The Advantages of Using Natural Substrate-Based Methods in Assessing the Roles and Synergistic and Competitive Interactions of Barley Malt Starch-Degrading Enzymes

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ABSTRACT

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Existing methods of assay of malt starch-degrading enzymes were critically appraised. New methods based on natural substrates, namely starch and its natural intermediate-derivative, were developed for all the enzymes, except limit dextrinase for which pullulan was used. Thermostability, optimal temperatures and pHs were established. α-Amylase and limit dextrinase were the most thermostable and β-amylase, α-glucosidase and maltase were the least stable while diastase occupied an intermediate position. The optimal temperatures were congruent with thermostability, β- amylase having the lowest (50°C) and α-amylase the highest (65°C) with the remaining enzymes, including diastase, falling in between. In contrast, α-amylase has the lowest optimal pH (pH 4.5) and β amylase the highest (pH 5.5) while the others have pHs in between the two values. The roles of the enzymes were evaluated taking into account the level of activity, thermostability, optimum pH, the nature of the product(s), and the relevance to brewing. β-Amylase production of maltose was synergistically enhanced, mostly by α-amylase but also limit dextrinase. α-Glucosidase and maltase are unimportant for brewing, because of their low activity and the negative impact on β-amylase activity and the negative effect of glucose on maltose uptake by yeast. The starch-degrading enzymes (diastase) in a gram of malt were able to degrade more than 8 g boiled starch into reducing sugars in 10 min at 65°C. The latter, suggests that it will be possible to gelatinise most of the malt starch at a higher temperature and ensure its hydrolysis to fermentable sugars by mixing with smaller portions of malt and mashing at lower temperatures e.g. 50 – 60°C.

Key words: Barley malt, enzymes, starch degradation, thermostability.

INTRODUCTION

Barley malt is a rich source of starch-degrading enzymes, particularly α- and β-amylases. The latter is present in both barley and malt in high concentrations[11,17]. The other enzymes, namely, limit dextrinase, the debranching enzyme, and α-glucosidases are present in relatively low levels compared to α- and β-amylases. All the enzymes contribute to degrade starch to sugars in a concerted and sequential manner, to varying degrees. The total activities of barley malt starch-degrading enzymes, generally known as diastase or diastatic power (DP) are considered to be an important quality trait for malting and brewing[2,3,5]. Any attempt to manipulate or control the activities of these enzymes would require a good knowledge and understanding of their individual, synergistic and competitive inhibitory roles in starch degradation.

There is more than one method of assay for each enzyme[1-3,10-12]. Most of these methods are based on modified starch or starch-derivatives such as starch-dye complex (Phadebas, Azo-starch) or *p*-nitrophenyl glycosides[1,7,10,11]. These substrates are specific and for most purposes are satisfactory for the assay of each individual enzyme. However, they are inadequate to use for the study of the individual contribution and the synergistic role of each individual enzyme in starch degradation. The main weakness of the colour-incorporated derivative-substrates is that they do not reflect the sequential action of these enzymes on the same substrate, such as the starch and its intermediate products. For example, α-amylase rapidly converts starch into dextrin and oligosaccharides, which then become substrates for limit dextrinase and β-amylase. In turn the maltose and the short chain oligosaccharide produced by α- and β-amylases become the substrate for α-glucosidases[4,7,9]. α-Amylase continues acting on oligosaccharides feeding more substrates to β-amylase and α-glucosidases. This sequential action of the enzymes with the entailing synergistic and competitive interactions cannot be portrayed in synthetic coloured substrate assays.

The objective of this work was to determine the roles, and the positive and negative interactions of barley malt starch-degrading enzymes by developing new methods of assay, wherever applicable.

MATERIALS AND METHODS

Malt

Franklin and Schooner barley varieties were micromalted in a Phoenix machine according to the following procedure: samples were steeped at 17°C for 6:8:6 h, in the sequence steep: air-rest: steep followed by 96 h germination. The samples were then kilned over 20 h gradually

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ramping temperature from 17 to 55°C over 4 h, from 55 to 65°C over 6 h, from 65 to 75°C over 6 h and from 75 to 82°C over 4 h.

Chemicals

All chemicals were of analytical grade supplied by Sigma-Aldrich, Australia. The standard starch used in this study was the product manufactured by Pfanstiehl Laboratories and certified by ASBC. Amyloglucosidase, α-glucosidase, reagents for glucose assay and the assay of α- and β-amylases and limit dextrinase by McCleary methods were purchased from Megazyme, Australia. Prepacked Sephadex G-25 columns were purchased from Sigma-Aldrich, Australia.

Extraction of enzymes from malt

The four commonly used extraction media (water, 0.5% NaCl, 10 mM L-cysteine or 50 mM Na-phosphate buffer pH 8) were investigated. Routinely, 0.75 g malt flour was weighed in duplicate into centrifuge tubes and 4 mL extraction media was added with mixing. Extraction was performed for 30 min at 30°C with regular vortexing for 5 s at 5 min intervals and was terminated by centrifugation for 10 min at 2826*g*. The supernatants were filtered through wet muslin into measuring cylinders and the volumes were recorded. These volumes were used in the calculation of enzyme activities. Further, the extracts were filtered through a Sephadex G-25 column (8 × 1.5 cm) for the assay of α-glucosidases by glucose measurement and limit dextrinase by reducing sugars.

Enzyme assays

α-Amylase assay: α-Amylase Assay using 3,5-dinitrosalicylic acid (DNSA) method of reducing sugars. To assay α-amylase by measuring the reducing sugars produced by its action on soluble starch, β-amylase was inactivated by heat treatment for 7 min at 70°C, in the presence of 20 mM $CaCl_2$. To pre-equilibrated soluble starch solution (0.25 mL 50 mg/mL in 0.1 M malate buffer pH 5.5 containing 10 mM $CaCl_2$) 0.05 mL heated extract (appropriately diluted) was added with mixing. The reaction was continued for 10 min and terminated by adding 2 mL 0.1 M NaOH. The reducing sugars were measured by adding 1.0 mL 3,5-dinitrosalicylic acid solution freshly prepared as described by Palmmer[16], mixing and boiling for 5 min. Standards and controls were treated similarly with the exception that the enzyme extract was added to the controls after NaOH. The samples, controls and the standard were read at 540 nm after cooling to room temperature. One unit of α-amylase activity was defined as the quantity of enzyme that released the amount of reducing sugars per minute equivalent to one μmole of maltose, under the above defined assay conditions.

The enzyme activity was calculated according to the following formula:

U/g malt = ODt/ODs*600*EV*DF/10/0.05/0.75/342.3

in which,

ODt = the optical density of test sample at 540 nm

ODs = the optical density of the maltose standard of 600 μg

EV = mL extraction volume. It has been established that 4 mL used to extract 0.75 g malt flour, yielded on average 3.188 mL (3.15-3.3), the difference being retained by the grist.

DF = dilution factor (100 in this study)

10 = min, incubation time in minutes

0.05 = mL, volume of diluted enzyme extract used

0.75 = g, weight of malt flour used in enzyme extraction

342.3 = molecular weight of maltose

The formula can be further simplified as

U/g malt = ODt/ODs*600*3.188*100/10/0.05/0.75/342.3
= 1490.2*ODt/ODs.

Assay with a modified Ceralpha method. The following modifications were introduced to the Ceralpha method described by McCleary and Sheehan[12] to ensure the enzyme saturation with substrate during the reaction. The substrate was prepared by dissolving the contents of one vial of the Ceralpha substrate plus the content of another vial which contained only the blocked *p*-nitrophenyl maltoheptaoside ($BPNPG_7$) in 10 mL 0.1 M malate buffer pH 5.5. The reaction medium in a final volume of 0.3 mL contained 0.25 mL substrate and 0.05 mL extract (appropriately diluted, 100 times under the above described extraction condition). The final substrate concentration was 6.67 mM, instead of 2 mM in the original method. The rest of the procedure was the same as in the original Ceralpha method[12]. When α-amylase was assayed at higher temperatures in the range 50-80°C, the assay reaction was split into two sequential steps. First, α-amylase was incubated with $BPNPG_7$ only for 5 min and heated in a boiling water-bath for 5 min to stop the reaction. After cooling, 0.1 mL mixture of amyloglucosidase and α-glucosidase was added to the α-amylase reaction medium and incubated for 10 min at 40°C. Further treatment was the same as in the Ceralpha method[12]. Controls were included and treated similarly, as mentioned above, without allowing the enzymes to act on the substrate.

β-Amylase assay: β-Amylase assay with 3,5-dinitrosalicylic acid method of reducing sugars. β-Amylase was assayed using soluble starch as the substrate at 65°C in the presence of 20 mM ethylenediaminetetraacetic acid (EDTA) to exclude α-amylase, which is inactive under these conditions. Soluble starch solution (0.25 mL 50 mg/mL) in 0.1 M malate buffer pH 5.5 containing 20 mM EDTA was pre-equilibrated at 65°C before 0.05 mL extract (appropriately diluted, usually 200 times) was added with mixing. After 10 min, adding 2 mL 0.1 M NaOH stopped the reaction. The maltose produced was assayed using DNSA as described above under α-amylase. A unit (U) of β-amylase activity was defined as the amount of enzyme required to produce, per minute, an equivalent to one μmole of maltose under the specified conditions. Controls were treated as described under α-amylase.

Activity was calculated, as described under α-amylase above, according to the formula:

U/g malt = 2980.4*ODt/ODs

Assay with p-nitrophenyl maltopentaose (PNPG5). β-Amylase activity was measured according to McCleary

and Codd[11] method with the following modifications. The substrate was prepared by dissolving the betamyl reagent (Megazyme, Australia) in 8 mL 0.1 M malate buffer pH 5.5 containing 5 mM EDTA. The reaction medium, 0.3 mL final volume, was composed of 0.25 mL substrate and 0.05 mL extract (properly diluted). As in the case of α-amylase, when β-amylase activity was assayed at higher temperatures it was carried out in two steps. All the remaining steps were as described by McCleary and Codd[11].

Limit dextrinase assay: *Assay with 3,5-dinitrosalicylic acid of reducing sugars.* Limit dextrinase was assayed using pullulan as the substrate and measuring the reducing sugars produced with DNSA. Pullulan solution (0.25 mL 30 mg/mL in 0.1 M malate buffer pH 5.5 containing 15 mM L-cysteine) was pre-equilibrated and 0.1 mL heated (to inactivate β-amylase) extract filtrate was added with mixing. The reaction was continued for 10 min and stopped by adding 2 mL 0.1 M NaOH followed by 1 mL DNSA solution, mixing and boiling for 5 min. After cooling, 2 mL distilled water was added, mixed and the absorbance was read at 540 nm. A unit (U) of activity was defined as the amount of limit dextrinase required to produce reducing sugars equivalent to one μmole of maltotriose/min under these conditions. Controls were included and treated in the same manner except that the enzyme was added after sodium hydroxide.

Limit dextrinase activity was calculated using the same procedure and the basic formula described under α-amylase with the necessary alterations, as follows:

$$\text{U/g malt} = \text{ODt/ODs}*600*3.188*1.4/10/0.1/0.75/342.3$$
$$= 10.43*\text{ODt/ODs}$$

Assay with azure cross-linked pullulan (limit-dextriZyme tablets). Limit dextrinase activity was assayed according to the McCleary[10] method using limit-dextriZyme tablets (Megazyme, Australia) with the following modifications. Enzyme extract (0.2 mL) plus 0.1 mL 0.5 M malate buffer containing 0.15 M L-cysteine was pre-equilibrated before adding limit-dextriZyme tablets and incubating for 10 min after the addition of the tablets. In controls the extract was replaced by 0.2 mL distilled water, at the start. After the reaction was terminated by adding 5 mL trizma solution, 0.2 mL extract or distilled water was added to controls and samples, respectively. The developed colour was measured at 590 nm and the enzyme activity was calculated using the standard curve provided with the tablets adjusting for the changes in extract preparation and extract volume used in the assay.

Diastase (total amylolytic activities) assay: *Assay with 3,5-dinitrosalicylic acid method of reducing sugars.* Diastase activity was assayed using soluble starch as the substrate and measuring the reducing sugars released with DNSA. The procedure was the same as described under α-amylase except that the fresh extract (not heated) was used. A unit (U) of diastase activity was defined as the amount of enzymes required to release reducing sugars equivalent to one μmole of maltose/min under the above conditions. Alternatively, diastase activity was expressed in maltose equivalent as grams per 100 grams dry malt per 10 min (g/100g dry malt/10 min), similar to Windisch-Kolbach units.

Diastase activity expressed in units was calculated according to the same formula as β-amylase because the same dilution factor was used.

$$\text{U/g malt} = 2980.4*\text{ODt/ODs}$$

However, when the activity is expressed as maltose equivalent in grams per 100 g malt as in Windisch-Kolbach units, the formula is changed, depending on the desirable time duration used in the measurement e.g., 10 min, into the following:

$$\text{Maltose (g/100 g malt/10 min)}$$
$$= 2980.4*\text{ODt/ODs}*10*100*342.3/10^6$$
$$= 1020.19*\text{ODt/ODs}$$

α-Glucosidases and maltase assay. These enzymes were differentially measured by the methods described previously, including the sensitive method of glucose oxidase peroxidase (GOPO) method and methods using synthetic substrates[14].

Soluble protein assay

Soluble proteins in malt extract and its Sephadex G25 filtrate were assayed by the Lowry method using bovine serum albumin as the standard[6].

Sugar/oligosaccharides analysis by high performance liquid chromatography (HPLC)

The products of starch degrading enzymes: α-amylase, β-amylase and diastase were analysed by HPLC. These enzymes were incubated with pre-boiled starch for 10 min at 65°C, dipped into ice-water, appropriately diluted and loaded on a Dionex Carbopac PA1 analytical column (4 × 250 mm). Sugars and oligosaccharides were detected and identified using a Water Pulsed Amperometric Detector.

Thermostability, optimal temperatures and optimal pHs

Thermostability, optimal temperatures and optimal pHs of starch-degrading enzymes were determined using similar procedures as described for endoproteinases[13].

RESULTS AND DISCUSSION

Extraction of barley malt enzymes

The main objective of this study, as mentioned earlier, was to investigate the role of the starch-degrading enzymes and their interactions of synergistic and competitive inhibitory nature during commercial mashing. For research purposes, almost every enzyme is extracted by a different and presumably specific method[5,8,10-12,20-22]. However, industrial mashing is performed in water (some breweries use deionised and deaerated water others as it is, with or without added ions) in which the enzymes, the products of their actions and other soluble materials are extracted. Although water was the obvious choice for the extraction of these enzymes, three other media commonly used for the extraction of individual enzymes were tested. These were, 0.5% NaCl, 10 mM L-cysteine and 50 mM phosphate buffer pH 8. As part (about 20%) of the initial medium added to the grist was retained and not fully re-

covered in the extract, the extract volume measured after the centrifugation, and not the initial volume added to the grist, was used in the calculation.

It is clear that all starch-degrading enzymes were extracted reasonably well with all four media tested (Table I). However, phosphate buffer pH 8.0 seemed to be the best medium for extracting starch-degrading enzymes despite the L-cysteine added medium having the highest protein extract. However, the latter is due to the contribution of the extraction medium L-cysteine, which is eliminated by the Sephadex filtration. The fact that the phosphate buffer (pH 8) is the best medium suggests that either the high pH or the added phosphate ions (higher concentration) enhance the release of more enzymes. Indeed, the phosphate buffer extract-filtrate (G-25 Sephadex) has the highest soluble protein concentration, as seen in Fig. 1. The increase in extracted enzymes was particularly evident in the case of maltase activity which was 3-fold higher in phosphate buffer extract than in water. However, although phosphate buffer pH 8.0 seems to be the best medium for extraction of starch-degrading enzymes, it is far displaced from the optimal pHs (4.0-5.5) of all the enzymes, rendering it unsuitable for mashing and starch degradation. The general pattern was consistently the same with all four media, showing α- and β-amylases as the two enzymes exhibiting the highest levels of activity, constituting more than 99% of the total activity.

Assay of starch-degrading enzymes

Assay with synthetic substrates. McCleary and others[1,10-12,20] have developed some specific and simple methods for the individual assay of all malt starch-degrading enzymes using synthetic substrates. A critical appraisal of some of these methods has revealed some minor weaknesses. For instance, the test for linearity with blocked *p*-nitrophenyl maltoheptaoside ($BPNPG_7$) as the substrates for α-amylase has shown that the linearity was restricted to the low levels of enzyme extracts (Fig. 2 A). Moreover, the enzyme activity declined steeply with the increase in incubation time (Fig. 2 B), suggesting insufficient level of substrate saturation. Indeed, the increase in substrate concentrations improved the linearity and exhibited higher activity levels for the same amount of α-amylase extract used, as seen in Fig. 2. These results indicate that though the method is fine for general qualitative assay, special care should be given to the amount of enzyme used and the duration of the reaction. Similar results (not presented) were obtained with β-amylase and *p*-nitrophenyl maltopentaoside ($PNPG_5$). Furthermore, the comparison of pH optima of these enzymes with natural and synthetic substrates (Fig. 3) revealed that the pH optima for α- and β-amylases were higher with synthetic than with natural substrates. This may be insignificant for general purposes of enzyme assay but it reveals that changes in substrate structure affect the interaction between the enzyme and the substrate.

The solubility of *p*-nitrophenyl glucose (PNPG) for the assay of α-glucosidases was found to be very low at room temperature, but improved with warming. Consequently, the activity measured with this synthetic substrate was much lower than with the natural substrates[9,22]. A similar

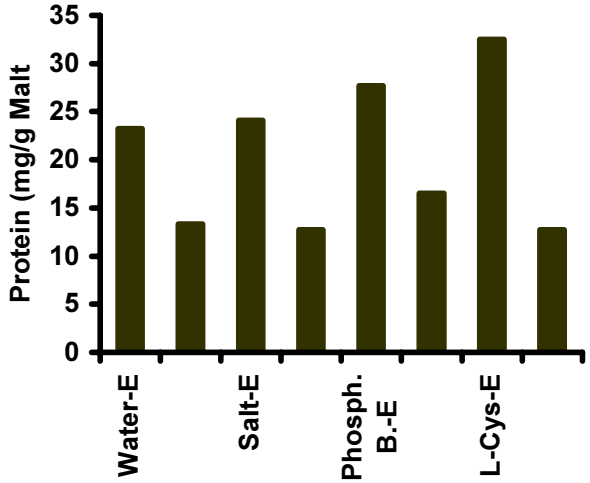

FIG. 1. Comparison of malt soluble proteins extracted in four media: water, salt, L-cysteine solutions and phosphate buffer. Extracts in the corresponding media: Water-E, Salt-E, L-Cys.-E and Phosph.B.-E; and the Sephadex G-25 filtrates of the same media extracts: Water-EF, Salt-EF, L-Cys.-EF and Phosph.B.–EF.

TABLE I. Activities of starch-degrading enzymes assayed individually and collectively (diastases) under different conditions in common extracts.

		Activities (U/g dry malt)[a]								
	Enzyme	Maltase	α-Glucosidase	Limit dextrinase	α-Amylase		β-Amylase		Diastase[b]	
	Substrate[c]	Maltose	Boiled starch	Pullulan	BPNPG7	Boiled starch	PNPG5	Boiled starch	Boiled starch	
Extraction media	Assay T °C	40	40	40	40	65	40	65	65	WK[d]
Water		0.1	2.1	0.31	188	1195	507	853	2354	805
Salt (0.5% NaCl)		0.14	2.4	0.35	275	1261	682	1086	2508	858
10 mM L-Cysteine		0.08	1.82	0.37	175	1203	553	1082	2458	841
50 mM Phosphate buffer (pH8)		0.34	2.22	0.66	277	1590	522	1023	2901	992

[a] Mean of 8 estimates.
[b] Diastase is the common name for all starch-degrading enzymes.
[c] $BPNPG_7$: blocked p-nitrophenyl maltoheptaoside, PNPG5: p-nitrophenyl maltopentaoside.
[d] WK activities expressed in maltose equivalent (g/100 g dry malt/10 min/65°C).

situation was also observed with limit dextrinase. To eliminate some of these limitations, modifications were introduced, mainly to increase the final substrate concentrations in the reaction media. These changes were described under Materials and Methods. In addition attempts to improve existing methods, new alternative methods based on natural substrates were developed and are discussed in more detail below.

Assay with natural substrates

Assay of α-amylase, β-amylase and diastase using starch as the substrate and the DNSA method of reducing sugars. The most accurate means of evaluating the roles of individual starch-degrading enzymes during hydrolysis is to follow (1) starch disappearance or (2) the appearance of the products released. However, this is not always pos-

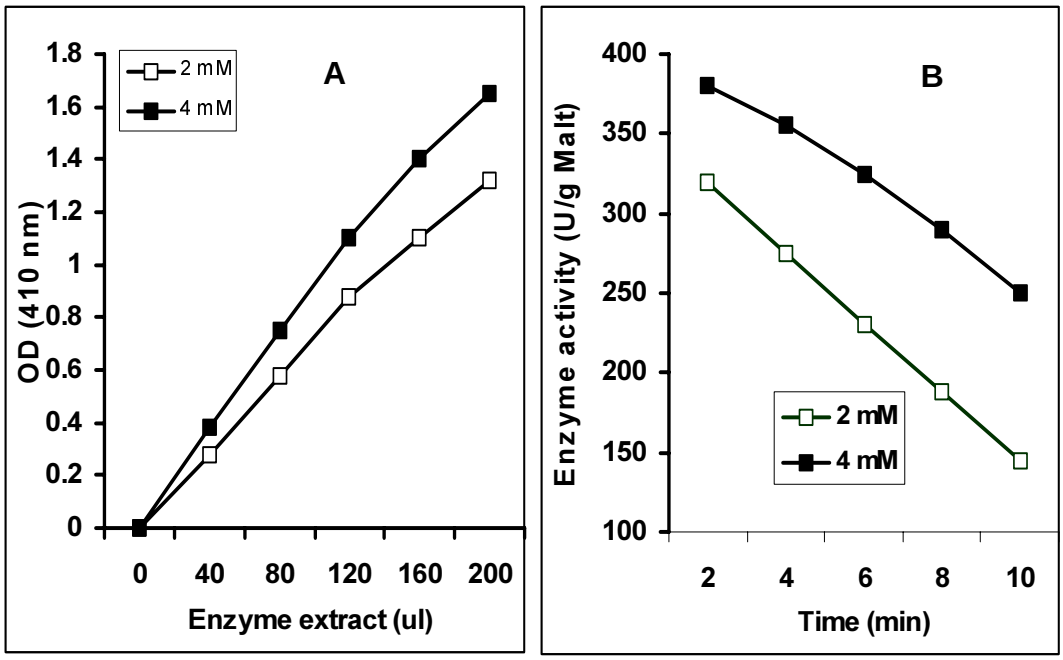

FIG. 2. Effects of enzyme and substrate $BPNPG_7$ concentrations [2 mM (-□-) and 4 mM (-■-)] on the linearity of the α-amylase reaction (A) and the effects of substrate concentration and the length of incubation time on the enzyme activity measured (B). The enzymes were extracted and assayed as described under Materials and Methods. The extract was diluted to obtain different enzyme concentrations in the same final reaction medium.

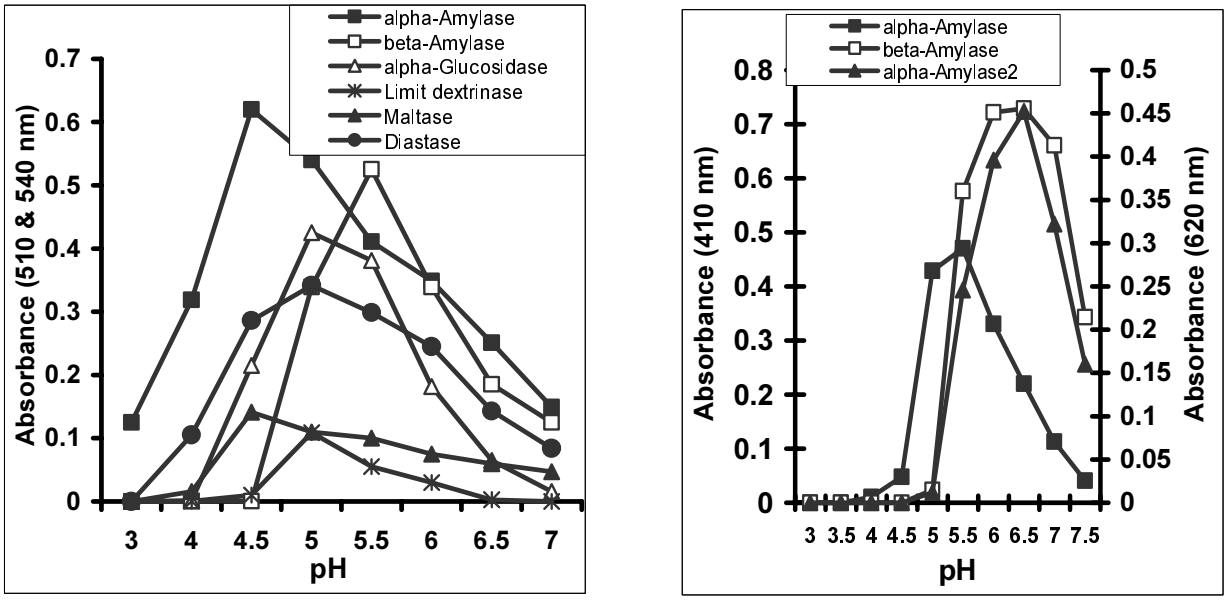

FIG. 3. The pH profiles of barley malt starch-degrading enzymes assayed with (A) natural substrates and (B) synthetic substrates Ceralpha (410 nm) and Phadebas (620 nm). Enzymes were extracted and assayed as described under Materials and Methods. α-Amylase2 (▲ 620 nm) was heated at 70°C for 7 min to inactivate β-amylase, before incubating with Phadebas substrate.

sible because all the enzymes are present together and act simultaneously on starch as well as the intermediate products, ultimately releasing some common products.

To surmount this problem, firstly, the existence and the level of each enzyme in the malt mixture was assessed (Table I). It was found that α-glucosidases and limit dextrinase were present in such small levels (< 1%), compared to α- and β-amylases, that their effects on the latter two could be ignored. Secondly, the conditions under which both α- and β-amylases could be measured without mutual interference were established. α-Amylase was assayed after inactivation of β-amylase by heating at 70°C in the presence of calcium ions and β-amylase was measured at 65°C in the presence of EDTA to inhibit the former. The validity of these assays and their specificity were verified by the analysis of the profiles of the sugar products by high performance liquid chromatography (HPLC) (see Fig. 4). It is evident from the HPLC profiles that the products of α-amylase (Fig. 4A) were different from those of β-amylase (Fig. 4C), and both were different from that of diastase (Fig. 4B), which is basically the result of their combined action.

The products of α-amylase reflected the endo-amylolytic nature of the action of this enzyme and were mainly large oligosaccharides, eluted later (> 28 min). In contrast, the product of β-amylase was maltose (17 min). The profile of the products of diastase (Fig. 4B) reflected the combined action of these two enzymes. Nevertheless, they were similar in some aspects and different in others. The peak for maltose observed with β-amylase remained and increased while in contrast, the peaks for larger oligosaccharides, the products of α-amylase diminished and the peaks for maltotriose (25 min) and maltopentaose increased (28 min). The increase in the maltotriose peak was most noticeable and suggested that this sugar was probably produced by the alternating/combined action of α- and β-amylases on oligosaccharides.

α-Amylase activity is only one-third that of β-amylase when measured with synthetic substrates, but is higher measured with starch. These results reflect the vulnerability of β-amylase to heat and its inability to degrade starch beyond the branching points. It also demonstrates the importance of α-amylase activity and its synergistic effect on β-amylase for an efficient and complete degradation of starch during mashing.

TABLE II. Summary of statistical analysis of the mean enzyme activities (U/g dry malt), n = 8.

Enzymes	Substrate	Mean[a]	SD[b]	SE[c]	CV[d] %
α-Amylase	$BPNPG_7$[e]	188	12.24	4.33	6.52
	Starch	1195	29.07	10.28	2.43
β-Amylase	PNPG5[f]	507	11.42	4.04	2.25
	Starch	853	26.43	9.34	3.1
Diastase	Starch	2354	70.97	25.09	3.01
Limit dextrinase	Pullulan	0.305	0.035	0.012	11.29
α-Glucosidase	Starch	2.07	0.09	0.03	4.39

[a] Mean activity (U/g dry malt).
[b] Standard deviation.
[c] Standard error.
[d] Coefficient of variation.
[e] Blocked *p*-nitrophenyl maltoheptaoside.
[f] *p*-Nitrophenyl maltopentaoside.

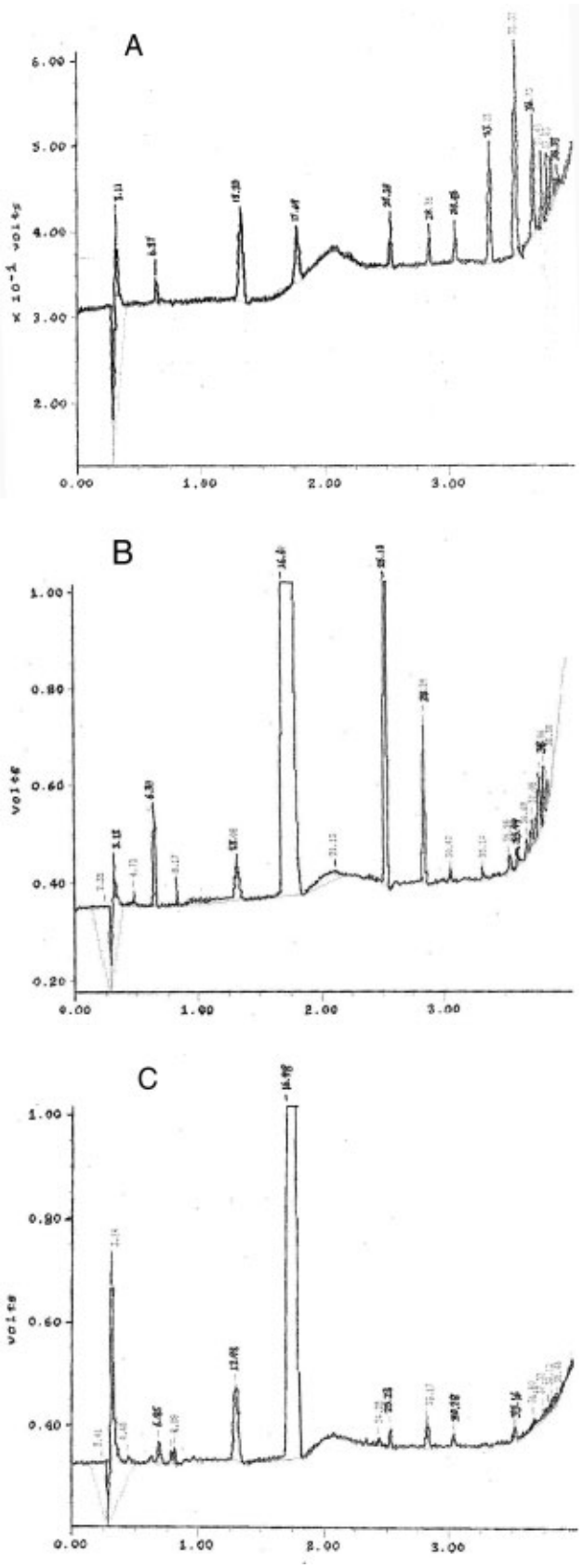

FIG. 4. HPLC profiles of the oligosaccharides and sugars produced by the action of α-amylase (A), diastase (B) and β-amylase (C) on boiled starch in 10 min incubation at 65°C. The X – axis is time (min) × 10. The peaks at 17, 25 and 28 min are maltose, maltotriose and maltopentaose, respectively.

Diastase or diastatic power (DP) measurement, as it is popularly known is very important for assessing the activity of starch-degrading enzymes in malting and brewing industries. However, the lack of a close correlation between diastatic power and α-amylase activity, in contrast to that of β-amylase, is a cause for concern. It appears that our new method may resolve that discrepancy because both α- and β-amylases as well as diastase are measured with the same method. The results in Table I support this contention, as diastase activity seems to be equal to the sum of α- and β-amylase activities, plus the contribution of the other enzymes and the synergistic effects. The results indicate a similar contribution by α- and β-amylases plus a very minor contribution by limit dextrinase, maltase and α-glucosidase and the rest can be attributed to the synergistic effect of α- amylase on β-amylase action.

We present the diastase activity expressed in two different forms or two types of units qualitative and quantitative. First expressed qualitatively in the normal conventional enzyme units and second expressed quantitatively as grams of maltose equivalent, produced in 10 min of mashing of 100 grams of malt (--g maltose equivalent/100 g malt/10 min). The latter is very similar to the popular Windisch-Kolbach and the American Society of Brewing Chemists (ASBC) units. The procedure for the enzyme unit's calculation, described here, allows the inter-conversions of the units for easy comparison and assessment. Furthermore, the use of the two types of units allows assessing, on the one hand, as to how active the enzymes are and how much activity is available and, on the other hand, how much sugar they can produce from starch in a specified period of mashing time.

Choice of 3,5-dinitrosalicylic acid

The 3,5-dinitrosalicylic acid (DNSA) was chosen over the similar reducing agent *p*-hydroxybenzoic acid hydrazide because it was less damaging on starch and produced much less background colour in the blank and controls. This was particularly evident when pullulan was used for limit dextrinase assay. The results in Fig. 5 demonstrate the linearity (r = 0.997) of the reaction between standard maltose and DNSA. Similar linearity curves were also obtained (Fig. 6) with the reducing sugars produced by the action of varying amounts of α- and β-amylases. Furthermore, the summary of the statistical analysis given in Table II shows that the coefficient of variation of means was very low and compared well with the synthetic substrate methods. Moreover, the methods using the natural substrates, are simple, reliable, precise, and most of all are much more economical. There are no special reagents required and no mandatory use of the pre-prepared standard curve is necessary.

Limit dextrinase assay

Limit dextrinase hydrolyses the α-(1-6) glycosidic linkages of the branching points in amylopectin, releasing oligosaccharides or most probably maltotriose from β-limit dextrins. There are some major constraints that hinder the development of specific assay methods for this enzyme. Among these are, lack of proper substrate, possible interference by α- and β-amylases and the coexistence of specific limit dextrinase inhibitors in the malt[8,20,21].

McCleary[10] has developed some methods using modified pullulan, red pullulan, Azurine-Cross-Linked and Limit-DextriZyme. It is given in that form by the supplier, tablets as substrates, the latter being the recommended by the author. All the methods of limit dextrinase assay require prolonged periods (2-16 h) of enzyme extraction from malt[8,10,18,20]. Such long extraction times are incompatible with the purpose of role evaluation of limit dextrinase in starch degradation during industrial mashing, which is less than 2 h. After intensive preliminary investigations, it was found that the assay with pullulan as the substrate and the measurement of reducing sugars released was more sensitive than other methods. Three factors contributed to this development. First, Sephadex G-25 filtration of the malt extract removed glucose and similar sugars. Second, L-cysteine included in the reaction medium was found to stimulate the enzyme and third, the substrate (pullulan) concentration was increased to ensure saturation

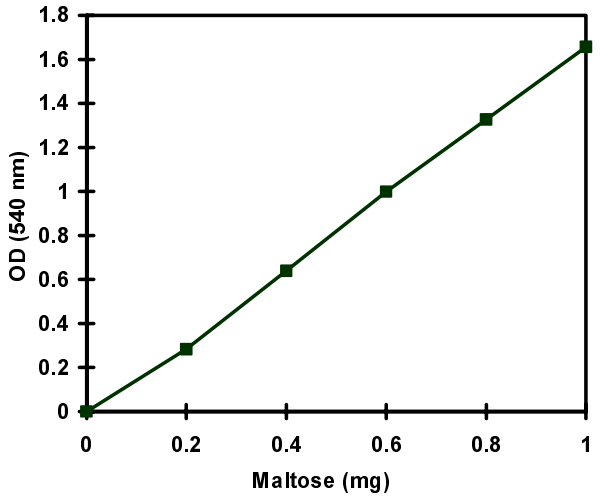

FIG. 5. Standard curve of maltose using 3,5-dinitrosalicylic acid reagent method.

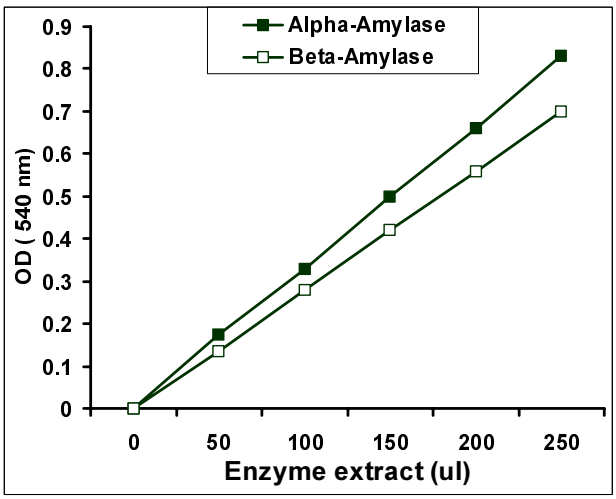

FIG. 6. Linearity of production of reducing sugars from boiled starch by different concentrations of α-amylase (-■-) and β-amylase (-□-) assayed with DNSA.

levels and maximum activity. The mechanism by which L-cysteine promotes limit dextrinase activity (Fig. 7) is not yet clear but it may have something to do with the inhibitors[8,18,21]. The main objection against the use of the reducing sugars to assay limit dextrinase is that β-amylase may further hydrolyse its products. This was eliminated by pre-inactivating β-amylase by heat treatment, as described under the α-amylase assay method.

Assay of α-glucosidases

The assay of α-glucosidases was greatly enhanced and simplified by the inclusion of a Sephadex G-25 filtration step in the extraction and preparation of malt extract[14]. This step secured the prompt and complete removal of glucose from the extract, thus opening the way for the use of natural substrates and the measurement of the glucose released by the sensitive glucose oxidase peroxidase method. Distinction has been made between maltase which specifically acts on maltose and other α-glucosidases which act on oligosaccharides because of their different impacts on other starch-degrading enzymes.

The choice of assay pH

The pH optima for individual enzymes and diastase assayed with natural substrates are shown in Fig. 3A and with synthetic substrates in Fig. 3B. It is interesting to note that the pH optima for α- and β-amylases were at least one point higher with synthetic substrates compared to starch. The optimum pH for diastase (pH 5.0-5.5) occupies a midway point between the pH optima of all individual starch-degrading enzymes. It also coincides with the recommended pH (5.5) of industrial mashing[17]. Therefore, pH 5.5 was chosen as the pH of assay for all enzymes to evaluate their individual and synergistic roles in starch degradation during mashing.

Thermostability and optimal temperatures

The results of thermostability and optimal temperatures of starch-degrading enzymes are shown in Figs 8A and 8B, respectively. Thermostability is an essential requirement of starch degrading enzymes for the industrial use of barley. This is because raw starch is difficult to hydrolyse and its gelatinisation requires temperatures above 60°C. These results indicate and confirm the reports that α-amylase is the most thermostable starch-degrading enzyme and that β-amylase is the least. Their optimal temperature also supported this fact, which is over 60°C for α-amylase and 50°C for β-amylase. Limit dextrinase exhibited a similar thermostability to α-amylase, whereas maltase and α-glucosidase were similar to β-amylase. Understandably, diastase that represents all the enzymes, displayed an intermediate position in both thermostability and optimum temperature. It is interesting to note that maltase and α-glucosidase, though labile and losing more than 80 % of their activity within 20 min of mashing, have an optimal temperature of 60°C. This is most likely due to (a) substrate protection against heat and (b) variation in the media density (i.e. the mashing medium having more solutes than the reaction medium). It is pertinent to add that the methods of enzyme assay used in determining thermostability and optimal temperatures were based on natural substrates, which more closely reflects what happens during mashing.

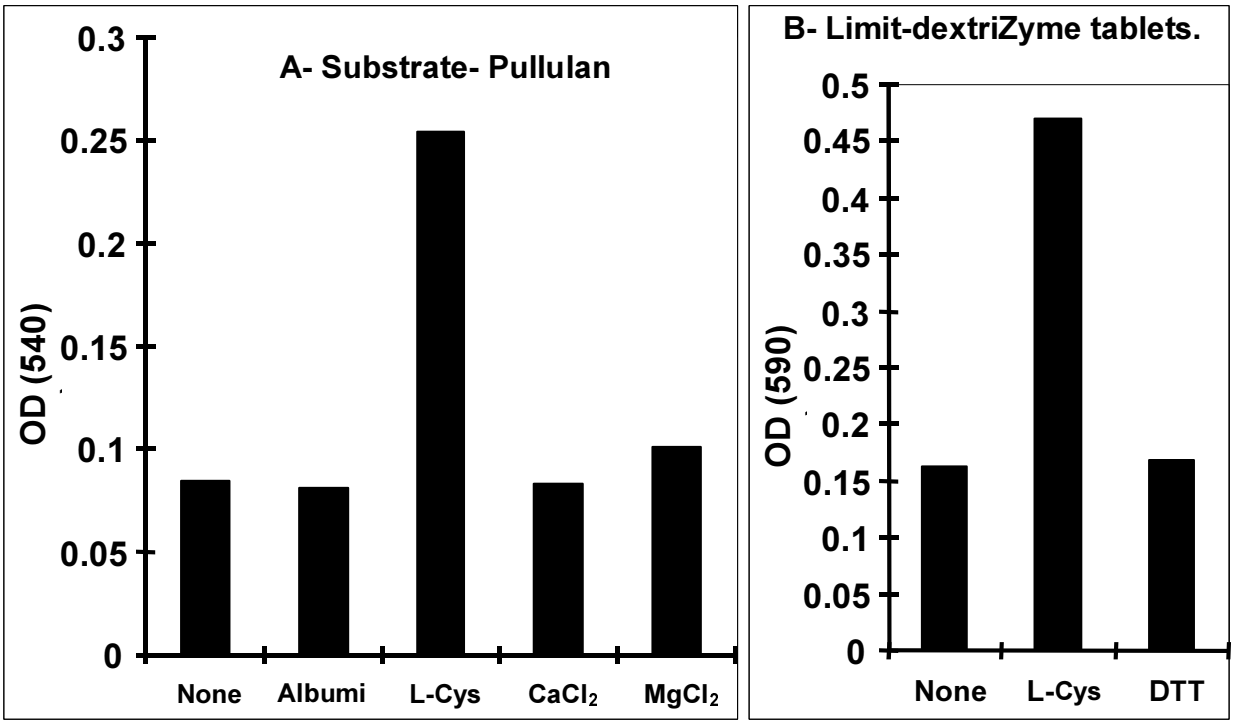

FIG. 7. Effect of adding albumin, L-cysteine, calcium, or magnesium to the reaction medium of limit dextrinase with pullulan (A), and L-cysteine and dithiothreitol (DTT) with limit-dextriZyme as substrate. The enzyme extraction and assay were as described under Materials and Methods.

General discussion

Malt starch-degrading enzymes are very important for the brewing and distilling industries. Malting barley with a high DP is always in great demand especially when mashing for brewing and distilling is carried out with starch containing adjuncts. Today's breeding science is capable of manipulating genes to produce barley with the desirable qualities for end users. However, a lot of details about the desirable qualities are not yet known. For instance, the diastatic power that is generated by the production of reducing sugars by the collective action of the four starch-degrading enzymes. These enzymes act in an orchestrated sequential manner driving the reaction to completion. How do these enzymes behave in a common mixture with the common substrates, do they compete, act synergistically or both? These are some of many questions that need answering, before any quality-defined specific attempts for transforming genes of starch-degrading enzymes can be approached.

To address these issues, we require specific methods of assay of these enzymes in a common extract that resembles and directly relates to their action during mashing. Hence, we evaluated the popular conventional methods and developed new methods using starch as the substrate for α- and β-amylases. Similarly, we developed methods for maltase, α-glucosidase and limit dextrinase using maltose, oligosaccharides and pullulan as substrates, respectively, as discussed above. The modified substrates, being specific for one enzyme, will only exhibit the initial and/or the successive steps of the actions of one enzyme and will not reflect the synergistic competitive phenomena discussed above. The new methods are more reflective, simple, economic and accurate (Table II).

The results indicate that α- and β-amylases are the two dominant enzymes contributing over 99 % of the total diastase activity of the malt. Limit dextrinase has a very low comparative activity. However, this does not diminish the importance of limit dextrinase as the only malt starch-degrading enzyme that hydrolyses the α-(1-6) glycosidic bonds in starch. In addition, its action is assumed to synergistically promote the actions of both α- and β-amylases. α-Glucosidase acts on starch and oligosaccharides to produce glucose from the reducing end, thus competing with β-amylase and inhibiting it. Maltase degrades maltose produced by the action of β-amylase, also releasing glucose. However, the predominant product of the collective action of all enzymes, except maltase, during mashing is maltose. Moreover, for the best uptake of maltose by the brewer's yeast, only a minimal level of glucose is tolerated[19]. Hence, neither α-glucosidase nor maltase is important for brewing and their presence and activities should be kept to the minimum required for germination and yeast growth.

It is evident that α- and β-amylases are the most important malt starch-degrading enzymes for brewing. They produce different products, when acting alone, compared to the products of their combined action (Fig. 4). However, more fermentable sugars were produced by their combined action (Fig. 4B), reflecting some synergistic interaction between these two. This fact was also confirmed by the results in Table I, showing the sum of their individual activities being less than that of the diastase activity.

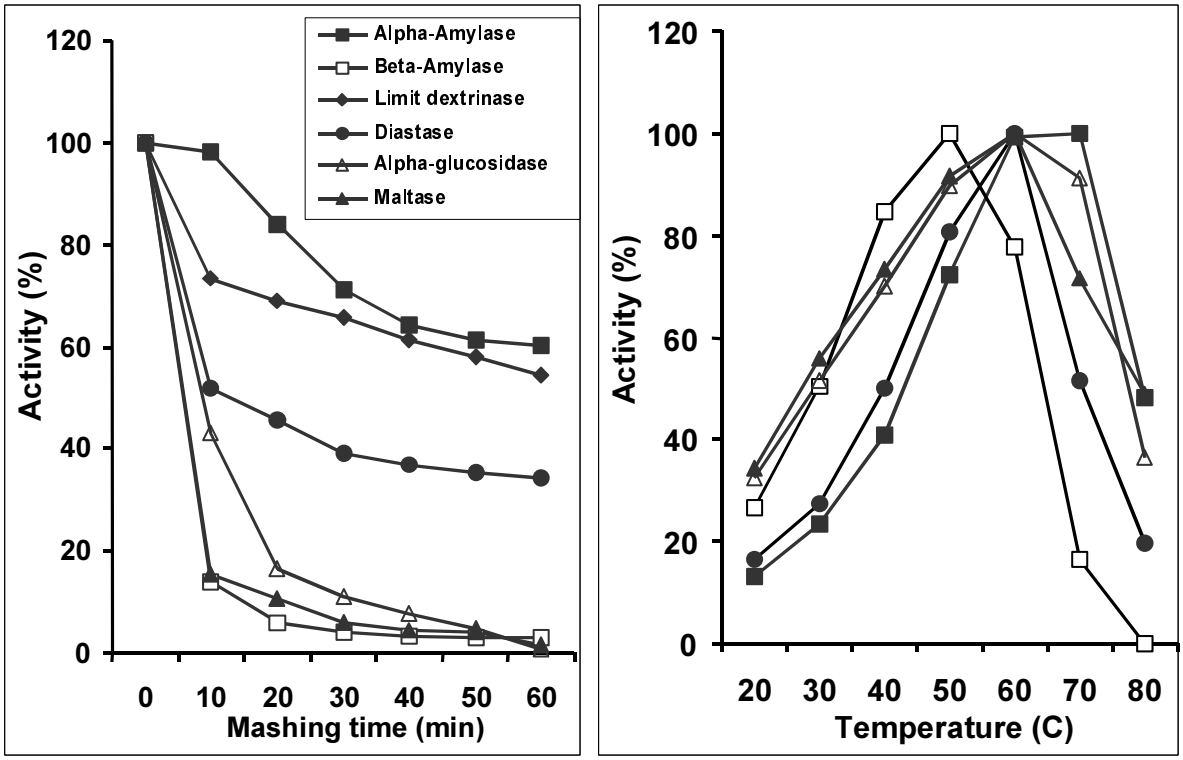

FIG. 8. Thermostability of starch-degrading enzymes measured with natural substrates, under simulated mashing conditions in 1:4 (grist/liquid) ratio (A) and optimal temperature determined with natural substrates and incubated for 5 min (B).

Although fermentable sugars are highly sought, there are some other beer quality attributes served by oligosaccharides, such as mouth feel, colour, foam formation and retention. Variability in the products of these two enzymes indicates that it is possible to manipulate the actions of theses two enzymes to suit the needs of the brewhouse. For instance, if more fermentable sugars are needed then more β-amylase activity and less α-amylase activity should be pursued and *vice versa*. Part of this regulation can be achieved in the brewhouse by adjusting pH, temperature, and the amounts of calcium and, if permissible, EDTA added to acquire the necessary equilibrium. Finally, equilibrium and the balance between these two enzymes is the key in any attempt to achieve the best outcomes from gene manipulation in breeding or the use of enzyme regulators in the brewhouse.

Maltose, produced by the action of β-amylase, is the predominant fermentable sugar of the wort. Most high quality malting barley varieties have a high content of β-amylase, however, its low thermostability is a problem that needs to be considered in gene manipulation. Both α-amylase and limit dextrinase have a synergistic effect on β-amylase action, but they are also competitive enzymes competing for the common substrate. α-Glucosidase is a competitive inhibitor of β-amylase. Hence for the best outcome, preference should be given to the conditions that promote β-amylase action by down-regulating the negative and up-regulating the positive effects of the other enzymes.

The total malt starch-degrading enzyme activity level, as represented by the Franklin barley variety, is high enough to degrade the starch in malt, provided it is free and can be gelatinised without damage to the enzymes. The activities in Table I show that one-gram of malt contains enzyme activity that can degrade 8 g of boiled starch in 10 min at 65ºC. As barley grain contains about 60-70 % starch that means the enzyme activity in one-gram malt is enough to degrade the starch in 12 g or more of malt. In other words, this means that the level of starch-degrading enzymes is not the limiting factor in starch degradation, even in mashing with starch adjuncts. These findings also indicate that it would be possible or even probable to pregelatinise the malt starch to enable the enzymes in smaller amounts of malt to hydrolyse it to fermentable sugars during mashing. Under these mashing conditions temperatures can be down regulated to the optimum temperature of β-amylase (50ºC) to maximise maltose production.

It is clear, that a lot can be achieved by understanding the roles and interaction of these enzymes. The suggestions to improve starch gelatinisation in conjunction with the high malt enzyme potential will greatly increase the mashing capability to produce the desirable fermentable sugars. Consequently, it will be possible to direct further breeding efforts towards improving other quality traits.

CONCLUSION

New methods were developed for the specific assay of starch-degrading enzymes in barley malt. All starch-degrading enzymes can be extracted in water in half an hour, resembling mashing and allowing a better assessment of the contribution of each enzyme in the whole process, in comparison to extracting each individual enzyme in a different medium. α-Amylase and β-amylase were specifically assayed using starch as the substrate and measuring the reducing sugars produced. The measurement of α-amylase and diastase activity (DP) with this new method incorporates β-amylase activity as the difference between diastase and α-amylase. This method is simple, sensitive, more economic and ensures more meaningful interpretation. The specific assays of α-glucosidases, maltase and limit dextrinase were greatly enhanced by Sephadex G-25 filtration of the malt extracts. Limit dextrinase can be specifically assayed with pullulan as substrate in the presence of L-cysteine and measuring the reducing sugars released after heat inactivation of β-amylase. The pH range of 5.0 to 5.5 is the optimum for diastase (DP) assay and coincides with the pH of the commercial mashing.

β-Amylase is the enzyme that produces the most dominant fermentable sugar of the wort, hence, it is the most important enzyme for brewing and the other enzymes complement its action. α-Amylase enhances malt starch degradation during mashing to maltose, by producing oligsaccharides and synergistically increasing the efficiency of β-amylase. The role of limit dextrinase is the hydrolysis of α-(1-6) glycosidic bonds of the branching points that facilitate the action of both α- and β-amylases. α-Glucosidases and maltase are unimportant for brewing because their product, glucose, in high quantity is detrimental to maltose uptake and fermentation by yeast. In addition, α-glucosidases competitively inhibit β-amylase.

The level of starch-degrading enzymes in malting barley varieties is not the limiting factor in starch degradation during mashing. The high malt enzyme potential enables the use of a combination of higher temperatures to gelatinise malt starch and hydrolyse it to fermentable sugars using a small amount of malt and lower mashing temperatures. Further attention and efforts should be directed towards improving starch-composition, accessibility to enzymes and other quality attributes.

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