge gained in recent years on structure and function of the plant limit dextrinases and related enzymes generates an excellent basis for exploitation and rational design of enzymes active on the branch points in starch and related oligo- and polysaccharides.

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