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Measurement of the content of limit-dextrinase in cereal flours*

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ABSTRACT

Procedures for the quantitative extraction, activation, and assay of limit-dextrinase in cereal flours have been developed. Extraction and activation require incubation in buffer containing 20mM cysteine for at least 16 h or with 25mM dithiothreitol for 5 h. Activity is assayed with a soluble, dyed substrate (Red-Pullulan) or an insoluble, dyed, and cross-linked substrate (Azurine-CL-Pullulan) which is dispensed in tablet form (Limit-DextriZyme tablets).

INTRODUCTION

Limit-dextrinase[1] [pullulanase, or α-dextrin 6-glucanohydrolase (EC 3.2.1.4)] catalyses the hydrolysis of the (1→6)-α-D-glucosidic bonds that occur in the inter-chain sequences of amylopectin and pullulan[2,3]. These enzymes have little action on highly branched glycogen and phytoglycogen. Plant limit-dextrinases have patterns of action similar to those of bacterial pullulanase (*e.g.*, from *Klebsiella pneumoniae*), but significantly different from those of isoamylases that cleave (1→6)-α-D-glucosidic bonds in amylopectin and glycogen, but unlike isoamylase, they rapidly hydrolyse pullulan[4].

Several methods for the assay of plant limit-dextrinase have been reported; these include reducing-sugar methods with limit-dextrins of starch (produced by using excesses of alpha- and beta-amylase) or pullulan as substrate[1], viscosimetric methods using pullulan[5], and procedures employing pullulan labelled with either ^{14}C (ref. 6) or a reactive dye[7,8]. The reducing-sugar methods require the enzyme extract to be partially purified in order to remove endogenous reducing sugars. Even with pullulan as the substrate, such endogenous enzymes as α-D-glucosidase (which hydrolyses the product of limit-dextrinase action, namely, maltotriose) can overestimate enzyme activity. This problem was resolved, in part, by employing a two-step reaction in which the maltotriose was hydrolysed completely (with yeast α-D-glucosidase) to D-glucose[9] which was then assayed. The major problem with this method is that the products of initial hydrolysis of pullulan by limit-dextrinase, an endo-acting enzyme, are linear oligosaccharides, not just maltotriose. Yeast α-D-glucosidase rapidly cleaves maltotriose, but has limited action on even the next higher oligosaccharide[10], *i.e.*, 6^3-α-D-maltotriosyl-D-maltotriose.

* Dedicated to Professor David Manners.

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Dyed-pullulan substrates for screening pullulanase-producing bacterial colonies on agar plates[11] and for the detection of enzymes in polyacrylamide gels[12] have been reported. Dye-labelled pullulan for use in assaying limit-dextrinase in crude extracts of plants has been the subject of a preliminary report[7] and independently described in detail[8]. In the former report, Procion Red MX-5B was employed and, in the latter, the dye was Reactive Red 120. However, in the latter work[8], the importance of activation of plant limit-dextrinases was not recognised.

Limit-dextrinase occurs in seed materials, mainly in an inactive form as demonstrated for pea[13] and rice[14]. The enzyme, like beta-amylase, can be activated either by treatment with specific proteases or such reducing agents as cysteine or dithiothreitol. Since this phenomenom was not recognised in the pioneering work on barley-malt enzymes, some of the conclusions[15] on the importance of limit-dextrinase on the fermentability of malt extract may need to be reassessed. Enevoldsen and Schmidt[16] demonstrated that wort and beer contain substantial quantities of branched α-dextrins, and, in fact, that the majority of the inter-chain linkages in amylopectin survive mashing. Thus, the limit-dextrinase in the malt has limited practical effect or value in the conversion of starch into fermentable sugars in the mashing process, but there is great potential for the use of pullulanase or limit-dextrinase in the production of beer. The source of this enzyme may be microbial. However, the possibility of selecting varieties of barley with increased potential to synthesise limit-dextrinase during malting, or the possibility of using limit-dextrinase from another plant source, such as rice seed, should not be discounted.

Procedures for the extraction, activation, and assay of limit-dextrinase in barley and malt and other cereal grains are now described.

EXPERIMENTAL

Materials. — Pullulan, soluble starch, oyster glycogen, and Reactive Red 120 were obtained from Sigma Chemical Co., and Procion Red MX-5B from Harcros Colours (Sydney).

Alpha-amylase (EC 3.2.1.1) from *Aspergillus oryzae*, beta-amylase (EC 3.2.1.2) from *Bacillus cereus*, amyloglucosidase (EC 3.2.1.3) from *Aspergillus niger*, isoamylase (EC 3.2.1.68) from *Pseudomonas* sp., and pullulanase (EC 3.2.1.41) from *Klebsiella pneumoniae* were from MegaZyme Pty. Ltd. The enzymes were homogeneous in electrophoresis and devoid (<0.001%) of each of the other enzyme activities. Red-Pullulan, Azurine-CL-Pullulan, and Limit-DextriZyme tablets were from MegaZyme Pty. Ltd.

Preparation of malt samples. — Samples collected during the commercial malting of Schooner barley were immediately frozen and then lyophilised. These and other samples of grain were milled to pass through a 0.5-mm screen (Tecator Cyclotec mill) before analysis.

Preparation of dye-labelled pullulan. — Red-Pullulan[17] and Azurine-CL-Pullulan[18,19] were prepared by the methods reported. Free dye was removed from the gelatinous dyed particles by repeated washing with demineralised water. The residue was dried by solvent exchange with EtOH and acetone, and dried *in vacuo*.

Assay of limit-dextrinase by reducing-sugar measurement. – Each suitably diluted enzyme preparation (0.2 mL) in 0.2M acetate buffer (pH 5 or 5.5) was incubated with a solution of pullulan (0.5 mL, 5 mg/mL) in the same buffer at 40°. The reaction was terminated after 0, 5, 10, 15, and 20 min by adding Nelson/Somogyi solution D[20] (0.5 mL) and the colour was developed as described[21]. One unit (U) of enzyme activity is defined as the amount of enzyme which releases 1 μmol of glucose reducing sugar equivalent per min at 40° and pH 5.0 (or pH 5.5).

Purification of limit-dextrinases. — (*a*) *Malt limit-dextrinase.* The limit-dextrinase was extracted from malt (1 kg) with 0.2M sodium acetate buffer (pH 5.0) containing 20mM cysteine (5 L) during 20 h. The supernatant solution recovered on centrifugation (3 000 r.p.m., 20 min) was stirred with ammonium sulphate (600 g/L) overnight at 4°. The pellet recovered after centrifugation (3 000 r.p.m., 20 min) was redissolved in the minimum volume of 20mM Tris buffer (pH 7.0) and dialysed against 10 L of the same buffer (twice) for 24 h. The solution was centrifuged (12 000*g*, 10 min) and the supernatant solution was applied to a column (3.5 × 15 cm) of DEAE-Sepharose FF and eluted with a linear gradient (0→0.5M) of KCl in 20mM Tris buffer. Fractions that contained limit-dextrinase activity were combined and purified further by chromatography on a column (4.5 × 90 cm) of Ultrogel AcA 54 and by affinity chromatography[22] on a column of cyclomaltoheptaose–epoxy-Sepharose 4B.

(*b*) *Rice limit-dextrinase.* The procedure in (*a*) was applied to flour (1 kg) from ungerminated rice.

Assay of limit-dextrinase using Red-Pullulan substrate. — (*a*) *Substrate dissolution.* Red-Pullulan (0.5 g) was stirred with 0.5M KCl (25 mL) at room temperature until dissolution was complete (~ 10 min). The substrate was stored at 4° and on ice during use.

(*b*) *Extraction buffer.* To 0.2M sodium acetate buffer (100 mL, pH 5.0) containing sodium azide (0.02%), cysteine (0.35 g) was added immediately before use (to give a cysteine concentration of 20mM), and the pH was adjusted to 5.0 by dropwise addition of 4M NaOH.

(*c*) *Extraction and activation of the enzyme.* Samples of grain and malt were milled to pass through a 0.5-mm mesh using a Tecator Cyclotec mill. A suspension of each sample (2.0 g) of flour in the extraction buffer (8.0 mL) was incubated for 16 h at 40°, with occasional stirring. The slurry was centrifuged at 1000 *g* for 10 min. The supernatant solution was not clear but this had no effect on the assay.

(*d*) *Assay procedure.* To a pre-equilibrated solution (0.5 mL, 2%) of Red-Pullulan was added a pre-equilibrated preparation of enzyme or flour extract (1.0 mL), and the mixture was incubated for 20 min at 40°. The reaction was stopped (and non-hydrolysed substrate was precipitated) by the addition of EtOH (2.5 mL) with stirring. The tubes were stored at room temperature for ~ 10 min and then centrifuged at 1 000*g* for 10 min. The enzyme reaction was monitored on the basis of increase in absorbance (510 nm) of the supernatant solution (the blank was prepared by adding EtOH to the substrate before the addition of the enzyme preparation).

Assay of limit-dextrinase using Limit-DextriZyme tablets. — (*a*) *Extraction buffer.*

To 0.1M sodium maleate buffer (50 mL, pH 5.5) containing sodium azide (0.02%) was added dithiothreitol (0.2 g) immediately before use (to give a concentration of approximately 25mM). This solution was used directly.

(*b*) *Enzyme extraction and activation.* Samples of grain and malt were milled to pass through a 0.5-mm mesh using a Tecator Cyclotec mill. A suspension of each sample (0.2 g) of flour in the extraction buffer (4.0 mL) was incubated at 40° for 6 h, with occasional stirring. The slurry was centrifuged at 1 000*g* for 10 min.

(*c*) *Assay procedure.* Aliquots (1.0 mL) of flour extract or enzyme solution were pre-equilibrated for 5 min at 40°. The reaction was initiated by the addition of a Limit-DextriZyme tablet to each tube (the tablet disintegrated in ~20 s without stirring). After incubation for 10 min at 40°, each reaction was terminated by vigorous stirring with Trizma base solution (10 mL, 1% w/v; Sigma Chemical Co., T-1503). Each mixture was stored for ~ 5 min at room temperature, then stirred again, and filtered through Whatman No. 1 filter circles (9 cm), and the reaction was followed by measuring the increase in absorbance (590 nm) of the filtrate (blanks were prepared by adding the Trizma base solution to the enzyme preparation before addition of the Limit-DextriZyme tablet). Enzyme activity (U/mL) was determined by reference to a standard curve.

Action of limit-dextrinase on Azurine-CL-Pullulan (the substrate component in Limit-DextriZyme tablets) was determined by adding pre-equilibrated and buffered enzyme preparation (1.0 mL) to the powdered substrate (10–50 mg) in pre-equilibrated test tubes and proceeding as for the Limit-DextriZyme tablets assay.

RESULTS AND DISCUSSION

Two substrates have been developed for the simple assay of limit-dextrinase in crude extracts of malt, namely, Red-Pullulan, a soluble dye-labelled substrate, and Azurine-CL-Pullulan, a dyed and cross-linked, insoluble substrate.

With Red-pullulan under the standard assay conditions (see Experimental), the maximum rate of hydrolysis with *Klebsiella pneumoniae* pullulanase was at a substrate concentration of 0.5%. However, the reaction curve was linear only to an absorbance value of ~0.6, consistent with the results of Serre and Laurière[8]. At a substrate concentration of 2%, the initial rate of increase in absorbance was ~50% of that at 0.5%. However, similar rates of reaction were obtained over the concentration range of 2–4%. In the final assay procedure, a concentration of 2% Red-Pullulan was used since the reaction curve was linear up to absorbance values of at least 1.4.

In the initial work with malt extracts, it was not possible to obtain linear reaction curves, and curves like that shown in Fig. 1 were obtained. The rate of reaction increased with the time of incubation. This effect may reflect either progressive activation of the limit-dextrinase or the removal of a competing substrate (*i.e.*, partially degraded starch) in the enzyme extract. Since activation of pea-seed[13] and rice[14] limit-dextrinase has been reported, the effect of reducing agents on limit-dextrinase activity was evaluated. Samples of a malt flour were extracted with 0.2M sodium acetate buffer (pH 5.0) containing cysteine, dithiothreitol, or 2-mercaptoethanol at concentrations of 5–50mM

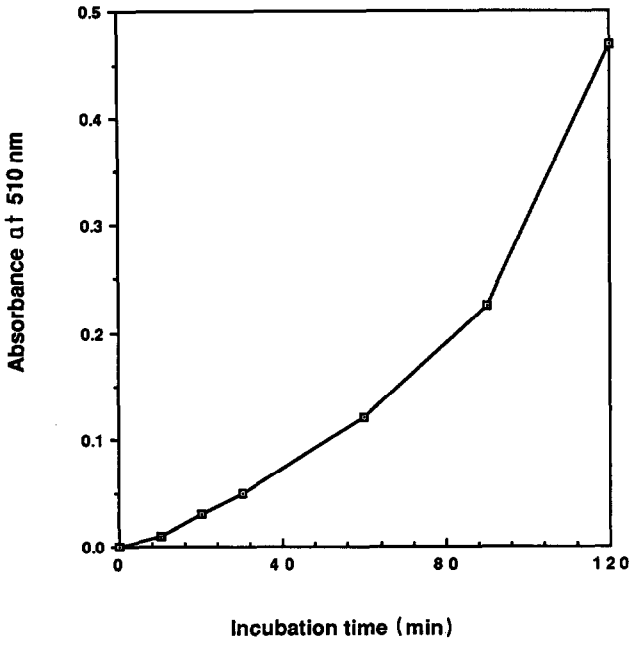

Fig. 1. Assay of malt limit-dextrinase using Red-Pullulan. Malt flour (2 g) was extracted with 0.2M acetate buffer (pH 5.0) for 15 min at room temperature. The slurry was centrifuged and the assay was performed immediately.

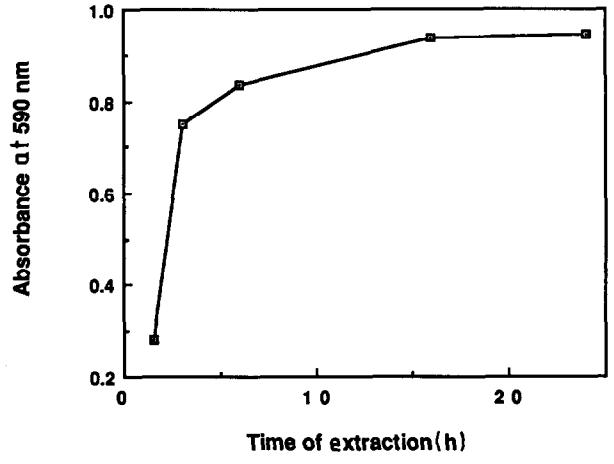

Fig. 2. The effect of extraction/activation time in buffer containing 20mM cysteine on the measured limit-dextrinase activity in a sample of malt flour.

for 1, 5, and 16 h at 40°. For maximal activity, 20–25mM reducing agent was required. In order to obtain complete activation with cysteine or 2-mercaptoethanol, an activation time of ~ 16 h was required. However, with 25mM dithiothreitol, activation required only 5 h. Cysteine was preferred as the reducing agent for routine use with the Red-Pullulan assay format, but with Limit-DextriZyme, the optimal extraction/activation conditions involved incubation for 5 h in 0.1M maleate buffer (pH 5.5) containing 25mM dithiothreitol.

The effect of the time of extraction/activation of a sample of malt flour in buffered 20mM cysteine at 40° on the limit-dextrinase activity is shown in Fig. 2 (the activity was assayed with Limit-DextriZyme tablets containing Azurine-CL-Pullulan). Complete activation of limit-dextrinase required incubation for 16–20 h. Similar time requirements were reported by Yamada[14] for rice-flour limit-dextrinase.

In Fig. 3, the time course of hydrolysis of Red-Pullulan by limit-dextrinase, extracted from malt for 20 h with different buffers plus amyloglycosidase, is shown. Cysteine is required to ensure complete activation, but inclusion of amyloglucosidase (to remove branched starch dextrins) is not necessary. Without added cysteine, the measured limit-dextrinase is high, but is still significantly less than the values obtained with cysteine. Without added cysteine, activation of limit-dextrinase is most probably due to the action of malt proteases[13].

Having optimised the conditions for the extraction and activation of malt limit-dextrinase, it was then possible to develop assays for this activity using Red-Pullulan

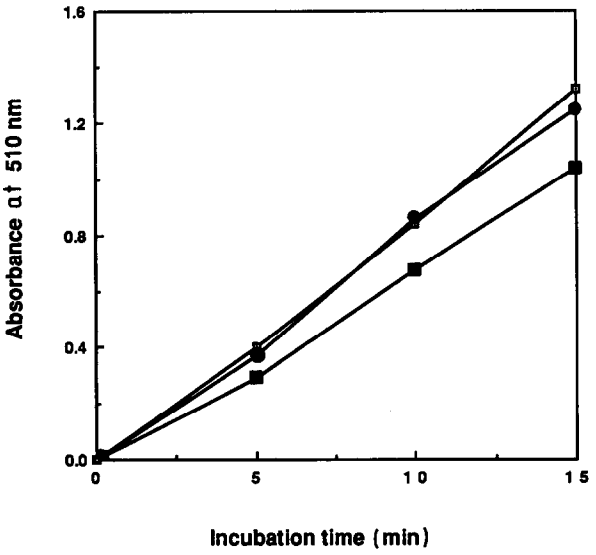

Fig. 3. Effect of extraction conditions on measured limit-dextrinase activity. Malt flour was extracted for 16 h at 40° with 0.2M acetate buffer (pH 5.0) (■), acetate buffer plus 20mM cysteine (□), or acetate buffer plus cysteine plus amyloglucosidase (200 U/g of flour) (●). Assays were performed on Red-Pullulan substrate at 40°.

and Azurine-CL-Pullulan. The major disadvantage of Red-Pullulan was the high blank absorbance (~0.2 absorbance unit at 510 nm with a substrate concentration of 2%). This absorbance was reduced by solvent precipitation of the soluble substrate followed by redissolution. However, if the substrate was dried by lyophilisation and redissolved at room temperature, the blank absorbance value was again ~0.2. A solution of Red-Pullulan in 0.5M KCl was relatively stable for several weeks at 4°. However, in 0.2M sodium acetate buffer (pH 5.0), blank absorbance values doubled within 2 weeks at 4°. This instability was much more pronounced at room temperature. The concentration of salt in the final assay mixture affects both the blank absorbance value (on addition of ethanol and centrifugation) and that of the reaction mixture. In order to minimise this effect, the Red-Pullulan was dissolved in 0.5M KCl. Varying this concentration from 0.4 to 0.6M did not change the absorbance values. The optimal pH for activity of limit-dextrinase on Red-Pullulan was pH 5.0 (0.2M sodium acetate buffer), whereas for Azurine-CL-Pullulan it was pH 5.5 (0.1M sodium maleate buffer).

A standard curve relating the activity of purified malt-flour limit-dextrinase on Red-Pullulan to that on pullulan is shown in Fig. 4. The curve is linear over the absorbance range (0.1–1.4 absorbance units), but it does not intersect the origin. The limit-dextrinase activity was standardised on pullulan (5 mg/mL) in 0.2M sodium acetate buffer (pH 5.0), using the Nelson/Somogyi reducing-sugar procedure[20] with

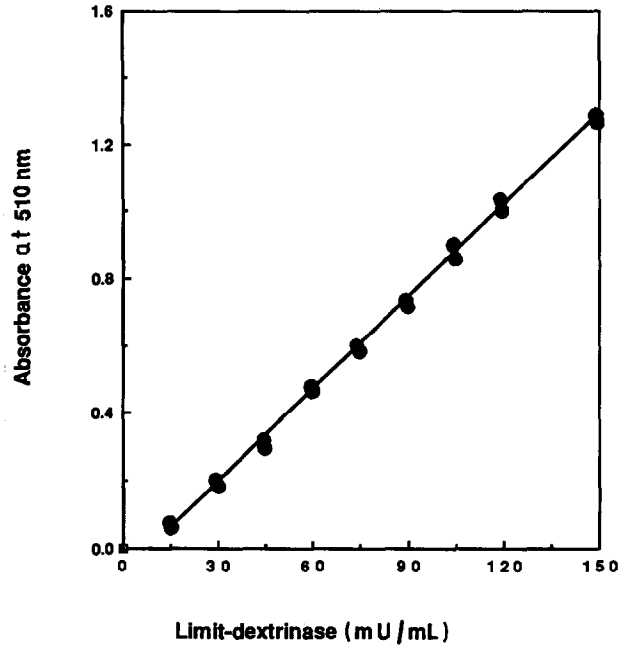

Fig. 4. Standard curve relating the activity of malt limit-dextrinase (U/mL) to increase of absorbance at 510 nm on hydrolysis of Red-Pullulan. Incubations were performed under standard conditions at pH 5.0 for 20 min at 40°. Limit-dextrinase activity (U/mL) was determined on pullulan using a reducing-sugar assay[20].

D-glucose as the standard. Purified rice-flour limit-dextrinase gave a standard curve on Red-Pullulan, which was identical to that obtained with the malt-flour enzyme. However, preliminairy results suggest that curves with different slopes are obtained with microbial pullulanases, suggesting subtle differences in enzyme action patterns.

The reproducibility of Red-Pullulan based assays for the measurement of limit-dextrinase activity in malt flours is shown in Table I. Flours from 4 different barley varieties at three different times of malting were analysed in duplicate on four separate days (an average of the duplicate assay values is reported.) Good reproducibility was obtained for samples giving absorbance values of >0.3, but the accuracy diminished rapidly at lower absorbances possibly due to the high blank values. These results are consistent with those found subsequently on inter-laboratory evaluation of this procedure[23].

The limitations in the use of Red-Pullulan for the assay of limit-dextrinase led to the evaluation of other dyed substrates. Pullulan dyed with Remazolbrilliant Blue R did not have the required sensitivity, but cross-linked, dyed-pullulan substrates were superior to Red-Pullulan. Assay procedures based on the use of the best of these substrates, Azurine-CL-Pullulan, had 10–15 times the sensitivity of those based on Red-Pullulan. Moreover, the substrate was stable, with blank absorbance values of ~ 0.08. Assays employing this substrate were reproducible (Table II) even though the reaction curve was not linear (Fig. 5). This lack of linearity may be related to the particle size of the

TABLE I

Reproducibility[a] of the Red-Pullulan[b] assay for the measurement of limit-dextrinase in freese-dried green malt samples

Barley variety	*Malting time (days)*	*Absorbance at 510 nm*					*S.d.*[c] (%)
		Day 1	*Day 2*	*Day 3*	*Day 4*	*Mean*	
Ark Royal	3	0.288[d]	0.281	0.272	0.287	0.282	2.5
	5	0.557	0.491	0.506	0.484	0.509	6.5
	8	0.882	0.822	0.868	0.812	0.846	4.1
Forrest	3	0.058	0.053	0.025	0.062	0.049	34.3
	5	0.322	0.287	0.316	0.287	0.303	6.1
	8	0.626	0.664	0.701	0.671	0.665	4.7
Schooner	3	0.334	0.342	0.360	0.329	0.341	4.1
	5	0.473	0.463	0.501	0.459	0.474	4.0
	8	0.732	0.725	0.777	0.718	0.738	3.6
WW870-10	3	0.263	0.248	0.229	0.287	0.257	9.6
	5	0.429	0.445	0.454	0.429	0.439	2.9
	8	0.762	0.796	0.806	0.749	0.778	3.5

[a] S.e.m. = 0.012 (standard error of the mean based on a model assuming fixed samples and random days and duplicates). C.v. (%) = 5.0; coefficient of variation expressing s.e.m. as a percentage of the mean. [b] Lot MRP90201. [c] Standard deviation of the four individual assays for each malt sample. [d] Average of duplicate analyses.

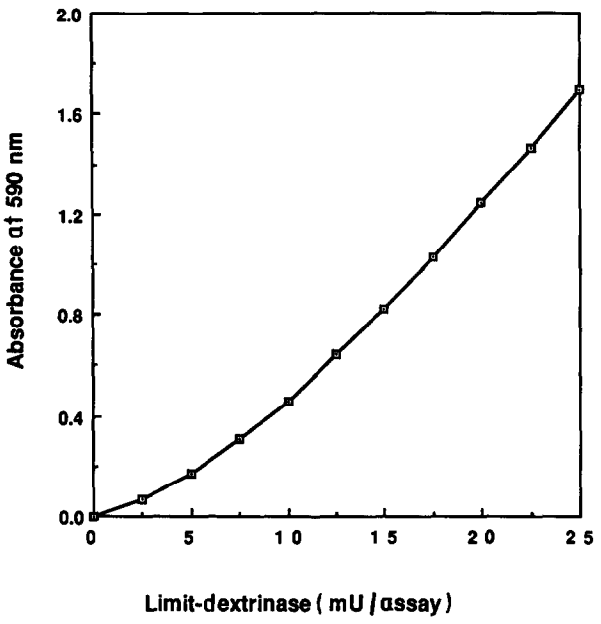

Fig. 5. Standard curve relating the activity of malt limit-dextrinase (U/mL, *i.e.*, U/assay) to increase of absorbance at 590 nm on hydrolysis of Azurine-CL-Pullulan (in Limit-DextriZyme tablets, Lot LDZ10801). Incubations were performed under standard conditions at pH 5.5 for 10 min at 40°. Limit-dextrinase (U/mL) was determined on pullulan using a reducing-sugar assay[20].

substrate and the degree of cross-linking and dyeing of the substrate. These parameters are under further evaluation.

Azurine-CL-Pullulan substrate consists of insoluble, gelatinous particles, which are charged and sensitive to the concentration of the buffer salt. The effect of the concentration of maleate buffer on the absorbance values is shown in Fig. 6; 0.1M maleate was adopted for routine use. The concentration of substrate required to give

TABLE II

Reproducibility[a] of Limit-DextriZyme (Lot LDZ10101) in malt samples

Sample	*Absorbance at 590 nm*								
	Run 1		*Run 2*		*Run 3*		*Run 4*		*Average*
Malt 8	0.828	0.810[b]	0.886	0.879	0.880	0.904	0.911	0.917	0.877
9	1.163	1.104	1.169	1.159	1.186	1.191	1.149	1.187	1.164
10	1.518	1.539	1.655	1.682	1.663	1.756	1.751	1.829	1.674
11	1.261	1.200	1.240	1.175	1.165	1.155	1.231	1.121	1.200

[a] S.e.m. = 0.022 (based on model assuming fixed samples and random occasions and duplicates); c.v. (%) = 5.03. [b] The duplicate analyses were made on four separate occasions

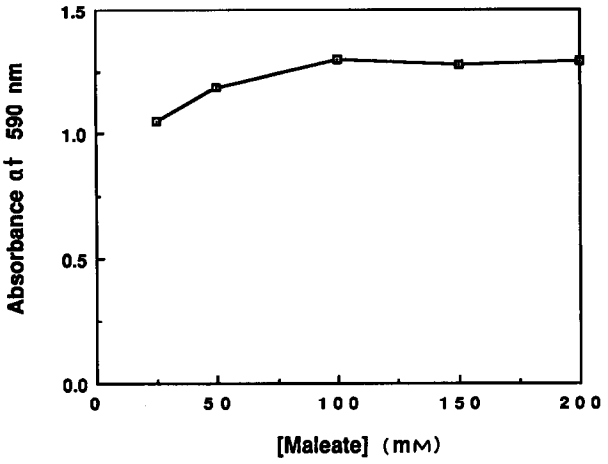

Fig. 6. Effect of concentration of maleate buffer on the measured limit-dextrinase activity (absorbance at 590 nm) with Limit-DextriZyme tablets. Incubations were performed with a malt extract under standard assay conditions.

maximal reaction velocity was > 50 mg/assay (*i.e.*, per mL), but such concentrations are difficult to achieve and a concentration of 20 mg/mL was employed routinely. At this concentration, the reaction rate was ~ 70% of that at a substrate concentration of 40 mg/mL.

Azurine-CL-Pullulan is resistant to hydrolysis by other enzymes present in malt as well as microbial starch-degrading enzymes. Purified malt alpha-amylase (50 U), wheat-flour beta-amylase (100 U), and amyloglucosidase (100 U) released no soluble dyed fragments under the standard assay conditions (pH 5.5, 10 min, 40°). *A. niger* alpha-amylase (20 U) gave an absorbance increase of 0.16 in 10 min due to its ability to cleave the maltotetraosyl structural elements in pullulan[6]. However, this effect does not affect the usefulness and specificity of this substrate for the assay of limit-dextrinase in cereal extracts.

Limit-DextriZyme tablets were used to measure limit-dextrinase activity in a range of cereal flours and in barley samples at different stages of malting (Table III). The highest activity values were found in ungerminated rice grains, but oat seed also had detectable levels. Ungerminated barley grain contained undetectable levels of limit-dextrinase, but activity increased steadily during malting. The change in activity of alpha-amylase[24], beta-amylase[25], and limit-dextrinase during steeping and malting of the barley variety Schooner is shown in Fig. 7. Some activity of limit-dextrinase is lost during kilning. This enzyme has thermostability properties similar to those of beta-amylase, being intermediate between alpha-amylase and malt beta-glucanase.

Thus, both Red-Pullulan and Limit-DextriZyme tablets (containing Azurine-CL-pullulan) can be used to measure limit-dextrinase activity specifically in malt flour. However, Limit-DextriZyme tablets have the advantages of greater sensitivity, low blank absorbance values, greater ease of use, and superior stability. The availability of

TABLE III

Limit-dextrinase activity of cereal grain samples and malted barley

Sample		*Limit-dextrinase activity (U/kg)*
Grain	*Variety*	
Barley	Corvette	u.d.[a]
	Parwan	u.d.
	Schooner	u.d.
	Forrest	u.d.
Malted barley (120-h malting)	Corvette	4.20
	Parwan	348
	Schooner	408
	Forrest	228
Oats	Carbeen	40
	Hill	24
	Mortlock	36
Rice	Amaroo paddy	2200
	Amaroo white	3200
	Amaroo white bran fraction	328
	Amaroo white hull fraction	u.d.

[a] Undetectable, *i.e.*, <5 U/kg of flour.

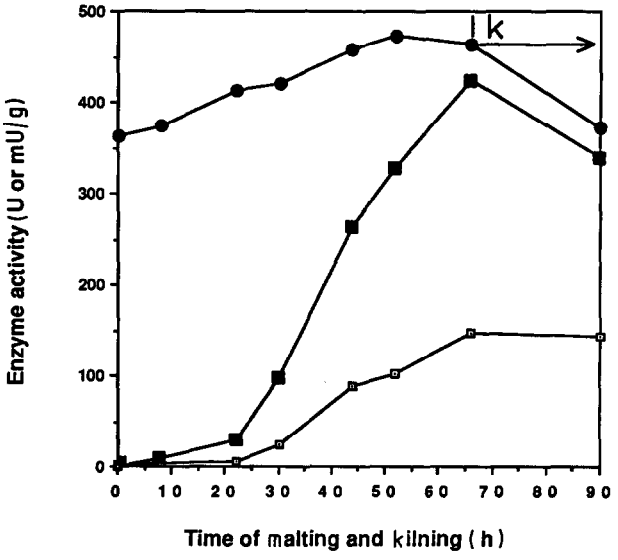

Fig. 7. Changes in activity of limit-dextrinase (■, mU), alpha-amylase (□, U), and beta-amylase (●, U) activities on malting of the barley variety, Schooner; k, initiation of kilning.

this substrate should assist cereal chemists to define the importance of limit-dextrinase in the conversion of malt saccharides into fermentable sugars. This substrate can also be used for the assay of microbial pullulanases, and, in powder form, should be useful in locating pullulanase-producing microbial cultures on agar plates.

In measuring limit-dextrinase activity in flour samples, we routinely obtain values with the Limit-DextriZyme procedure which are 1.5 times that obtained with Red-Pullulan substrate. The reason for this is not yet clear, but it could be related to the different pH optima values with the two substrates or to the much greater ratio of extraction buffer to flour sample with the Limit-DextriZyme format (leading to better activation of the enzyme).

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REFERENCES

1 E. Kneen and J. M. Spoerl, *Proc. Am. Soc. Brew. Chem.*, 20 (1948).
2 D. J. Manners and D. Yellowlees, *Staerke*, 23 (1971) 228–234.
3 E. Y. C. Lee, J. J. Marshall, and W. J. Whelan, *Arch. Biochem. Biophys.*, 143 (1971) 365–374.
4 D. J. Manners, *Cereals Food World*, 30 (1985) 722–727.
5 D. G. Hardie and D. J. Manners, *Carbohydr. Res.*, 36 (1974) 207–210.
6 B. J. Catley, *Carbohydr. Res.*, 61 (1978) 419–424.
7 B. V. McCleary and R. Codd, *Proc. Int. Symp. Cereal Carbohydrates*, Edinburgh, 1988.
8 L. Serre and C. Laurière, *Anal. Biochem.*, 186 (1990) 312–315.
9 W. J. Lee and R. E. Pyler, *Brewers' Digest*, (1982) 24–27.
10 S. Chiba, S. Sugawara, T. Shimomura, and Y. Nakamura, *Agric. Biol. Chem.*, 26 (1962) 787–793.
11 M. Kanno and E. Tomimura, *Agric. Biol. Chem.*, 49 (1985) 1529–1530.
12 S. S. Yang and R. D. Coleman, *Anal. Biochem.*, 160 (1987) 480–482.
13 Y. Shain and A. M. Mayer, *Physiol. Plant.*, 21 (1968) 765–776.
14 J. Yamada, *Carbohydr. Res.*, 90 (1981) 153–157.
15 G. N. Bathgate, *J. Inst. Brew., London*, 84 (1978) 22–29.
16 B. S. Enevoldsen and F. Schmidt, *J. Inst. Brew., London*, 80 (1974) 520–533.
17 H. Rinderknecht, P. Wilding, and B. J. Haverback, *Experientia*, 23 (1967) 805.
18 M. Ceska, *Eur. J. Biochem.*, 22 (1971) 186–192.
19 F. M. Rombouts, A. K. Wissenburg, and W. Pilnik, *J. Chromatogr.*, 168 (1979) 151–169.
20 M. Somogyi, *J. Biol. Chem.*, 195 (1952) 19–23.
21 B. V. McCleary and M. Glennie-Holmes, *J. Inst. Brew., London*, 91 (1985) 285–295.
22 D. Lacommandeur, A. W. MacGregor, and J. Daussant, *J. Chromatogr.*, 441 (1988) 436–442.
23 R. J. Henry and D. Butler, *J. Inst. Brew., London*, 98 (1992) in press.
24 B. V. McCleary and H. Sheehan, *J. Cereal Sci.*, 6 (1987) 237–251.
25 B. V. McCleary and R. Codd, *J. Cereal Sci.*, 9 (1989) 17–33.