Application of β-Amylase Thermostability Analysis In A Barley Improvement Program

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Introduction

Australian barley varieties, with a few exceptions, are relatively low in the diastatic power (DP) enzymes which degrade starch into fermentable sugars. In particular, the activity of β-amylase, one of the key enzymes of DP, is limited both by the amount of β-amylase in malt and by its inherent thermolability. In cultivated barley, β-amylase is synthesised during the development of the barley grain. During brewing, extraction of soluble malt components (mashing) is performed either isothermally at approximately 65°C or using a ramped temperature profile from approximately 48°C to 70°C. These temperatures are required for starch gelatinisation, and subsequent degradation. However, β-amylase rapidly loses activity at temperatures above 55°C.

β-amylase exists in three allelic forms in cultivated barley, *Bmy1*-Sd1, -Sd2L and -Sd2H, which exhibit significant differences in thermostability (Eglinton et al. 1998). These workers have also suggested that high thermostability is associated with high malt fermentability (the yield of fermentable sugars produced from the hydrolysis of starch). Fermentability affects the level of alcohol produced and is a critical parameter for brewing. Thus the ability to select for barley β-amylase with enhanced thermostability would be highly desirable.

Current laboratory methods for analysing β-amylase do not give an indication of its thermostability. In this study, an assay recently developed to distinguish between *Bmy1*-Sd1, *Bmy1*-Sd2L and *Bmy1*-Sd2H alleles was assessed for its potential application in a barley improvement program.

Materials and Methods

Rationale for a rapid, small scale β-amylase thermostability assay

The rapid small scale β-amylase thermostability assay used in this study was adapted from the Megazyme Betamyl-based assay described by Eglinton et al. (1998), in which relative β-amylase thermostability was determined using a two point assay where both initial activity and residual activity after 10 min incubation at 60°C were measured. For the method described in the current study, a scaled-down version of the Betamyl assay (Megazyme, Ireland) was tested in conjunction with Beckman blocks (deep well, 1ml, 96 well, polypropylene titre plate, Beckman, USA) for dilution and performing the assay, and ELISA plates for reading absorbance. The results of this trial showed that reproducibility across the microplate was excellent (mean cv. of 2.6%), and that a one point assay, in which only β-amylase activity remaining after 10 min incubation at 60°C was measured, was sufficient to correctly assign breeding lines to one of the three allelic classes.

Samples
Samples of 180 entries from 1998 Stage 3 Waite breeders trials from Clinton in South Australia, and 14 entries from 1998 South Australian Research and Development Institute (SARDI) Stage 4 trials at Mundulla, Lameroo, Mangalo and Brentwood in South Australia, were used in this study.

β-amylase thermostability assay
Extracts were prepared by incubating 100mg samples of barley flour with 1.0mL extraction buffer containing 100mM maleic acid, 1mM disodium EDTA, 0.02% sodium azide, 1mg/mL BSA (Sigma, St Louis, U.S.A.), 0.02% NaN_3 and 143mM 2-mercaptoethanol for 1hr at room temperature with constant mixing. Samples were then incubated at 60°C for 10 min, chilled immediately on ice and centrifuged at 10,000g for 5 min at 4°C. A 1/150 dilution (using extraction buffer minus reducing agent) was performed in a Beckman block. 50μl from each cell was then transferred to a second Beckman block for enzyme assay. Enzyme activity was determined by the addition of 50μl of the substrate *p*-nitrophenyl maltopentaoside (PNPG5, Megazyme). One unit of β-amylase activity is defined as the amount of enzyme required to release 1μmol of *p*-nitrophenol per min in the presence of excess α-glucosidase under the defined assay conditions. After 10 min, the assay was stopped by the addition of 750μl 1%Trisma base. 300μl aliquots of each sample were transferred to an ELISA plate, and absorbance values read at 405nm.

Adjustment for percentage grain protein
Near infrared spectroscopy (NIR) was used to measure grain protein for 180 lines from 1998 season Waite breeders trials. These lines were chosen to represent a wide range of grain protein levels. A correlation between β-amylase activity and grain protein (measured using NIR) was established, and residual β-amylase activity was expressed per milligram of grain protein to correct for variation in initial β-amylase activity between samples.

Results and Discussion

Relationship between β-amylase activity and protein, and its effect on β-amylase thermostability testing.
An excellent regression was obtained between grain protein and β-amylase activity for the 180 lines from 1998 Experiment 1 ($r^2 = 0.92$) (Figure 1). Based on this regression, residual activity was expressed per milligram of grain protein to correct for variation in initial β-amylase activity between samples. This allowed all genotypes tested to be successfully resolved into one of the three allelic forms of β-amylase (Table 1). This genotyping was validated using the original 2 point assay (data not shown), and in addition was found to be consistent with the pedigrees of the entries. Of the 180 entries, 127 were classified as Sd1 (intermediate thermostability), 50 were classified as Sd2L (low thermostability) and 11 as Sd2H (high thermostability). The high representation of the Sd1 allele is due to the large proportion of progeny from the varieties Sloop and Franklin in the South Australian Barley Improvement Program.

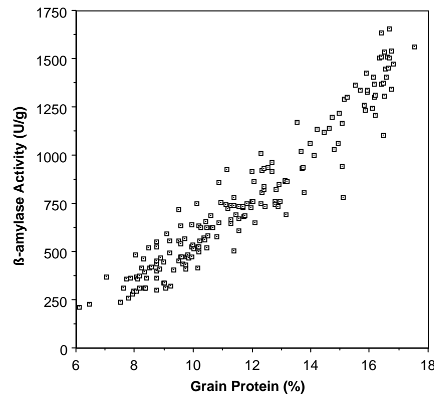

Figure 1: Initial β-amylase activity as a function of grain protein for 1998 Experiment 1 (r^2 = 0.92).

Table 1: Distribution of the three β-amylase alleles (*Bmy*-Sd1, *Bmy*-Sd2L *and Bmy-Sd2H) in 1998 Waite Experiment 1.*

***Bmy*-Sd1**				***Bmy*-Sd2L**		***Bmy*-Sd2H**
88S28-1-1	WI3158	WI3195	WI3140	Barque	WI3044	Arapiles
B%1302	WI3159	WI3196	WI3141	Chebec	WI3050	Sapporo 93013
Fitzgerald	WI3160	WI3197	WI3142	Dash	WI2976	Sapporo 93110/8
Franklin	WI3161	WI3198	WI3143	CMOKoru123	WI2978	Sapporo 93113/15
Gairdner	WI3162	WI3199	WI3144	Fr*Che/1016	WI3102	Sapporo 95203
Jubilant	WI3163	WI3200	WI3145	Galleon	WI3184	Sapporo 95274
Molloy	WI3164	WI3203	WI3146	Mundah	WI3184	Unicorn
Monarch	WI3165	WI3204	WI3147	Schooner	WI3185	VB9536
Profit	WI3166	WI3205	WI3181	Skiff	WI3185	VB9720
Sapporo	WI3167	WI3206	WI3182	Tantangara	WI3187	WI3188
95223	WI3168	WI3207	WI3183	VB9613	WI3187	WI3245
Sloop	WI3169	WI3210	WI3186	VB9614	WI3189	
Tilga	WI3170	WI3211	WI3190	VB9726	WI3189	
VB9527	WI3171	WI3212	WI3191	VB9728	WI3193	
VB9610	WI3172	WI3213	WI3236	VB9729	WI3201	
VB9615	WI3173	WI3214	WI3237	WA0563	WI3202	
VB9623	WI3174	WI3215	WI3238	WB146-14	WI3208	
VB9703	WI3175	WI3216	WI3239	WB213	WI3217	
VB9710	WI3176	WI3218	WI3240	WB217	WI3222	
VB9725	WI3177	WI3219	WI3241	WB219	WI3229	
VB9727	WI3178	WI3220	WI3242	WB223	WI3230	
Venture	WI3179	WI3221	WI3244	WB227	WI3235	
Vic 9524	WI3180	WI3225	WI3246	WB228	WI3243	
WB220	WI3181	WI3226	WI3247	WI2986	WI3254	
WB226	WI3182	WI3227	WI3248	WI3084		
WB229	WI3183	WI3228	WI3249	Wyalong		
WB230	WI3186	WI3157	WI3250			
WI-3071	WI3190	WI3194	WI3251			
WI-3072	WI3191	WI3231	WI3252			
WI-3073	WI3192	WI3232	WI3253			
WI-3076	WI3223	WI3233	WI3255			
WI-3099	WI3224	WI3234	WI3139			

Thermostability testing of 1998 SARDI Stage 4 Trial Entries

The results from the thermostability testing of the 1998 SARDI Stage 4 trials showed that the assay clearly resolved the entries into the *Bmy*-Sd1, -Sd2L and -Sd2H alleles of β-amylase (Figure 2) across the 4 sites tested (individual site data not shown). Of the 14 entries, 7 were classified as Sd1, including the malting varieties Franklin, Sloop and Gairdner, and the three Sloop CCN backcrosses, WI 3140, WI 3141 and WI 3148. Four entries were classified as Sd2L, including the feed variety Barque and the hulless breeding line WI 3107. Three entries were classified as Sd2H, including the malting variety Arapiles, the Sapporo breeding line SA93013, and the hulless variety Namoi.

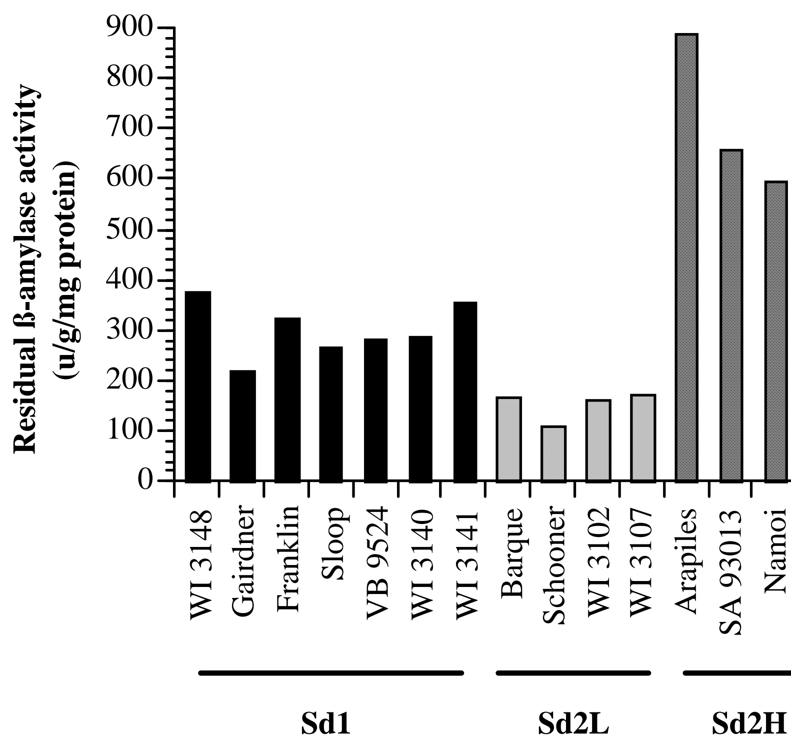

Figure 2: Residual β-amylase activity in barley extracts after incubation at 60°C for 10 min, for 1998 SARDI Stage 4 entries averaged over 4 sites (Mundulla, Lameroo, Mangalo and Brentwood).

Strategies for Implementation in Barley Improvement Programs

The results from this study indicate that the β-amylase thermostability assay can be successfully used to discriminate between the three β-amylase alleles, *Bmy*-Sd1, *Bmy*-Sd2L and *Bmy*-Sd2H.

Eglinton *et. al*. (1998) investigated the relationship between thermostability and wort fermentability in 42 commercial malt samples. Their results showed a strong positive correlation between diastatic power (DP) and fermentability (apparent attenuation limit [AAL]). However, the malts with the high thermostability *Bmy*-Sd2H allele did not strictly follow this relationship, generating high AAL from relatively low DP. These workers suggested that an increase in thermostability may result in more efficient starch degradation, compared with Sd1 and Sd2L malts with similar levels of DP. This relationship has also been reported in a survey of Japanese breeding lines Kihara et. al. (1997).

The implication of these findings is that the β-amylase thermostability assay may offer an alternative selection method for breeding barley with higher levels of fermentability. Current laboratory methods for determining wort fermentability are based on labour intensive assays with very small sample throughtput (e.g. AAL assay), and thus, only lines in the most advanced stages of quality evaluation can be tested. The thermostability assay requires only a small sample and there is no requirement for either micromalting or mashing. In addition, large numbers of samples can be handled (in the order of 100/day including duplicates) at relatively low cost (cost of materials in the order of $0.33 per sample), making it amenable to early generation screening.

The *Bmy*1-Sd2L allele confers low fermentability, and is also associated with low ß-amylase activity (Coventry et al. 1999). The use of the ß-amylase thermostability assay as an early generation selection tool to cull the *Bmy*1-Sd2L allele would apply strong selection pressure for high DP and high fermentability. These characteristics are highly desirable for the bulk of Australia's export markets that use starch based adjuncts in brewing.

However some members of the domestic brewing industry currently prefer the low DP and fermentability conferred by the *Bmy*1-Sd2L allele. In order to produce varieties suitable for domestic brewing, the assay can be used as a late generation informative tool. This would facilitate the division of germplasm suitable for either domestic or export markets. The strategy used for implementing β-amylase thermostability screening is therefore dependent upon the objectives of the barley breeding program.

Acknowledgments

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