

# The (1-3,1-4)- $\beta$ -Glucanases in Malting Barley: Enzyme Survival and Genetic and Environmental Effects

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## ABSTRACT

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Eighteen barley genotypes used in Brazilian malting barley breeding programs were characterized in relation to (1-3,1-4)- $\beta$ -glucanase activity in green and kilned malt. They were tested to determine the loss of enzyme activity during kilning in the malting process and the environmental effects on enzyme activity were measured. The genotypes analyzed showed great variation regarding the enzyme activity in both kinds of malt, in a range from 531.94 to 934.31 U/kg in green malt, and from 187.02 to 518.40 U/kg in dry malt. The mean enzyme activity loss during kilning was close to 60%, very similar to the results obtained in other studies. The loss among genotypes varied from 8.04% to 71.54%. The enzyme activity varied significantly under the different environments tested, showing existence of environmental effects on the genotypes analyzed. Embrapa 127 was the genotype that exhibited the highest enzyme activity in finished malt although it had shown a low activity in green malt, reflecting a negligible loss of activity during kilning. The data indicate promising results to malting barley breeding due to the wide variability exhibited by genotypes as to enzyme activity and levels of isoenzyme with high thermostability.

**Key words:** Barley, (1-3,1-4)- $\beta$ -glucanases, environmental, enzyme activity, kilning, malting.

## INTRODUCTION

During the germination of barley (*Hordeum vulgare* ssp. *vulgare* L.) grains, hydrolytic enzymes secreted from the aleurone layer and scutellum depolymerise the starch and the reserve proteins stored in the cells of the starchy

endosperm. An important preliminary event in this process is the removal of the walls of the endosperm cells because they act as a physical barrier that restricts access of hydrolytic enzymes to their substrates within the cell. Cell walls of the starchy endosperm of barley consist of approximately 70% (1-3,1-4)- $\beta$ -D-glucans and 20% arabinoxylan, together with smaller amounts of mannose-containing polysaccharides and cellulose<sup>4</sup>.

During the first stage in the cell wall breakdown,  $\beta$ -glucans are brought into solution. As revised by Palmer and Agu<sup>19</sup>, in general, it has been proposed that enzymes such as (1,3)-endo- $\beta$ -glucanases, carboxypeptidases, phospholipases,  $\beta$ -glucan solubilases, or (1-4)-endo- $\beta$ -glucanases may be involved in the release of  $\beta$ -glucan into solution. Although the principles of this release are not understood, it is accepted that the  $\beta$ -glucans so dissolved are then attacked by the (1-3,1-4)- $\beta$ -glucan endohydrolases (EC 3.2.1.73), also named (1-3,1-4)- $\beta$ -glucanases.

Two isoenzymes of (1-3,1-4)- $\beta$ -glucanases, EI and EII, have been purified from extracts of germinating barley. These isoenzymes exhibit almost identical substrate specificities and kinetic properties<sup>27</sup>, and act specifically on  $\beta$ -(1,4) linkages but only if there is an adjacent (1,3)- $\beta$ -glucosyl residue toward the non-reducing end of the substrate<sup>27</sup>. However, EI and EII can be readily distinguished by their apparent molecular weight, isoelectric point, carbohydrate content, and thermal stability. Enzyme I, codified by the gene *Glb1*<sup>3</sup>, has  $M_r$  28000, an isoelectric point of 8.5, and contains approximately 0.7% associated carbohydrate<sup>26</sup>. Enzyme II, codified by the gene *Glb2*<sup>21</sup>, has  $M_r$  33000, an isoelectric point > 10, and contains approximately 4% carbohydrate<sup>26</sup>. Isoenzyme EI is far less stable at elevated temperatures than the more heavily glycosylated isoenzyme EII. It has been suggested that the differences in carbohydrate content of the isoenzymes are responsible for dramatic differences in their respective thermostabilities<sup>12</sup>.

Based on the high degree of amino acid sequence homology (approximately 90%), Woodward et al.<sup>28</sup> suggested that the enzymes are derived from different genes, which originated by duplication of a common ancestral gene. Molecular analyses have shown that the isoenzyme EI gene is located on barley chromosome 5, while the isoenzyme EII gene is carried by chromosome 1<sup>13,21</sup>. Litts et al.<sup>11</sup> suggested the existence of several other sequences related to the gene family encoding (1-3,1-4)- $\beta$ -glucanase

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enzymes in barley that could be identified by Southern blot analyses, apart from genes for isoenzymes EI and EII. Genes encoding two barley (1-3,1-4)- $\beta$ -glucanase isoenzymes have been cloned and sequenced<sup>11,21,25</sup>.

Endosperm cell wall degradation during germination is considered important in malt production for the brewing industry, where barley is germinated under conditions designed to maximize endosperm modifications while limiting seedling growth<sup>24</sup>. Insufficient degradation of cell walls reduces malt extract because it limits the diffusion of germination enzymes and the mobilization of kernel reserves. Moreover, the presence of residual  $\beta$ -glucans may lead to highly viscous wort and beer, giving rise to difficulties in filtration in the brewery. Residual  $\beta$ -glucans may also play a role in beer maturation, promoting the formation of undesirable precipitate and hazes<sup>16</sup>. Problems associated with residual  $\beta$ -glucans depend not only on initial  $\beta$ -glucan levels but also on the amount of (1-3,1-4)- $\beta$ -glucanases produced during malting. Consequently, a major aim of most barley breeding programs is the identification and development of varieties that produce and maintain high levels of the (1-3,1-4)- $\beta$ -glucanases.

Since (1) the (1-3,1-4)- $\beta$ -glucanases play a key role in the depolymerization of barley  $\beta$ -glucans during malting, (2) the activity of these enzymes is partially lost during kilning in the malting process, and (3) environmental conditions affect their activity, 18 barley genotypes used in Brazilian breeding programs of malting barley were characterized in relation to (1-3,1-4)- $\beta$ -glucanase activity in green and kilned malt, tested to determine the loss of enzyme activity during kilning in the malting process, and environmental effects on the activity of these enzymes were measured.

## MATERIALS AND METHODS

### Plant material

Seed samples of 18 barley genotypes, comprising commercial cultivars and experimental lines ( $F_6$  generation), used in Brazilian malting barley breeding programs (Table I) were supplied by AmBev Brewery (Brazil). Each geno-

**Table I.** Barley genotypes (cultivars and  $F_6$  lines) analyzed with data of their genealogies.

Genotypes	Genealogies
<b>Cultivars</b>	
BR 2	FM 424/NORBERT
Robust	Morex/Manker
MN 698	MN 599/MN 635
Simona	—
Morex	CREE/Bonanza
Embrapa 127	Alexis/BR 2
Menuet	L 92/Minerva/Emir/3/Zephyr
<b><math>F_6</math> lines</b>	
CEV 97004	—
CEV 96025	Stirling/MN 599
CEV 97068	Embrapa 43/PFC 9114
CEV 97047	(Alexis/Embrapa 43)/BRS Borema n° 9089 Uruguay 1980
Ana/B-99-90	Antártica 5/(FM 437/Villa);
MN 662/Triumph	Hadmersieben24566//Diamant/ ST140264/3/ALSA/ABYSSINIAN// UNION
MN 677/Alexis	Antártica 5/(FM 424/FM429); Proctor/Carlsberg II/Heine 480/St. vLP
BR 2 <sup>x2</sup> /MN 610	FM 424/NORBERT; FM 434/Duvekot
BR 2/MN 682	FM 424/NORBERT; MN 635/MN 599
MN 691/Guimpel	(Bonita/MN 377)/MN 599; Gerlinde/Karat
MN 657/Guimpel	FM 519/Antártica 5; Gerlinde/Karat

type was grown at Encruzilhada do Sul, Brazil, in 1999, in blocks without replications. Two genotypes with contrasting levels of (1-3,1-4)- $\beta$ -glucanase activity in green malt and two others in dry malt were grown, in 2002, in three sites in South Brazil. In two locations (Passo Fundo and Victor Graeff, RS) the plants were arranged in a randomized block design with two replications and an unreplicated randomized block in the third location (Guarapuava, PR). Data regarding these sites are shown in Table II.

### Malting procedure

Barley grain samples of 18 genotypes were harvested in 1999 and an amount equivalent to 340 g dry weight per genotype was malted in a micromalting unit at AmBev Brewery, Navegantes Malting Subsidiary. Extraneous matter and small grains (< 2.2 mm) were removed. The malting conditions, allowing an average degree of steeping

**Table II.** Soil type, average monthly temperatures ( $T$  °C) and rainfall, latitude, longitude and altitude of each site where seeds were harvested in 1999 (Encruzilhada do Sul) and 2002 (Passo Fundo, Victor Graeff, Guarapuava).

Site	Soil	Climate	Growing season							
			May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Encruzilhada do Sul Rio Grande do Sul 30°33'S; 52°33'W Altitude 432 m	Typical dystrophic red yellow clay "Camaquã" group	$T_{min}$	10.5	8.7	8.7	9.0	10.8	12.0	13.5	16.3
		$T_{max}$	18.6	16.6	16.1	19.2	20.4	21.0	25.1	27.4
		Rainfall	211.7	144.4	126.9	53.3	112.4	137.1	41.3	134.2
Passo Fundo Rio Grande do Sul 28°15'S; 52°24'W Altitude 687 m	Typical dystrophic red Latsol	$T_{min}$	12.9	9.3	8.6	11.3	9.1	14.8	15.1	16.8
		$T_{max}$	22.4	18.6	18.2	20.4	20.0	24.1	26.5	27.3
		Rainfall	192.4	241.9	146.3	233.8	253.6	372.3	205.0	329.5
Victor Graeff Rio Grande do Sul 28°55'S; 52°74'W Altitude 455 m	Dystrophic with clay deep dark red Latsol	$T_{min}$	12.9	9.3	8.6	11.3	9.1	14.8	15.1	16.8
		$T_{max}$	22.4	18.6	18.2	20.4	20.0	24.1	26.5	27.3
		Rainfall	192.4	241.9	146.3	233.8	253.6	372.3	205.0	329.5
Guarapuava Paraná 25°23'S; 51°27'W Altitude 1.100 m	Typical Bruno Latsol with aluminum	$T_{min}$	12.1	11.7	8.0	10.9	8.8	14.9	14.2	15.9
		$T_{max}$	21.1	21.8	19.1	21.4	21.2	25.2	25.3	26.3
		Rainfall	245.4	22.8	75.2	144.2	194.4	197.4	239.6	157.4

**Table III.** The malting process used.

	Step	Time	Temperature
Steeping	Steep 1	4 h	
	Air step 1	5 h	
	Steep 2	2 h	19°C ± 1°C
	Air step 2	5 h	
	Steep 3	2 h	
Germination	Air step 3	96 h	16–18°C
Kilning	Kiln 1	20 min	Room T to 55°C
	Kiln 2	5 h	55°C
	Kiln 3	5 h	55°C to 65°C
	Kiln 4	5 h	65°C
	Kiln 5	3 h	65°C to 82°C
	Kiln 6	3 h	82°C

of 44%, are given in Table III. Green malt samples were taken just before raising the temperature for kilning. The grains were frozen by contact with dry ice and freeze-dried. The remaining material gave finished malt after kilning. The malt, green and kilned, was ground with a roller mill (Buhler-Miag, Braunschweig, West Germany) with a roller separation of 0.2 mm. For the four genotypes grown and harvested in 2002, samples of malt were developed separately, one for each replication per site, in the same conditions described above.

### β-Glucanase activity assay

β-Glucanase activity in green malt (before kilning) and finished malt (after kilning) was measured according to McCleary and Shameer<sup>15</sup> with the commercial kit of 'Azo-barley glucan method' (Megazyme Ltd., Ireland), but using half of the recommended quantity for the flour and all reagents of the kit and following the protocol of Zwickert-Menteur et al.<sup>30</sup> Preliminary tests indicated no differences between the recommendation of the manufacturer and this condition. For green malt, a further dilution was made due to higher levels of β-glucanase activity when the kilning step was omitted. The two replications of material from 2002, which were malted independently,

were analyzed for β-glucanase activity separately. The β-glucanase activity was expressed in U/kg of dry malt, where U is the International Unit of enzyme activity. One unit (U) of activity is the amount of enzyme that releases one micromole of reducing sugar equivalent per minute under defined conditions of temperature and pH (30°C and pH 4.6 in this assay). All assays were performed in triplicate or, in few cases, in duplicate, and the mean activity was calculated.

### Statistical analysis

The data were analyzed by One Way ANOVA using the SAS program (SAS Institute).

## RESULTS

### Genetic effect

Levels of (1-3,1-4)-β-glucanase activity analyzed in the green and finished malt from the 18 genotypes grown in Encruzilhada do Sul, in 1999, are shown in Table IV. The varieties analyzed showed great variation with regard to the enzyme activity in both kinds of malt. For green malt, the highest value of activity was 934.31 U/kg observed in the line obtained by the cross BR 2<sup>x2</sup>/MN 610, while the lowest value was 531.94 U/kg in line CEV 97047. For dry malt, values ranged from 187.02 U/kg to 518.40 U/kg observed in the line CEV 96025 and in the cultivar Embrapa 127, respectively.

### Survival of (1-3,1-4)-β-glucanases during kilning

Although (1-3,1-4)-β-glucanase activity decreased during drying, part of the initial activity in the green malt (mean of 41.38%) was retained in the finished malt (Table IV).

The varieties also showed a wide range of (1-3,1-4)-β-glucanase activity survival during kilning (Table IV). Thus, the variety that exhibited the highest activity level in green malt was not the same that had the highest level

**Table IV.** The (1-3,1-4)-β-glucanase activity before and after the kilning process of genotypes grown at Encruzilhada do Sul in 1999. U equals one micromole of glucose reducing sugar equivalent released per minute at 30°C and pH 4.6. Mean activities were obtained from triplicate or, in few cases, from duplicate assays.

Genotypes	Mean (1-3,1-4)-β-glucanase activity and limit values		
	Green malt (U/kg)	Dry malt (U/kg)	Loss (%)
CEV 97004	803.43 (557.77–1049.08)	308.29 (293.80–322.78)	61.62
ANA/B-99-90	764.25 (733.75–815.23)	292.96 (276.16–307.66)	61.67
CEV 96025	559.66 (548.95–570.35)	187.02 (174.73–199.30)	66.58
MN 662/Triumph	748.37 (643.50–827.76)	235.84 (228.28–249.70)	68.49
MN 677/Alexis	820.66 (793.92–860.36)	284.56 (273.01–296.32)	65.33
BR 2	651.94 (553.99–749.89)	366.41 (273.01–503.59)	43.80
Robust	746.62 (712.92–780.32)	268.60 (259.15–278.05)	64.02
MN 698	817.73 (726.24–909.22)	398.38 (376.33–420.43)	51.28
BR 2 <sup>x2</sup> /MN 610	934.31 (825.26–1043.37)	329.29 (318.37–347.35)	64.76
Simona	710.77 (664.81–782.64)	314.80 (303.25–320.89)	55.71
BR 2/MN 682	818.15 (780.13–862.86)	317.11 (301.99–331.60)	61.24
Morex	899.00 (809.47–988.53)	266.71 (247.18–286.24)	70.33
MN 691/Guimpel	777.63 (753.81–790.16)	221.35 (205.60–237.10)	71.54
Embrapa 127	563.70 (506.74–614.23)	518.40 (447.52–560.29)	8.04
CEV 97068	725.37 (718.13–732.60)	262.93 (248.44–277.42)	63.75
MN 657/Guimpel	864.95 (756.31–988.22)	356.17 (329.71–405.31)	58.82
CEV 97047	531.94 (521.86–542.02)	244.98 (236.47–253.48)	53.95
Menuet	750.04 (698.65–798.93)	267.97 (254.74–264.82)	64.27
Mean	749.36	302.32	58.62

**Table V.** Variance analysis of (1-3,1-4)- $\beta$ -glucanase activity in green and kilned malts in relation to the four sites where the plants were grown: GUA (Guarapuava, 2002), PF (Passo Fundo, 2002), VG (Victor Graeff, 2002), and ENC (Encruzilhada do Sul, 1999).

Malt type	Genotype	GUA	PF	VG	ENC	Genotype mean
Green	MN-698	1222.7	886.9	1014.9	817.7	985.6 <sup>a</sup>
	CEV-97047	882.4	741.5	741.4	531.9	724.3 <sup>b</sup>
	Local mean	1052.6 <sup>A</sup>	814.2 <sup>B</sup>	878.2 <sup>B</sup>	674.8 <sup>C</sup>	
Kilned	Embrapa-127	1064.1	779.1	694.4	518.4	720.9 <sup>a</sup>
	CEV-96025	485.6	402.5	492.2	187.1	410.1 <sup>b</sup>
	Local mean	773.9 <sup>A</sup>	590.7 <sup>B</sup>	593.3 <sup>B</sup>	352.7 <sup>C</sup>	

Means followed by the same letter are not significantly different by Tukey test at  $\alpha = 0.05$ .

in the dry malt. The highest loss of enzyme activity due to drying was 71.54% observed in the line derived from cross MN 691/Guimpel. The lowest loss of activity was 8.04%, in the cultivar Embrapa 127.

### Environmental effects

Levels of (1-3,1-4)- $\beta$ -glucanases generated during micromalting were tested for the four varieties grown in 2002 in three different sites to verify the environmental effects on the activity of these enzymes. For the varieties analyzed in relation to green malt, the activity levels were highest in Guarapuava, PR, in relation to Passo Fundo and Victor Graeff, RS. These differences were statistically significant (Table V). For kilned malt, the results were less clear because each variety generated higher values in different sites: line CEV 96025 in Victor Graeff, RS, and Embrapa 127 in Guarapuava, but the local mean too showed activity levels significantly higher in Guarapuava than in the other two places (Table V).

When Encruzilhada do Sul, where the plants were cultivated in 1999, is compared to the other sites, the activity levels observed in that local were significantly lower than the levels obtained for the other three sites analyzed in 2002 (Table V). In fact Encruzilhada do Sul presents characteristics of soil type and average monthly temperatures (T °C) and rainfall very different from the other three locations analyzed (Table II), which could explain the differences observed in the enzyme activity, although the year effect cannot be discarded. As the plants were not grown in Encruzilhada do Sul in 2002, it was not possible to verify whether these differences were due to local differences or year effect.

## DISCUSSION

The mean loss of 60% of (1-3,1-4)- $\beta$ -glucanase activity observed in this study is similar to the results obtained by others authors that used temperatures between 45–65°C at the beginning of the kilning step<sup>1,30</sup>. Changes in the kilning temperatures and/or duration alter these results. Brunswick et al.<sup>3</sup> kilned the malt at 60°C for 16 h, with resulting losses from 80 to 90% in (1-3,1-4)- $\beta$ -glucanase activity. Barber et al.<sup>2</sup>, using a malting schedule with a more progressive temperature increase (from 30 to 65°C), observed a better survival of the (1-3,1-4)- $\beta$ -glucanase activity with losses from 16 to 26%.

The difference in the loss of enzyme activity between genotypes reflects dissimilarities in the content of the two kinds of isoenzymes of (1-3,1-4)- $\beta$ -glucanases since one

is more thermolabile than the other. Loi et al.<sup>12</sup> showed that the activity in finished malt is attributable entirely to isoenzyme II and that isoenzyme I disappears totally during initial drying at 60 to 65°C. Similar results were reported by other authors<sup>1,3,6</sup>.

Several reports on the (1-3,1-4)- $\beta$ -glucanase activity during germination have been published. These studies have shown that the initial activity of these enzymes is very low, but increases rapidly from the 2nd to the 4th day of the malting process. Nevertheless, the maximum values of enzyme activity ranged from the 4th, or 5th and 6th days, or even to the 11th day, in different experiments<sup>1,3,14,20</sup>. McFadden et al.<sup>17</sup> verified that no mRNA encoding (1-3,1-4)- $\beta$ -glucanase is detected in ungerminated grain. Expression of these genes is first detected in the scutellum after one day and only at the epithelial layer. At this stage, no expression is apparent in the aleurone. After the second day, levels of mRNA decrease in the scutellar epithelium but increase in the aleurone.

The role of each (1-3,1-4)- $\beta$ -glucanase isoenzyme and their tissular and temporal control on germination and malting quality have also been investigated. Studies have shown that the isoenzymes are differentially synthesized by action from gibberellic acid and auxins<sup>22,23</sup>. Isoenzyme EI is synthesized predominantly in the scutellum, while isoenzyme EII exclusively in the aleurone<sup>18,23</sup>. Høj et al.<sup>7</sup> verified that isoenzyme EI consistently appears somewhat earlier than isoenzyme EII. The mRNA for isoenzyme EII accumulates preferentially (70%) when compared to the mRNA for isoenzyme EI (30%) in the three days following imbibition<sup>11</sup>, but isoenzyme EI was the major component (63 to 82% of the total activity) after steeping and 48 h of germination<sup>6</sup>. Thus, as mentioned by Henry<sup>6</sup>, isoenzyme EI is quantitatively important in the early stages of malt but isoenzyme EII is equally important in determining (1-3,1-4)- $\beta$ -glucanase levels in malt due to its greater heat resistance, being therefore the only isoenzyme detectable in kilned malt.

In the present analyses, the genotype that exhibited the highest enzyme activity in finished malt was Embrapa 127, which, nevertheless, had low activity in green malt. The negligible loss of activity during kilning was the reason for the high final enzyme activity in this cultivar, and this fact must be due to differences in the proportion of isoenzymes EI and EII<sup>30</sup>. Since isoenzyme EI is the predominant form in the early stages of malting, and as it is a thermolabile molecule, cultivar Embrapa 127 may be considered to have very little expression of this isoenzyme. Consequently, very little activity was lost during kilning

and the activity detected in green and finished malt is very similar because it is ascribed mainly to isoenzyme EII.

Although low levels of (1-3,1-4)- $\beta$ -glucanase during germination can lead to a lower rate of grain modification at the end of the malting process, this fact does not affect the malting performance of Embrapa 127, which is considered a top malting quality cultivar. Certainly, the high enzyme activity that remained after kilning was enough to ensure an effective role of these enzymes in the final degradation of the  $\beta$ -glucans in the wort.

Like other malt quality characteristics, the (1-3,1-4)- $\beta$ -glucanase present in malted barley depends on both varietal and environmental effects. Stuart et al.<sup>24</sup> reported that the activity of  $\beta$ -glucanases was influenced more strongly by environmental than the genotype conditions and cite other authors who suggest that environmental influence is very important, and this results in a lower heritability for (1-3,1-4)- $\beta$ -glucanase activity when compared with other malt characteristics.

The present study confirms these previous results because statistically significant differences were also found among different environments. Guarapuava differed from Passo Fundo and Victor Graeff, but these two last sites were similar. In fact, the environmental conditions of Passo Fundo and Victor Graeff were acceptably similar in the year 2002, differing only in the soil type. The environmental influence can also be verified when the results are compared with previously published data. An example of this is Morex, a North American malting cultivar. Zwickert-Mentour et al.<sup>30</sup> observed an enzyme activity of 480.80 U/kg in finished malt, while in the growing conditions of Encruzilhada do Sul, Morex exhibited an enzyme activity of only 266.71 U/kg. It is also important to emphasize that cultivar Embrapa 127 showed an enzyme activity of 518.40 U/kg, a value two times superior to that of Morex, which is considered a good malting type barley, in the same environment conditions of Encruzilhada do Sul.

Alternative strategies to increase the thermostability of the (1-3,1-4)- $\beta$ -glucanases during malting are being developed to improve malt quality. Transgenic barley plants that express a transgene encoding heat stable (1-3,1-4)- $\beta$ -glucanase are being produced<sup>8,10</sup> and the use of commercial enzymes with high thermostability during malting is being considered, but these procedures can be very expensive and not practical<sup>5,9</sup>. Moreover, the results obtained in this and in other studies indicate a wide variability in barley concerning activity levels of this enzyme and enzyme survival during kilning, which suggests this variability should be explored before attempting more sophisticated technologies. The most important aspect may be that, as already cited by Zhang et al.<sup>29</sup>, each barley breeding program should contemplate estimates about the ability and stability of malting barley grain to synthesize (1-3,1-4)- $\beta$ -glucanases rapidly under a wide range of environmental conditions, side by side with a cultivar fit to particular locations.

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