A New Approach to Limit Dextrinase and its Role in Mashing*

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Limit dextrinase (EC 3.1.2.41) is a debranching enzyme catalyzing the hydrolysis of α-1,6-glucosidic linkages in starch. The role of this debranching enzyme in beer brewing has been questioned due to its assumed heat lability. In the present work the effectiveness of limit dextrinase was studied under conditions mimicking brewery practice rather than in a buffer solution. It was demonstrated that typical conversion temperatures of 63-65 °C and a mash pH of 5.4-5.5 favour the action of malt limit dextrinase. The temperature optimum for the limit dextrinase of a malt extract was 60-62.5 °C, as opposed to 50 °C for purified limit dextrinase. Lowering the mash pH from 5.8 to 5.4 increased wort fermentability due to increased limit dextrinase activity. Wort fermentability was more strongly correlated to the free limit dextrinase activity of malt than to the α- and β-amylase activities.

Key Words: *Limit dextrinase, dextrins, mashing, fermentability.*

INTRODUCTION

Starch consists of essentially unbranched amylose and highly branched amylopectin. In mashing, amylose is rapidly and extensively degraded by α-amylase and β-amylase to a mixture of maltose and smaller amounts of glucose and maltotriose. The hydrolysis of amylopectin yields a mixture of branched dextrins in addition to fermentable sugars. A debranching enzyme is required to convert these dextrins into linear maltosaccharides, which can be degraded by amylases. The final stage in starch hydrolysis is catalyzed by α-glucosidase, which hydrolyses maltose and maltotriose to glucose[10].

The presence of one or more starch-debranching enzymes in malt has been recognised for more than 50 years, but the importance of the debranching activity in brewing is still under debate. Research on plant debranching enzymes in 1970-1975 demonstrated that only one debranching enzyme is present in malt. The natural substrates for this enzyme are limit dextrins produced during starch hydrolysis by α- and β-amylase, and therefore the enzyme was given the name limit dextrinase. Limit dextrinase is present in very small amounts in malt and is therefore laborious to purify. Suitable substrates for analysis of limit dextrinase activity became commercially available only recently. For these reasons little research on malt limit dextrinase has been carried out. Furthermore, many earlier results need to be re-evaluated now that the shortcomings of the techniques used previously have been recognised[19].

The aim of the present work was to investigate the properties of malt limit dextrinase that influence its effectiveness in mashing and to evaluate the importance of limit dextrinase in brewing. Most of the experiments were performed under conditions mimicking brewery practice. For comparison, some experiments with purified limit dextrinase were also carried out.

MATERIALS AND METHODS

Malt samples

A commercial Kymppi malt (brewer's malt), a commercial Pokko malt (high enzyme malt) and 12 Alexis malts malted in a Seeger micromalter were used. The amylolytic activities of the malts are listed in Table I.

TABLE I. Enzyme activities of malt samples

Sample	α-amylase activity (U/g)	ß-amylase activity (U/g)	Free limit dextrinase activity (U/kg)	Total limit dextrinase activity (U/kg)
Kymppi	240	670	40	380
Pokko	410	1350	140	1170
Alexis (n = 12)	110-390	400-800	40-240	290-540

Purified malt limit dextrinase

Limit dextrinase was purified from a commercial Pokko malt using the method of MacGregor[8] with slight modifications as described previously[19].

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Malt extracts

Malt extracts were prepared by extracting 50 g of malt with 200 ml of water (containing 1.5ml/litre 0.5 M H_2SO_4 and 375 mg/litre $CaCl_2$ $2H_2O$) for 30 minutes at 48°C. At the end of the extraction, the mixture was filtered through a Macherey-Nagel filter paper. The protein content of the malt extracts was determined using the Sigma Microprotein-PR™ protein assay (Sigma 611-2) using BSA as a standard.

Mashing

Mashing experiments were carried out in a Bender & Hobein 3M mashing bath according to the VTT high gravity mashing procedure mimicking brewery practice (malt:water ratio 1:4, pH 5.5, temperature programme 48°C/63°C/72°C/80°C)[18,20]. Samples taken during mashing were immediately cooled (<5°C) in ice-water and centrifuged (8000 g, 10 minutes, 4°C).

Enzyme assays

Free and total limit dextrinase (at 40°C, pH 5.5 unless else stated), α-amylase (at 40°C, pH 5.2) and free β-amylase (at 40°C, pH 6.2) activities of malts were determined using Megazyme methods[13-15] with slight modifications as described earlier[19]. Enzyme activities of the purified enzyme, malt extracts and mash samples were determined using the same methods as for malts except that the samples were diluted in 0.1 M sodium acetate buffer, pH 5.5 (mash pH). For limit dextrinase assays the buffer was supplemented with 0.5 mg/ml BSA, for α-amylase assays with 1 mM calcium chloride, for the β-amylase assays with 1.0 mg/ml BSA.

Wort analyses

Wort apparent fermentability was determined according to Analytica-EBC[2]. The pH of wort samples was measured at 20°C.

Experimental procedures

Temperature optimum of limit dextrinase

The effect of reaction temperature on the limit dextrinase activity of a Pokko malt extract and of purified malt limit dextrinase (from Pokko) was studied in the temperature range 30-70°C (pH 5.5, incubation time 10 min).

Heat stability of limit dextrinase

Heat stability of limit dextrinase of a Pokko malt extract and of purified limit dextrinase (from Pokko) in sodium acetate buffer (0.1 M, pH 5.5, containing 0.5 mg/ml BSA) was studied by incubating the samples for 60 min at 40-65°C.

pH optimum of limit dextrinase

The effect of reaction pH on the limit dextrinase activity was examined in the pH range 4.0-5.5 using 0.1 M sodium acetate buffers and in the pH range 4.0-7.0 using McIlvain buffers (0.1 M citric acid/0.2 M Na_2HPO_4).

Effect of mash pH on limit dextrinase activity and wort fermentablity

Mashings with a commercial Kymppi malt were carried out with the addition of variable amounts of 0.5 M sulphuric acid (1-12.5 ml/litre) to give mash pH values in the range 4.9-5.8 (measured at 20°C). Limit dextrinase activity of samples taken at the end of the mashing-in (48°C/30 minutes) was determined both at the actual mash pH and at a constant pH of 5.5. For comparison, the α-amylase and β-amylase activities of the samples were also measured. Fermentability was determined from samples taken at the end of the mashing.

Correlations between amylolytic activities and wort fermentability

The correlations between the amylolytic activities of 12 Alexis malts and the fermentabilities of the corresponding high gravity worts were calculated.

RESULTS AND DISCUSSION

Temperature optimum of limit dextrinase

The temperature dependence of limit dextrinase activity is shown in Figure 1. The highest activity was measured at 50°C for the purified enzyme and at 60-62.5°C for the malt extract. The incubation temperature

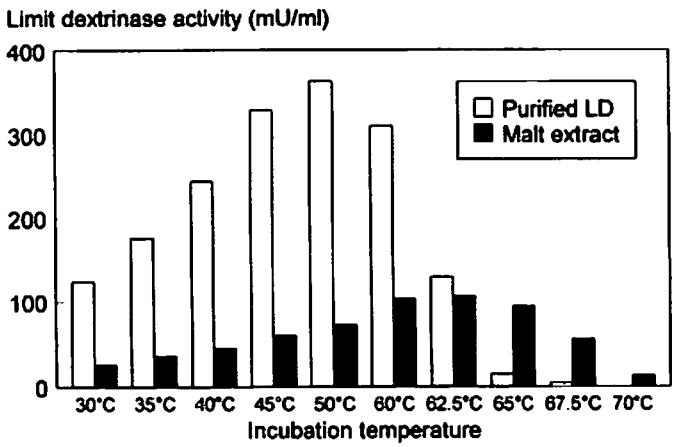

FIG.1 Effect of assay temperature (30-70°C) on limit dextrinase activity of a Pokko malt extract and purified malt limit dextrinase (from Pokko) (pH 5.5, incubation time 10 min). LD = limit dextrinase. Results are means of three determinations. Maximum standard deviation within replicates was 4 mU/ml for purified LD and 2 mU/ml for malt extract.

in the original Limit-Dextrizyme method is 40°C[13]. At this temperature the activity of purified limit dextrinase was only 65% and that of the malt extract only 40% of the maximal value. On the basis of these results it can be concluded that limit dextrinase activity at common saccharification temperatures (60-65°C) is considerably higher than the activity measured at 40°C.

The recorded temperature optimum of 50°C for purified limit dextrinase is in accordance with the results of Maeda *et al.* [9] but higher than that reported by Manners and Rowe[11]. The stabilising effect of a malt extract on the heat stability of limit dextrinase has been reported previously[1,5], but the temperature optimum of limit dextrinase in a malt extract has not been reported. Since the extract was prepared with the same malt:water ratio as used in commercial mashings, the result provides useful information for evaluation of the role of limit dextrinase in mashing.

Heat stability of limit dextrinase

When evaluating the heat stability of limit dextrinase under mashing conditions by measuring the activity in the liquid phase of the mash[17,21], simultaneous dissolution and inactivation makes interpretation of the results difficult. Under such experimental conditions limit dextrinase might appear more heat stable than it really is, since gradual dissolution of activation could mask inactivation in the liquid phase. In order to overcome this artefact, the heat stability of malt limit dextrinase was studied by incubating a Pokko malt extract rich in limit dextrinase for one hour at 40-65°C. Samples were taken at 10, 20, 30 and 60 minutes and the limit dextrinase activity was measured at 40°C. For comparison, purified malt limit dextrinase was incubated at the same temperature.

The limit dextrinase activity of the malt extract was stable at temperatures up to 62.5°C (Fig. 2A). At 65°C the malt extract lost 40% of its activity during one hour. When pure limit dextrinase was incubated in acetate buffer (0.1 M, pH 5.5, 0.5 mg/ml BSA) the activity began to decrease already at 50°C (Fig. 2B). At 55°C 70% of the activity was lost in 60 minutes. At 60°C the activity was totally lost in 20 minutes. The purified limit dextrinase was thus much more heat labile than the limit dextrinase of the malt extract. The protein content of the malt extract was 3.4 mg/ml. The same amount of BSA added to the purified limit dextrinase was not sufficient to increase its heat stability to that of limit dextrinase in the malt extract[19]. This indicates that the malt extract contains some other protective factor than the protein. The protective function of malt extract has been reported previously[5].

The experiments with malt extract confirmed the heat stability results of limit dextrinase from the mashing experiments presented previously[17,21]. At 62.5°C the limit dextrinase of the malt extract appeared to be even slightly more heat stable than the limit dextrinase in the liquid phase of the mash at the same temperature. It is possible that part of the decrease in the limit dextrinase activity during mashing is not due to heat inactivation but to limit dextrinase complexing with limit dextrinase inhibitor, which is slowly solubilized[7,19].

Experimental conditions strongly affect the behaviour of limit dextrinase and the present work thus provides new information on the importance of limit dextrinase during mashing. On the basis of the results in Figure 2 it can be concluded that pure limit dextrinase cannot be used to evaluate the role of limit dextrinase in mashing in terms of temperature stability.

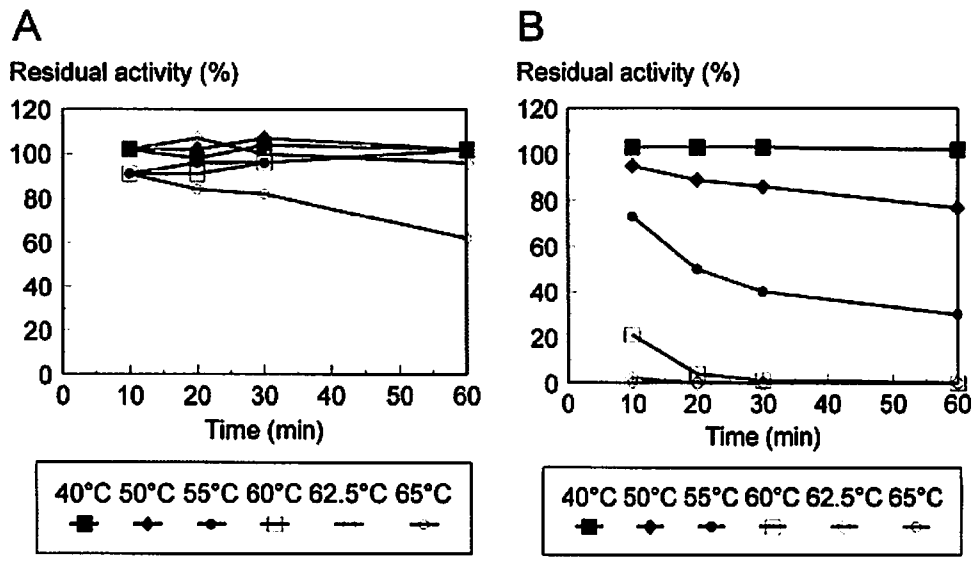

FIG. 2 Heat stability of limit dextrinase of a Pokko malt extract (A) and purified limit dextrinase (from Pokko) in sodium acetate buffer (0.1 M, pH 5.5, 0.5 mg/ml BSA) (B). Samples were incubated at 40-65°C for 10-60 min before activity measurement at 40°C. Activities are expressed as percentages of the activities measured at 40°C without pre-incubation.

pH optimum of limit dextrinase

The highest limit dextrinase activity for purified limit dextrinase was recorded at pH 5.5 and for limit dextrinase of a malt extract at pH 5.0-5.5 (Fig. 3). For purified limit dextrinase the activity was more than 90%

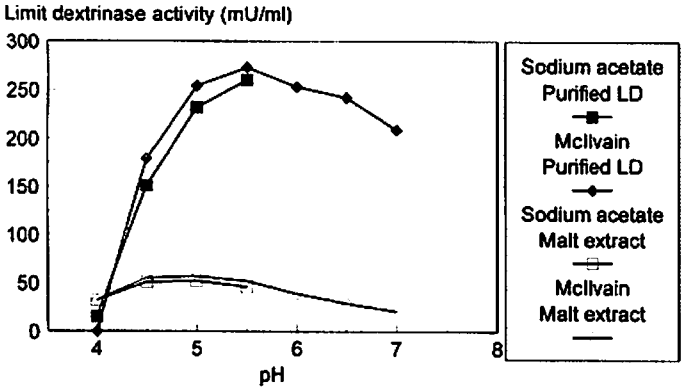

FIG. 3 Effect of assay pH (4.0-7.0) on limit dextrinase activity of a Pokko malt extract and purified malt limit dextrinase (from Pokko). Samples were diluted in 0.1 M sodium acetate buffers (pH 4.0-5.5) and McIlvain buffers (pH 4.0-7.0) containing 0.5 mg/ml BSA. Activity was measured at 40°C (incubation time 10 min). LD = limit dextrinase. Results are means of three determinations. Maximum standard deviation within replicates was 6 mU/ml for purified LD and 2 mU/ml for malt extract.

of the maximum value at pH 5.5 over the whole pH range relevant to mashing (pH 5.0-6.0). However, the malt extract results indicate that acidification of the mash to about pH 5.0-5.5 (measured at 20°C) might promote starch hydrolysis by increasing the limit dextrinase activity by 30-40%. The recorded pH optimum of 5.5 for purified limit dextrinase is in accordance with previously reported results[9,12,16]. MacGregor *et al.* [8] reported a slightly higher pH optimum (pH 5.5-6.5) for purified limit dextrinase in the presence of BSA. The pH optimum of limit dextrinase in a malt extract has not been reported previously.

Effect of mash pH on limit dextrinase activity and wort fermentability

Mash pH might affect the dissolution of malt limit dextrinase in addition to the activity of the free enzyme. In order to investigate this possible effect, mashings with a commercial Kymppi malt was carried out with the addition of variable amounts of 0.5 M sulphuric acid (0-12.5 ml/litre) to give mash pH values in the range 4.9-5.8 (measured at 20°C). Samples were taken at the end of the mashing-in (48°C/30 min) and the limit dextrinase activity was determined both at the actual mash pH and at a constant pH of 5.5.

When the pH of the mash decreased from 5.8 to 5.4, the limit dextrinase activity increased by 45% when measured at pH 5.5 and by 110% when measured at mash pH (Fig. 4). The activity measured at the actual mash pH (4.9-5.8) was not markedly different from that at pH 5.5, except at pH 5.8. The results indicate that the mash pH influences the solubilisation of limit dextrinase and support suggestions that the endogenous endopeptidases (pH optimum 3.8-4.5)[22] take part in the activation of latent limit dextrinase and the release of bound limit dextrinase[6].

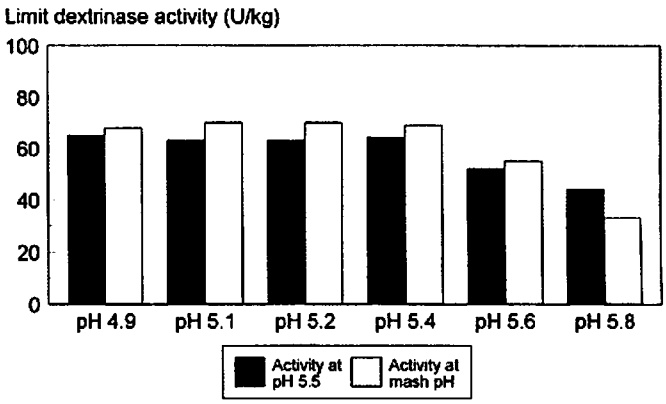

FIG. 4 Effect of mashing pH on limit dextrinase activity. High gravity mashings at pH 4.9-5.8, Kymppi malt. Samples for analysis were taken at the end of the mashing-in rest at 48°C (30 min). Limit dextrinase activity of the liquid phase of the mash was measured at pH 5.5 and at mash pH. Results are means (single mashing, two determinations per sample).

In a second set of high gravity mashings with the same Kymppi malt the mashes were taken through the mashing programme of the high gravity mashing procedure and the apparent fermentabilities of the worts were determined (Fig. 5). Lowering the mash pH from 5.7 to 5.4 increased the apparent fermentability, but as the pH decreased below 5.4 a decrease in the fermentability value was observed. Lowering the pH from 5.7 increased the limit dextrinase activity (Fig. 4), but did not affect the α-amylase activity and slightly decreased the β-amylase activity (results not shown). When the mash pH dropped below 5.4 the increase in limit dextrinase activity was counteracted by a marked decrease in both α-amylase and β-amylase activities resulting in lower apparent fermentabilities. When comparing the mashing results with those in Figure 3, it should be borne in mind that at mashing temperatures the pH is lower than that of cooled wort[4].

It is common brewery practice to adjust pH by acid addition in order to promote starch hydrolysis. On the basis of these results, it appears that the practice of adjusting the mash pH is effective not because of the

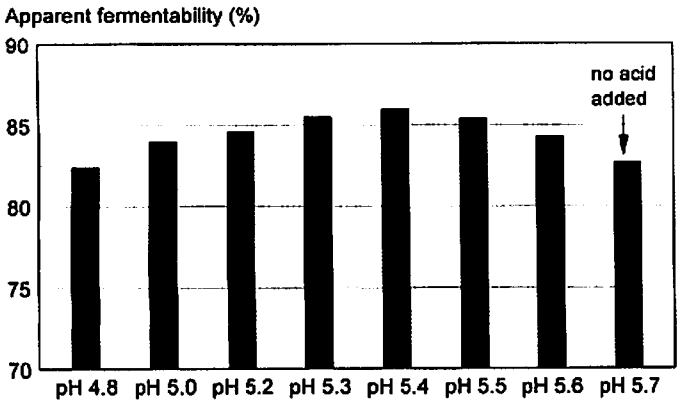

FIG. 5 Effect of mashing pH on the apparent fermentabilty of wort (Kymppi). Results are means (duplicate mashing, one determinations per wort sample).

effect on the major starch degrading enzymes, i.e. α-amylase and β-amylase, but due to the more efficient dissolution or activation of limit dextrinase.

Correlations between amylolytic activities and wort fermentability

α-amylase and β-amylase are the main enzymes responsible for the formation of fermentable sugars during wort production. In modern malting barley varieties, the activities of these enzymes are rather high and they are unlikely to be the limiting factors in starch hydrolysis in all-malt mashings. However, Enevoldsen and Schmidt[3] demonstrated that the majority of the α-1,6-linkages persist in mashing and that addition of pullulanase alters the wort carbohydrate profile, resulting in higher ethanol yields in fermentation.

In order to elucidate the influence of malt limit dextrinase activity on wort fermentability, a series of high gravity mashings was carried out with 12 Alexis malts varying in amylolytic activities. There was a statistically highly significant correlation between the free limit dextrinase activities of the malts and the apparent fermentabilities of the corresponding high gravity worts (Table II, Fig. 6). β-Amylase activities did not correlate with wort fermentability, whereas the correlation between α-amylase and wort fermentability was statistically almost significant (Table II). On the basis of these results the free limit

TABLE II. Correlation coefficients between amylolytic activities of Alexis malts and the apparent fermentabilites of the corresponding high gravity worts

	Alexis malts (n = 12)
Malt α-amylase	0.58 *
Malt β-amylase (free)	0.37
Malt limit dextrinase (free)	0.84 ***
Malt limit dextrinase (total)	0.31

*** = statistically highly significant, ** = statistically significant, * = statistically almost significant.

dextrinase activity of the malts was more limiting than the α-amylase and β-amylase activities in starch hydrolysis.

CONCLUSIONS

Purified limit dextrinase was rapidly inactivated at temperatures above 50°C, whereas the limit dextrinase of a malt extract retained 100% of its activity at 60°C and 60% of its activity at 65°C for one hour. For this reason purified malt limit dextrinase cannot be used to evaluate the role of this enzyme in mashing.

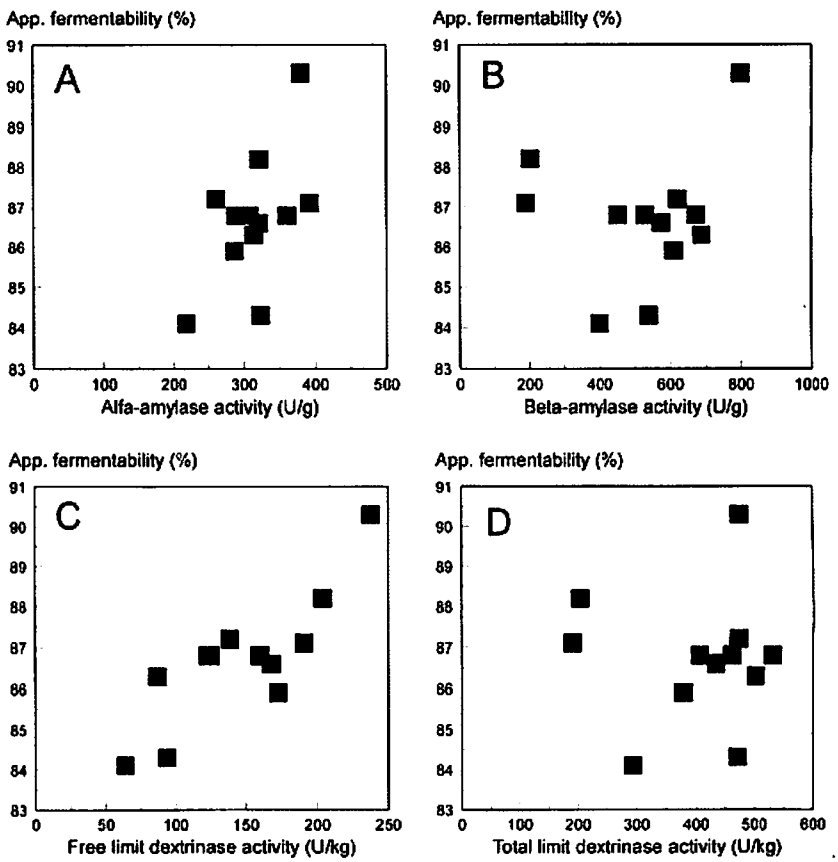

FIG. 6 Relationship between the α-amylase (A), β-amylase (B), free limit dextrinase (C) and total limit dextrinase (D) activities of Alexis malts (n=12) and the apparent fermentabilities of the corresponding high gravity worts.

The temperature optimum of the limit dextrinase of a malt extract was 60-62.5°C and the pH optimum 5.0-5.5. In the pH range 5.4-5.8, the limit dextrinase activity was the higher, the lower the mash pH. The free limit dextrinase activity of brewer's malts appeared to be more limiting that the α-amylase and β-amylase activities in hydrolysis of starch to fermentable sugars. The higher the free limit dextrinase activity was, the higher was the fermentability.

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