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In Vivo Modeling of β-Glucan Degradation in Contrasting Barley (*Hordeum vulgare* L.) Genotypes

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An important determinative of malt quality is the malt β-glucan content, which in turn depends on the initial barley β-glucan content as well as the β-glucan depolymerization by β-glucanase (EC 3.2.1.73) during malting. Another enzyme, named β-glucan solubilase, has been suggested to act prior to β-glucanase; its existence, however, has not been unequivocally proven. We monitored changes in β-glucan levels and in the development of β-glucan-degrading enzymes during malting of five lots of contrasting barley genotypes. Two models of in vivo kinetics for β-glucan degradation were then compared as follows: (i) a biphasic model based on the sequential action of β-glucan solubilase and β-glucanase and (ii) a monophasic model assuming that all β-glucans are depolymerized by β-glucanase without the previous intervention of another enzyme. Confirmatory regression analysis was used to test the fit of the models to the observed data. Our results show that β-glucan degradation is mostly monophasic, although some enzyme other than β-glucanase seems to be required for the early solubilization of a small fraction of insoluble β-glucans (on average, 7% of total β-glucans). Furthermore, the genotype-dependent kinetic rate constant (indicating β-glucan degradability), in addition to β-glucanase activity, is suggested to play a major role in malting quality.

KEYWORDS: Barley (*Hordeum vulgare* L.); malting quality; β-glucan solubilase; β-glucan degradability; in vivo kinetics

INTRODUCTION

Highly polymeric β-glucans make up 70% of the cell wall of the amylaceous barley endosperm (*1*). During malting, β-glucans have to be depolymerized to allow storage proteins and starch to become, in turn, fully accessible to hydrolyzing enzymes (*2*, *3*). An extensive modification of the native physical−chemical endosperm structure is therefore required to produce a good malt (*1*, *4*). Since the pioneer work of Preece and Ashworth in the 1950s (*5*), it has been suggested that, during malting, the breakdown of barley endosperm cell walls polysaccharides can be described in terms of two successive steps: solubilization and splitting into small molecules. A number of well-characterized endo- and exohydrolases act concurrently in the latter step, but the major depolymerizing activity is due to two isozymes of the barley (1→3),(1→4)-β-D-glucan endohydrolase (EC 3.2.1.73) (*1*, *6*, *7*), commonly known as β-glucanase. On the contrary, the existence of a specific solubilizing enzyme (or enzymes) is still debated (*1*, *4*, *6*). In an early work, Scott (*8*) reported an apparent heat-stable enzymatic activity of green malt that catalyzed the initial release of insoluble β-glucans from inactivated, undermodified malt at 65 °C. Forrest and Wainwright (*9*) supported that a close binding of insoluble β-glucans and structural protein prevents the former from dissolving in water, so that proteolysis, rather than β-glucan depolymerization, would be the first step in β-glucan degradation. Thereafter, the presence of a thermostable enzyme, called β-glucan solubilase (commonly referred to as solubilase), has been suggested to be required in the mash to free the insoluble β-glucans from a cell wall protein matrix so that they are available for depolymerization by β-glucanase and other hydrolases (*10*). Since then, many enzymes have been claimed to be responsible for the "freeing" of insoluble β-glucans into water (*1*, *4*) and/or shown to promote the solubilization of β-glucans by overcoming the restriction caused by the pentosan component of the cell wall (*11*, *12*). However, none has definitively shown an in vivo capacity for complete β-glucan solubilization (*6*, *13*). In addition, β-glucanase has been shown to be able to account for β-glucan solubilization in vitro (*12*, *14*). Nonetheless, poor correspondence with in situ microscopy observations (*14*) and the report that about 7−8% of the insoluble β-glucans would not be accessible even to β-glucanase (*12*) would make β-glucanase itself a pre-eminent but not unique agent of β-glucan solubilization. Hence, the existence of a purported, unknown enzyme placed at the beginning of the working model for β-glucan degradation still remains an open question (*1*, *4*, *6*).

In recent years, the two-stage model for β-glucan degradation has received renewed attention, particularly in relation to the

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cell-wall breakdown that occurs during malting (*15*). The presence of a peak of soluble β-glucans during endosperm modification of the malting cultivar Chariot (*15*) was in fact suggested to reveal an unbalance between solubilization and depolymerization and then the existence of a solubilase activity distinct from that of the hydrolyzing enzymes such as β-glucanase. We considered this issue worthy of further study.

To appraise the role of a solubilase activity distinct from that of β-glucanase, we adopted an indirect approach based on the overall kinetics of β-glucan degradation. Both the two-stage (biphasic) model and another model dismissing any solubilase activity (i.e., monophasic) were expressed in terms of kinetic equations to test their fit to experimental data. Specifically, we used extended time−course kinetics (*16*) to model curves of β-glucan degradation predicted from measures of both initial β-glucan content and development of enzyme activities. For this purpose, we optimized the analytical procedures for the determination of β-glucan solubility and β-glucan solubilase activity, targeting them to the conditions required to validate the model.

Modeling of the kinetic behavior of enzymes is usually applied to strictly controlled, in vitro reactions that follow the deterministic rate laws (i.e., are determined by the law of mass action), like the classical Michaelis−Menten kinetics (which assumes a constant reaction rate). However, especially for cooperative kinetics (i.e., where more enzymes cooperate in a process), modeling can be valuably studied with natural substrates in physiological conditions (*17*). Quite often, the classical Michaelis−Menten equation has to be substantially modified to approximate the actual extended time−course kinetics (*18*). Anyway, it has been demonstrated (*19*) that when considering reaction kinetics in vivo, the deterministic approach can indeed be a valid one to use even in highly microscopic environments (like cell walls).

In this work, changes of acid−extract viscosity (considered to be indicative of changes in β-glucan levels) were first compared during malting of five barley genotypes harvested in the same environment and selected for their contrasting malting quality and β-glucan-related parameters. In the second and main experiment, β-glucan degradation was monitored during malting, and the fitting of in vivo kinetic regression models to actual data of β-glucan degradation was studied. In a third experiment, a single Scarlett batch was used to test the heterogeneity of β-glucans' enzymic degradability.

MATERIALS AND METHODS

Five two-rowed barley genotypes, three spring (Scarlett, Extra, and CDC Candle) and two winter (Fior 6315 and Fior 7054), grown at Fiorenzuola (Northern Italy), were harvested in 2002. The seed lots were conditioned for 1 week at 25 °C to a 10−12% moisture content. The five genotypes were chosen, based on their qualitative and viscosimetric traits, as being representative of the variability existing in an original set of 35 widely differing barleys (*20*). Scarlett is a good malting quality variety, Extra is a feeding barley, CDC Candle is a hulless waxy barley, and Fior 6315 and Fior 7054 are experimental lines developed from crosses between elite feed and malting cultivars (*20*). Samples normally considered unsuitable for industrial malting were included in order to capture a wide range of structural variation and improve the inferential power of the experiments.

Each seed lot was analyzed in duplicate after removal of screenings (kernels < 2.0 mm) with an Octagon 200 test sieve shaker (Endecotts Ltd., London, England). To remove dormancy, samples were stored at room temperature (22−32 °C) for 3 months after harvest and then transferred to a cooled storage facility (9 °C) until malting. After storage, the samples had a 95 ± 5 % germination after 3 days (4 mL water test, 19 °C).

Malting Process. For each genotype, two replications, 100 g each, of sieved barley seeds were malted at 17 °C with an Automatic Micromalting System (Phoenix Biosystems, South Australia). Used was the following malting cycle: 8 h steep, 8 h air rest, 9 h steep, 6 h air rest, 0.5 h steep, 88.5 h germination, and 24 h kilning at 30−80 °C.

Analytical Determinations. The protein content was measured with an NIT instrument (Infratec 1241 Grain Analyzer, Foss Tecator, Höganäs, Sweden). Barley and malt β-glucans were determined with a streamlined enzymic procedure (mixed-linkage β-glucan kit, Megazyme, Bray, Ireland) according to McCleary and Codd (*21*). For the determination of insoluble β-glucans, soluble β-glucans were extracted before the enzymic assay [modified from Åman and Graham (*22*)]: 250 mg of milled barley/malt sample was hydrated with 2.5 mL of 70% aqueous ethanol in Falcon (15 mL), heated at 100 °C for 5 min, and cooled with 2.5 mL of 70% ethanol; the supernatant was discharged after 10 min of centrifugation at 2000*g*, and the pellet was washed twice with about 10 mL of 20 mM Na−phosphate buffer, pH 6.5 (1 h of agitation each time), at 37 °C. β-Glucans remaining in the pellet were measured with the streamlined method, starting with heating for 5 min at 100 °C. Soluble β-glucans were calculated as the difference between total and insoluble β-glucans (*22*).

Viscosity of Acid Extracts. In the first experiment, the acid−extract viscosity of barley and malt was determined with a thermostated (20 °C) rotational viscometer (LVDV-II+ with Ultralow Adapter, Brookfield Engineering Laboratories, Middleboro, MA) using the supernatant obtained (5 min, 3000 g) after extracting 2 g of milled sample (dwb) with 50 mL of 0.1M KCl/HCl, pH 1.5, for 1 h at 25 °C (*22*).

***β*-Glucan Degradation.** In the second and main experiment, β-glucan degradation was measured throughout the entire malting time−course. Soluble and insoluble β-glucans were determined as above, and the β-glucanase activity was measured according to McCleary and Shameer (*23*) with the azo-barley glucan method (β-glucanase assay kit, Megazyme). The β-glucan solubilase activity was measured by quantifying the insoluble β-glucans remaining in a β-glucan-rich substrate after incubation with an enzyme extract. This method was preferred to the direct measure of the β-glucans that are released during the reaction because, particularly during the late phases of malting, the crude enzyme extract may contain large amounts of β-glucan-degrading enzymes, which could interfere with the determination of soluble β-glucans. The method used to measure β-glucan solubilase activity is detailed hereafter in three schematic steps. First, denatured substrate was prepared from a β-glucan-enriched barley flour (cv. CDC Candle) obtained by air classification (manuscript in preparation). This fraction was gently boiled for 1 h in 70% ethanol under a reflux condenser. After it was cooled, the mixture was centrifuged for 5 min at 2000*g* and the supernatant was discarded. The pellet was washed twice with four volumes of distilled water, vortexed for 2 min before each centrifugation, and then transferred to 50 mL Falcon tubes. The pellet was resuspended in four volumes of 10 mM Na−phosphate (pH 6.5) containing 7.5 mM NaCl and 0.2 mL of α-amylase stock (0.5 g/mL in 10 mM Na−phosphate, pH 6.5, containing 9 mM NaCl); α-amylase type VI-B from porcine pancreas (Sigma Chemical Co., St. Louis, MO) was added to each tube (about 50 mL of mixture). The tubes were shaken overnight at 25 °C and centrifuged. The pellet was washed twice with distilled water as explained above. Finally, the pellet was freeze-dried overnight, coarse ground, and aliquoted in 1.5 mL vials. The moisture content, total, and insoluble β-glucans were assayed, and the vials were stored at +5 °C for subsequent assays. As a second step, crude enzyme extracts were prepared from each of the five milled barley/malt samples: 250 mg was weighed in 2 mL vials, and 1 mL of 40 mM Na−citrate, pH 5.5 (0.02% Na−azide), was added. After this was vortexed briefly and incubated for 15 min at room temperature, the mixture was centrifuged (10000*g*, 10 min) and the supernatant was transferred to a new vial. For each sample, three aliquots of crude enzyme extract were prepared immediately before the assay and subjected to different thermal treatments: One aliquot was immediately cooled on ice (not treated, NT), whereas the other two were heated, respectively, for 20 min at 62 °C and for 5 min at 100 °C before cooling. In the third and final step, the activity of each crude enzyme extract was assayed for its ability to solubilize β-glucans from the denatured substrate. For each crude extract, three determinations (one for each

thermal pretreatment) were performed as follows. In a 15 mL Falcon vial, 50 mg of denatured substrate was moistened with 100 μL of 50% aqueous ethanol and then washed with 10 mL of 40 mM Na−citrate, pH 5.5 (0.02% sodium azide). After 5 min of centrifugation at 3000*g*, 2 mL of citrate buffer and 0.2 mL of crude enzyme extract were added to the pellet. Following 2 h of incubation at 37 °C, the reaction was stopped by adding 4 mL of 95% ethanol, vortexed, and incubated for 5 min at 100 °C. This reacted mixture was kept at 4 °C overnight. The next day, the vial was centrifuged for 10 min at 2000*g*, the pellet was washed twice with 20 mM Na−phosphate, and the remaining insoluble β-glucans were measured as explained previously under analytical determinations. The blank assay was performed at pH 6.5, at which no activity was detected during preliminary experiments. The β-glucan solubilase activity was calculated as the milligrams of β-glucans solubilized per hour from the denatured substrate, referring to 1 g of the sample used to prepare the crude enzyme extract (dwb) and was therefore expressed as units of solubilization per gram of dry sample (U/g).

Enzymic Assay of β-Glucan Degradability. In a third experiment, a modification of the streamlined enzymic procedure of McCleary and Codd (*21*) was used to verify the effect of the crude solubilase extract on the β-glucan level actually measured in a single batch of Scarlett seed (taken from a different field at Fiorenzuola but treated as above). Barley flour (100 mg wetted with 50% ethanol) was suspended in 4 mL of 5 mM Na−citrate buffer, pH 5.5 (0.02% sodium azide), and then, the mix was heated for 5 min at 100 °C, and 0.2 mL of the crude enzyme extract (prepared as for the determination of solubilase activities and pretreated 20 min at 65 °C) was added. After the mixture was incubated for 2 h at 65 °C and reheated for 5 min at 100 °C, 5 mL of 40 mM phosphate buffer, pH 6.5, was added before the introduction of lichenase. All further steps were performed in accordance with the standard streamlined protocol (mixed-linkage β-glucan kit, Megazyme). A correction factor for the increased final reaction volume (14 mL) was applied according to the manufacturer's instructions. Five independent experiments, each with duplicate measures for both the standard streamlined protocol and the modified procedure described in this paragraph, were performed.

Statistics. All of the analytical determinations were replicated at least twice and were reported as mean values. The latter was used for correlation and regression analyses (Systat 9.0 software; SPSS Inc., Chicago). Principal component analysis (PCA) provided a synthesis of the overall correlations between the measured parameters: On the plot, each parameter is represented by a segment; closer segments correspond to a greater correlation between parameters.

Kinetic Models. In the second and main experiment, data on β-glucan degradation were used to model in vivo depletion of β-glucans. The enzymic degradation of β-glucans in the germinating barley grain is substantially an irreversible process that occurs in a heterogeneous medium. It lasts several days, during which high levels of enzymes develop and the reaction progresses to completion. Under these conditions, the Michaelis−Menten equation for enzyme-catalyzed reactions does not apply. However, a simplified approximation of the reaction kinetics can be adopted that considers the entire time−course of the process (*16*): $v = -\mathrm{d[S]}/\mathrm{d}t = k_{cat}/K_m \cdot [S] \cdot [E]$. Where [S] is substrate concentration, [E] is enzyme concentration, and k_{cat}/K_m is an apparent second-order rate constant for the reaction of E and S to form product (using this approximation, the kinetic parameters K_m and k_{cat} cannot be determined separately). For a given level of enzyme ([E] = constant), the rate is proportional to [S] and the reaction is said to be "pseudo-first order in S". However, enzyme levels increase during germination (independently of the reaction itself), so that [E] has to be progressively adjusted for the actual enzyme level and the subsequent substrate concentrations are predicted by a series of pseudo-first-order kinetic equations. Such a series was generated using, for each sampling time (t), the average enzyme level from 0 to t ($[E]_t$). The velocity equation can be integrated to predict the time−course for substrate depletion during malting:

$$[\mathrm{tot}\beta\mathrm{G}]_t = [\mathrm{tot}\beta\mathrm{G}]_0 \cdot \exp(-k_{tot}' \cdot [E]_t \cdot t) \quad (1)$$

where $[\mathrm{tot}\beta\mathrm{G}]_0$ and $[\mathrm{tot}\beta\mathrm{G}]_t$ are β-glucan concentrations, respectively, in the unmalted grain and at subsequent times of sampling, $[E]_t$ is the corresponding enzyme level, t is the day of sampling, and k' is the apparent rate constant ($k' = k_{cat}/K_m \cdot k_a$, where k_a is a correction factor that accounts for expressing enzyme concentration in terms of activity and β-glucans as a weight percentage). We used this equation to test the monophasic model's fit to the data, assuming β-glucanase as the sole responsible for total β-glucan degradation.

Analogously, a time−course for depletion of insoluble β-glucans can be predicted separately:

$$[\mathrm{ins}\beta\mathrm{G}]_t = [\mathrm{ins}\beta\mathrm{G}]_0 \cdot \exp(-k_{Insol}' \cdot [E]_t \cdot t) \quad (2)$$

This equation was used to test whether either the β-glucanase activity or the solubilase activities that were assayed after the three different thermal pretreatments fit to the measured values of β-glucan solubilization.

For soluble β-glucans, depolymerization of both the soluble β-glucans already present in the grain, $[\mathrm{sol}\beta\mathrm{G}]_0$, and the β-glucans subsequently solubilized during germination, are considered

$$[\mathrm{sol}\beta\mathrm{G}]_t = [\mathrm{sol}\beta\mathrm{G}]_0 \cdot \exp(-k_{sol}' \cdot [E_d]_t \cdot t) + [\mathrm{ins}\beta\mathrm{G}]_0 \cdot [1 - \exp(-k_{Insol}' \cdot [E_s]_t \cdot t)] \cdot \exp(-k_{sol}' \cdot [E_d]_t \cdot t) = \{[\mathrm{sol}\beta\mathrm{G}]_0 + [\mathrm{ins}\beta\mathrm{G}]_0 \cdot [1 - \exp(-k_{Insol}' \cdot [E_s]_t \cdot t)]\} \cdot \exp(-k_{sol}' \cdot [E_d]_t \cdot t) \quad (3)$$

This equation was used to test the fit when either β-glucanase or differently pretreated solubilase activities were considered as $[E_s]$ (solubilizing enzyme), while $[E_d]$ (depolymerizing enzyme) was always assumed to be β-glucanase because its action on soluble β-glucans is undisputed.

As, in the biphasic model, β-glucans are destroyed only as a consequence of the depolymerization phase, the term accounting for soluble β-glucan depletion should be the same as the one for total β-glucan depletion. The following equation can therefore be used as an additional test of the biphasic model:

$$[\mathrm{tot}\beta\mathrm{G}]_t = [\mathrm{tot}\beta\mathrm{G}]_0 - \{[\mathrm{sol}\beta\mathrm{G}]_0 + [\mathrm{ins}\beta\mathrm{G}]_0 \cdot [1 - \exp(-k_{Insol}' \cdot [E_s]_t \cdot t)]\} \cdot \exp(-k_{sol}' \cdot [E_d]_t \cdot t) \quad (4)$$

Two main assumptions were applied to formulate testable regression equations: (i) The substrate may have a variable number of sites for enzymatic attack depending on the degree of polymerization, so that it is properly measured as a percentage of dry matter rather than in mol/L; (ii) the enzyme concentration, [E], is assumed to be proportional to the measured activity, so that the latter can be used in place of [E]. The ability of these kinetic models to fit the measured enzyme activities was evaluated by means of confirmatory regression analysis. Equation coefficients were optimized with a nonlinear module (least-squares method). R^2 (mean-centered) was used as a relative measure of goodness-of-fit. It should be noted that although a more complex and rigorous kinetic model approximating the integrated form of the Michaelis−Menten equation has been proposed (*24*), the additional term included in that model was verified to be nonsignificant when applied to our data (not shown). Moreover, as under restrictions to free molecular diffusion, the conventional rate laws exhibit a characteristic reduction of the rate constant with time (*18*), we tested whether the apparent rate coefficient (k') was time-dependent, that is, whether adopting $-k_{tot}' \cdot [E]_t \cdot t^{(1-h)}$ (where h is the fractal kinetic exponent) in place of $-k_{tot}' \cdot [E]_t \cdot t$ as exponential term, would improve equation fitting (*25*).

RESULTS

Grain Traits and Acid−Extract Viscosity. The five barleys showed large differences for both protein and β-glucan traits (**Table 1**). The changes in acid−extract viscosity observed during malting in the first experiment are reported in **Figure 1** and are assumed to reflect major differences in β-glucan degradation among barley samples. The highest initial value was observed for CDC Candle at over 7 cP, followed by Extra (4 cP) and then the others, which leveled just above 2 cP. Acid−

Table 1. Origin, Grain Traits, and Malting Quality of the Five Barley Genotypes (Mean of Two Replicates ± SE)

genotype	origin	type	protein (%)	total β-glucans (%)	insoluble β-glucans (%)	soluble β-glucans (%)	insoluble β-glucan ratio (%)
CDC Candle	Canada	hulless waxy	10.6 ± 0.2	6.5 ± 0.3	3.7 ± 0.1	2.8 ± 0.2	57
Extra	Austria	feeding	11.8 ± 0.4	4.7 ± 0.2	3.6 ± 0.1	1.1 ± 0.2	76
Scarlett	Germany	malting	10.9 ± 0.1	4.1 ± 0.1	3.2 ± 0.4	0.9 ± 0.5	79
Fior 6315	Italy	experimental line	15.1 ± 0.4	4.2 ± 0.1	3.4 ± 0.1	0.8 ± 0.1	81
Fior 7054	Italy	experimental line	16.4 ± 0.4	3.2 ± 0.1	2.4 ± 0.1	0.8 ± 0.1	75

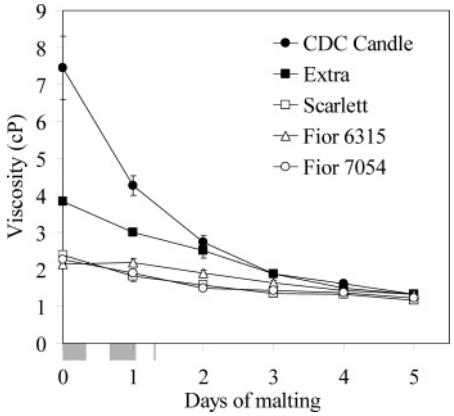

Figure 1. Viscosity of acid extracts during malting. Bars represent standard errors ($n = 2$). Gray bars on the *x*-axis indicate the steepings.

extract viscosities showed exponentially decreasing trends and, after 5 days of malting, converged to 1.2−1.3 cP for all of the barley samples.

The five genotypes captured most of the variability observed within the original set of 35 barleys from which they were selected (*20*): CDC Candle had high grain β-glucan levels and a high initial acid−extract viscosity; Extra showed moderately high grain β-glucan levels, with a slow loss of acid−extract viscosity; Scarlett, of good malting quality, had a low acid−extract viscosity profile; Fior 6315 had a β-glucan composition similar to that of Scarlett but had a very poor malting quality and a unique, although very small, increase of acid−extract viscosity after 1 day of malting; and Fior 7054, with a low β-glucan content and a corresponding low acid−extract viscosity profile, had a suboptimal malting quality.

***β*-Glucan Degradation.** In the second and main experiment, total, soluble, and insoluble β-glucans, as well as β-glucan solubilase and β-glucanase activities, were measured daily during malting in the five barley samples. The reduction in total β-glucan content (**Figure 2A**) was paralleled by a decrease in insoluble β-glucans (**Figure 2C**). For both total and insoluble β-glucans, after an initial peak or stationary phase, the amount decreased rapidly after the second day of malting. Although this decrease was expected because of the development of degrading enzymes during germination, the initial peak represented a puzzle (although not completely unexpected, because the biphasic model entails some sort of β-glucan release). No significant reduction in dry matter was observed during the first day of malting (not shown), so that an apparent "freeing" of previously unmeasured β-glucans was deduced. This trend was quite consistent among barley samples and, in some cases, (Extra and Fior 6315), corresponded to a peak in soluble β-glucans as well (**Figure 2E**). On the other hand, soluble β-glucans showed quite different trends among samples: Whereas the two aforementioned barleys maintained a constant level after the peak, Scarlett had no peak, and CDC Candle had a uniformly

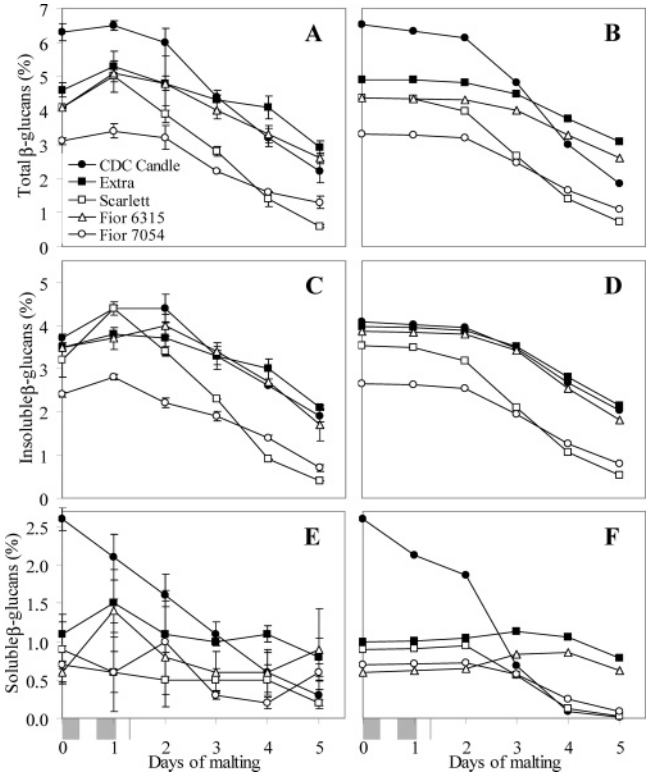

Figure 2. Total (**A**, **B**), insoluble (**C**, **D**), and soluble (**E**, **F**) β-glucans during malting. Left plots (**A**, **C**, and **E**) show measured values, whereas right plots (**B**, **D**, and **F**) are values predicted by the kinetic models considering degradation of β-glucan by β-glucanase alone. Bars in plots of measured values (**A**, **C**, and **E**) represent standard errors ($n = 2$). Gray bars on the *x*-axis indicate the steepings.

decreasing trend. The higher total β-glucan content of CDC Candle was essentially due to soluble β-glucans that were rapidly degraded so that its total β-glucan content soon decreased to the level of the other poor malting quality barleys (Extra and Fior 6315). Fior 7054 had the lowest initial content of total and insoluble β-glucans, and they were degraded at a speed similar to that of Extra and Fior 6315. Instead, Scarlett showed initial levels of total and insoluble β-glucans similar to those of the poor malting quality genotypes, but when Scarlett was malted, its β-glucans were degraded faster than in any other barley sample.

As expected, β-glucanase activity developed following 2 days of malting (**Figure 3**). The plots of activity showed similar trends, although the actual level of activity differed between gentotypes (**Figure 3**): Extra and Fior 6315 attained low levels of activity in green malt; CDC Candle and Fior 7054 reached higher levels, and their activity increased faster than in the previous poor malting quality barleys; finally, Scarlett showed both the highest final activity in the green malt and the steepest increment. Following kilning, the activities of all of the samples

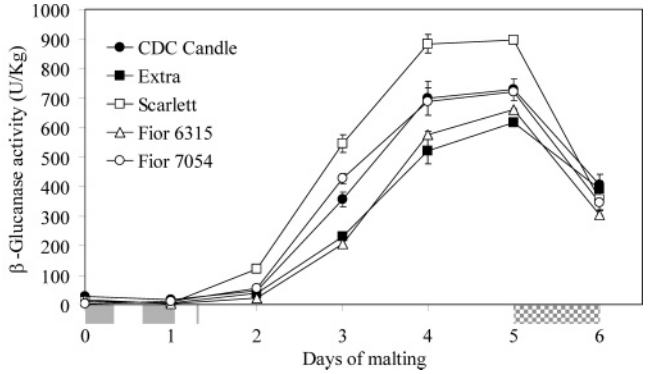

Figure 3. Activity of β-glucanase during malting. Bars represent standard errors ($n = 2$). Gray and checkered bars on the *x*-axis indicate steepings and kilning, respectively.

attested to a similar level, about 40−60% of the previous maximum activity.

With regard to β-glucan solubilase activity, the assay established in this work showed that the solubilase activity that was determined without having pretreated the crude enzyme extract derived from enzymes with different thermosensitivities. In fact, increasing the temperature of the pretreatment decreased the overall solubilase activity (**Figure 4**). However, it was surprising that a significant activity still remained after 5 min at 100 °C. This fact compelled us to use pH-dependent inactivation of the crude enzyme extract to prepare analytical blanks, rather than the more commonly preferred thermal inactivation. Preliminary work had in fact showed that in our conditions no solubilase activity could be detected at pH 6.5 (data not shown). This pH was thus selected for the sample blank, and pH 5.5 was used for the analyses, which should also be closer to the pH of the kernel apoplast. The assay was performed at moderate (*26*) rather than high (*10*, *12*, *13*) temperature. In addition, to reduce the temperature-induced loss of physiological substrate, the same moderate temperature (37 °C) as used in the assay was adopted during the initial removal of water-soluble β-glucans from the substrate.

Relevant differences in solubilase activity were observed among samples (**Figure 4**): Malting barleys had high peaks of activity, and Extra showed only small differences between activities measured after pretreating at low and high temperatures. The solubilase activities assayed after the three different pretreatments of the crude enzyme extract (not treated, 20 min at 62 °C, and 5 min at 100 °C) showed corresponding trends during malting, although increasing the temperature of pretreatment obviously reduced the activity. In three genotype samples (Scarlett, Fior 7054, and Extra), a relevant activity was already present in the ungerminated barley grain (**Figure 4**). In addition, Fior 7054 and, particularly, Extra, showed an apparent initial decrease of activity (0−2 days) before it increased again. In every case, the solubilase activity reached a maximum at 4−5 days of malting and decreased during kilning. **Figure 5** shows the overall correlation among parameters related to β-glucan degradation as measured during malting time−course. From this plot, it is evident that (i) correlation of β-glucan solubilase activity with β-glucanase activity decreased as the pretreatment temperature increased, so that solubilase activity measured in crude extract pretreated at 100 °C was independent of β-glucanase; (ii) enzyme and β-glucan levels were negatively correlated, as, in fact, they follow opposite trends during malting. The first result suggests that whereas some thermostable enzyme activity was effectively measured, β-glucanase was also involved in β-glucan solubilization. Anyway, solubilase activity, but not

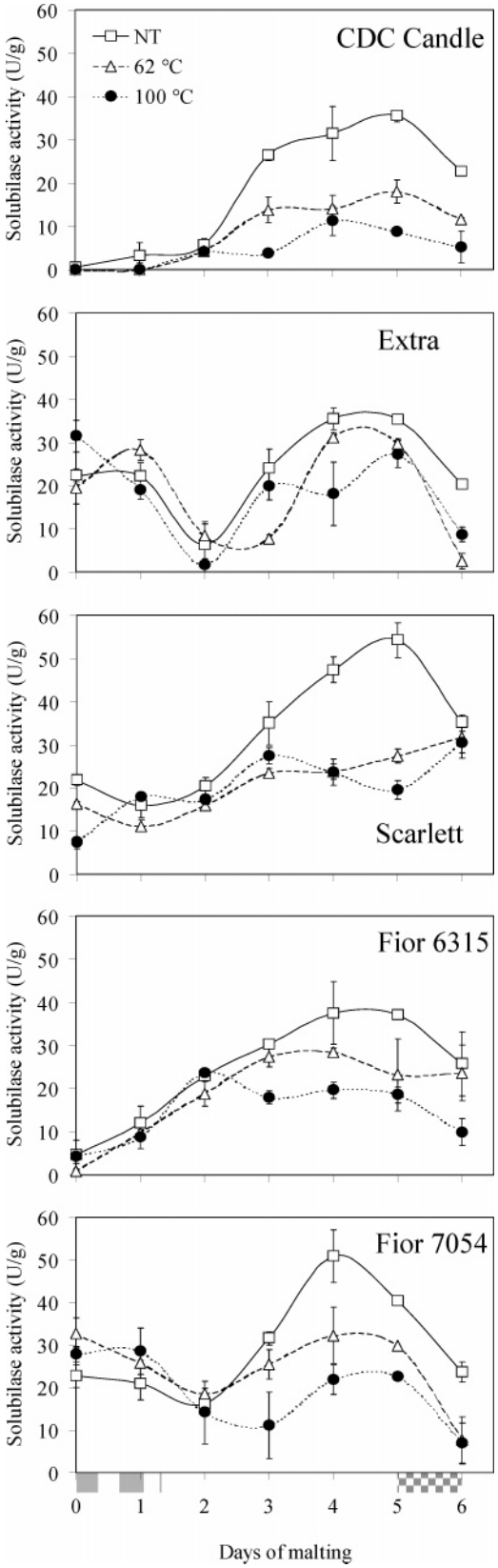

Figure 4. Activity of β-glucan solubilase measured during malting after pretreating the crude enzyme extract for 5 min at 100 °C, for 20 min at 62 °C, or without pretreating (not treated, NT). Bars represent standard errors ($n = 2$). Gray and checkered bars on the *x*-axis indicate steepings and kilning, respectively.

β-glucanase, was detected in the dry grain and during the first days of malting.

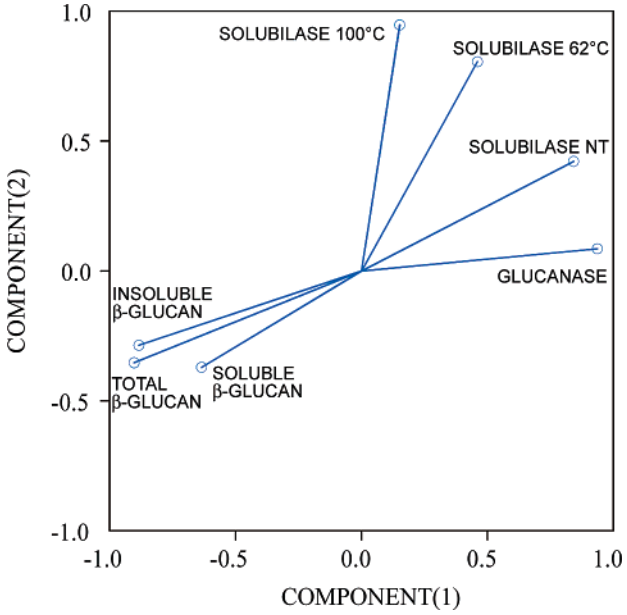

Figure 5. Principal component analysis plot showing overall correlation of the parameters measured during malting. The closer the segments are, the higher the correlation between parameters. The solubilase activity was assayed after pretreating the crude enzyme extract for 5 min at 100 °C, for 20 min at 62 °C, or without pretreating (not treated, NT).

Table 2. R^2 Values for the Fitting of Enzyme Activities into Kinetic Models ($n = 30$)

β-glucan model	β-glucanase	solubilase			no enzyme term[a]
		NT[b]	62 °C	100 °C	
total (eq 1)	0.958				0.831
insoluble (eq 2)	0.913	0.794	0.756	0.729	0.713
soluble (eq 3)[c]	0.635	0.419	0.354	0.301	0.339
total (eq 4)[c]	0.325	0.199	0.175	0.163	0.086

[a] On each row, lower R^2 values are joined to higher mean square residual. [b] Not treated at high temperature. [c] Alternative activities are considered in the $[E_s]$ term; the β-glucanase activity is kept as the $[E_d]$ term.

In Vivo Kinetics of β-Glucan Degradation. The data obtained in the malting experiment described above were used to compare the biphasic and monophasic models by modeling the corresponding enzyme activities measured in vivo and then testing the fit of the respective kinetic equations (**Table 2**). Specifically, the ability of the models to predict β-glucan levels during malting (days 1−5) from both the initial β-glucan level (day 0) and the appropriate enzyme activities (averaged over the corresponding periods) were evaluated. For the biphasic model, the best fit would be expected by separately regressing insoluble and soluble β-glucans on solubilase and β-glucanase activities, respectively. For the monophasic model, it would instead be expected that not only regression of insoluble β-glucans should have a better fit if performed on β-glucanase than on solubilase activity but that an optimal fit would be obtained when β-glucans as a whole (i.e., total β-glucans) were regressed onto β-glucanase activity. Thermally differentiable solubilase activities were also considered in testing the model for solubilization of insoluble β-glucans.

In performing modeling tests, a first difficulty arose due to the β-glucan peaks observed at the beginning of malting: Because they suggested a "freeing" of β-glucans, this additional amount had to be considered in the kinetic calculations. Thus, the initial levels of insoluble and total β-glucans were assumed to be greater than those actually measured in the kernel, and a further approximation was introduced into the regression model by multiplying the initial measured levels by $1 + x$, where x represents a fraction of β-glucans that had to be estimated by fitting because of an underestimation of kernel β-glucans.

Table 3. Optimized Regression Coefficients for Regressions Assuming β-Glucanase as the Sole Responsible for Depletion of Both Soluble and Insoluble β-Glucans[a]

regression coefficients	genotype	β-glucan model		
		total[b]	insoluble[c]	soluble[d]
x[e]		0.068	0.107	
K[f]	Scarlett	7.3×10^{-4} a	7.7×10^{-4} a	21.1×10^{-4} a
	Fior 6315	3.5×10^{-4} b	5.1×10^{-4} a	9.8×10^{-4} a
	Fior 7054	5.9×10^{-4} ab	6.4×10^{-4} a	18.1×10^{-4} a
	Extra	3.3×10^{-4} b	4.3×10^{-4} a	9.0×10^{-4} a
	CDC Candle	6.8×10^{-4} a	3.7×10^{-4} a	33.4×10^{-4} a

[a] All of the reported values were significantly different from zero ($P < 0.05$, Wald test). Within each column, K values marked with the same letter were not significantly different from each other ($P < 0.05$, Wald test). [b] From eq 1. [c] From eq 2. [d] From eq 3. In fitting this equation, the coefficients for depletion of insoluble β-glucan were fixed to the values already optimized from eq 2 (left column). [e] Estimation of the fraction of insoluble β-glucan assumed to be present in the grain but not directly measurable. [f] Coefficient representing degradability.

Confirmatory regression analysis (**Table 2**) indicates that the monophasic model considering degradation of total β-glucans by β-glucanase activity alone showed a good fit ($R^2 = 0.958$). Accordingly, a better fit for solubilization of insoluble β-glucans was obtained when β-glucanase activity, rather than any of the solubilase activities measured after the three different pretreatments of the crude extracts, was considered. The same was true for depletion of soluble β-glucans, even if the fit was poor in any case, possibly due to the great complexity of the underlying model (eq 3) and the large variation in values (as derived from two other analyses). It may also be that other enzymes (e.g., exohydrolases and β-glucosidases) affect the degradation of soluble short-chain β-glucans, so modeling the changes of the insoluble β-glucans should be a more proper way to assess any solubilase action. The good fits obtained when the first two equation models were tested without any enzyme term (**Table 2**) are most likely due to the temporally dependent build-up of β-glucanase during germination, which makes malting time a correlative of enzyme activity, so that time by itself can partially account for enzyme development. Finally, the assumption of the biphasic model that depletion of total β-glucans should essentially occur through soluble β-glucans (eq 4) was not supported. Instead, degradation of total β-glucans by β-glucanase alone was more consistent with the observed data.

Indeed, considering the monophasic model, plots of observed and predicted levels of β-glucans during malting (**Figure 2**) look very similar. Nonetheless, one difference is apparent in the measured data: a peak in β-glucan at the beginning of malting. Because the peaks appeared for both total and insoluble β-glucans, they seemed to reveal an "unmasking" of a fraction of insoluble β-glucans that was not initially detectable but was nevertheless promptly released during malting, apparently in the absence of β-glucanase (see **Figure 3**). As coefficient x (i.e., the estimated β-glucan fraction not directly measurable in the kernel) was significantly above zero ($P < 0.05$), the initial underestimation of β-glucans was supported (**Table 3**). Moreover, as the underestimated measure of initial β-glucans was based on lichenase (β-glucanase) activity, we concluded that such a fraction was not even solubilizable by β-glucanase.

Therefore, the in vivo kinetic models indicated that the initially measurable total β-glucans (including most of the insoluble ones) were degraded by β-glucanase, but a small additional fraction (on average, 10.7% of the insoluble β-glucans measured in the kernel) required some other enzyme activity; for this reason, it was not detected in the unmalted grain. Thus, although monophasic β-glucan degradation appeared to be the main route, a minor role for the biphasic model was supported too, but this was not evidenced in modeling because β-glucans insoluble in water (37 °C) turned out not to be exactly the substrate of solubilization. These findings provide useful information for further studies.

Although the monophasic model was not able to explain β-glucan peaks, it could be successfully applied after the peaks, especially for total β-glucans, which, in accordance with this model, were the substrate of β-glucanase (**Figure 2**). Thus, on the basis of the model of β-glucan degradation by β-glucanase alone, optimized values of the apparent rate constant k' were estimated for total, insoluble, and soluble β-glucans (**Table 3**). In fact, it can be hypothesized that k' varied among samples and solubility class because of physicochemical differences. These differences represent diverse degrees of degradability, so that k' can be interpreted as a relative measure of β-glucan degradability. Soluble β-glucans appeared to be more degradable than insoluble ones, and, accordingly, k' values of total β-glucans approached those of insoluble β-glucans, suggesting that the latter limited β-glucan degradation as a whole. Among the genotypes, total and insoluble β-glucans of Scarlett were the most degradable. Together with the high β-glucanase activity, its high β-glucan degradability explained why this variety performs quite well in malting even if it has medium (or, sometimes, high) grain β-glucan contents. On the other hand, Extra and Fior 6315, with the lowest overall degradability of β-glucans and β-glucanase activities, attained malt β-glucan contents similar to that reached by CDC Candle, notwithstanding the fact that CDC Candle had high grain β-glucan content. This latter variety, however, also had the best degradability of soluble β-glucans.

Finally, when the kinetic exponential term of eq 1 and 2 was modified to test time dependency of k' by including an additional fractal exponent ($-h$), the latter resulted not significantly different from zero (not shown), suggesting that k' did not change with malting time and therefore that the properties of the substrate (β-glucans) did not change significantly during its degradation.

"Masked" β-Glucans. To confirm the presence of a fraction of β-glucans that was not degradable by β-glucanase alone, in a third experiment, we tested an additional batch of Scarlett that showed an increase in detectable total β-glucans after 1 day of malting. β-Glucans were then measured in the unmalted barley sample with the usual streamlined enzymic procedure and with a modified assay that included the addition of a crude enzyme extract (from the same barley) to favor complete solubilization. Considering that the enzyme extract contributed about 0.8 mg of soluble β-glucans per 0.1 mL (determined with the streamlined procedure), a mean increment of 0.4 g β-glucans per 100 g of dry flour was obtained with the modified assay, with respect to the value established by the usual procedure (5.7% total β-glucans). This difference represented a proportion of β-glucans (about 7% of the measured amount of total β-glucans) that corresponded quite well to that estimated by fitting the x parameter in the in vivo kinetic equation for the monophasic degradation of total β-glucans. However, it is worth noting that this amount was quite small and required a number of replicated tests to be evidenced. Therefore, the presence of masked β-glucans should not significantly affect the values measured with the regular assay procedure. For the very same reason, only because of the consistence of peaks in different experiments, later confirmed by the significance of the x parameter, was the freeing of masked β-glucans considered factual.

DISCUSSION

Confirmatory regression analysis was used to test the fit of in vivo kinetic models for β-glucan degradation to the actual data monitored during malting. Two β-glucan degrading activities, namely, β-glucanase and β-glucan solubilase, were considered in formulating the kinetic equations used to compare the biphasic and monophasic degradation models. Better fitting of the monophasic model indicated that both soluble and insoluble β-glucans are essentially depolymerized by β-glucanase. Nevertheless, the presence of a β-glucan peak early during malting suggested that not all of the β-glucans in the barley grain were actually measured and a small masked fraction was released shortly after imbibition. Small but significant β-glucan peaks had already been found by Prentice and Faber (*27*) in two samples of germinating barleys assayed with *Trichoderma* β-glucanase. Thus, this small fraction of insoluble β-glucans appears to require an enzyme other than β-glucanase before it becomes available for depolymerization. This result confirms the finding of Kanauchi and Bamforth (*12*) that 7−8% β-glucans are not available even to β-glucanase. Indeed, a number of enzymes, β-glucanase among them, have failed to fully degrade β-glucan when added to structured cell walls (*12*, *14*). Therefore, the biphasic model involving an initial action of solubilase, could still hold, at least for this small fraction of insoluble β-glucans. An effect of solubilase (or similar enzyme) on the physical−chemical state of β-glucans is also suggested by the steep decrement of acid−extract viscosity observed during early malting (**Figure 1**), when the β-glucanase level was still close to zero.

Another issue emerging from our analysis is that the apparent kinetic rate constant (k') can be interpreted as a relative measure of degradability. Varietal differences in endosperm degradability are known (*2*−*4*, *28*, *29*), and in our work, differences among barleys were indeed linked to strong differences in β-glucan degradation (compare k' values in **Table 3** to the contrasting slopes in **Figure 2**). The total protein content was not linked to k' values (no significant correlation was found between the two variables). Indeed, specific protein fractions have been purportedly considered to affect endosperm modification during malting (*2*, *3*, *29*), but this point is still unsettled and needs further studies.

It is worth noting that the kinetic term $[E]_t \cdot t$ is equivalent to the sum of daily activities from time 0 to t; that is, it represents a measure of integrated enzyme activity. In other words, this term considers both the progressive development of enzyme activity (which showed relevant differences between samples) and the time such an activity operates. Therefore, the kinetic relationship, considering the combined effect of β-glucan degradability (k') and the integrated β-glucanase activity ($[E]_t \cdot t$), provides a more complete picture of β-glucan degradation than the simple level of enzyme activity at any point during malting. In this sense, the importance of having an early development of β-glucanase to achieve an high degree of endosperm modification was highlighted by Chandra et al. (*29*) who compared the enzyme levels in cultivars Chariot and Target, of good and poor malting quality, respectively.

One might be wonder why the solubilase activity measured in vitro did not fit the model for solubilization in vivo. β-Glucan solubilase has been suggested to be a type of endo-1,4-β-glucanase (cellulase) present in barley husks because of contamination of kernels with common field fungi (*30*). Indeed, the barley grain can harbor a wide variety of microorganisms producing various extracellular hydrolytic enzymes (*31*). However, because the majority of the microbes are present in the covering layers of barley (*32*), that is, not close to the substrate in vivo in the barley grain, we deem that microbial enzymes (present or not) are unlikely to access the substrate during barley germination and thereby have a solubilase role in vivo. Instead, we could have artifactually evidenced the contribution of some thermostable activity that is not effectively involved in β-glucan solubilization during malting because of a different compartmentalization within the grain (which is lost with milling). Indeed, at least one enzyme with such high thermostability, but localized outside the endosperm, has been reported (*33*). This could be a general issue in measuring solubilase activity and suggests that in vivo modeling is a more reliable approach to study β-glucan degradation in the germinating grain than the direct enzymic assay. On the other hand, original work on solubilase (*10*) was developed in the context of β-glucan release in the mashing stage of brewing and not as an expression of what was necessarily happening in vivo during germination. Thus, the fact that solubilase activity is detected in crude enzyme extracts implies that such an activity could be of importance in the mashing process in the brewery, where the substrate is indeed subjected to enzymes that would not have access to it in vivo.

The good agreement between predicted and observed levels of β-glucans confirms that the whole time−course deterministic kinetics provided an effective model of in vivo β-glucan degradation. As the classical kinetics are only valid in fully unrestricted three-dimensional environments (*18*), the main source of bias in in vivo biochemical reactions is the limited diffusion effect due to structural organization and macromolecular crowding (*34*). Thus, the good fit of β-glucan degradation curves suggests that in the barley endosperm cell walls there are no relevant obstructions to diffusion of enzyme molecules (β-glucanase) toward their substrate (β-glucans). Indeed, the presence of very large macromolecular structures can be expected to have no significant effect on the diffusion of small molecules (*34*). Furthermore, as, in this case, β-glucan macromolecules, which should constitute most of the obstructions to diffusion, are the very same substrate of the enzyme activity, it may be speculated that the frequency of active collisions, and then the reaction kinetics, are not affected. In fact, the apparent kinetic rate constant, k', varied between genotypes but did not change with time. This fact contrasts with findings for cellulose, for which a time-dependent change of k' was observed and explained as due either to restrains to a perfectly three-dimensional diffusion of cellulase (*25*) or to cellulose heterogeneity causing digestibility to decrease as the hydrolysis proceeds (*35*). In this respect, our in vivo experiments provide an indication that the kinetics of β-glucan hydrolysis are different from those of cellulose hydrolysis.

Altogether, we can hypothesize that after an initial stage when the levels of masked β-glucans and solubilase activity could establish the rate of demolition of the native cell walls (*10*), β-glucan degradability (**Table 3**), in addition to the balance between the levels of barley grain β-glucans and malt β-glucanase (*7*), should influence the rate of β-glucan degradation. Fast β-glucan degradation is a determinant of malting quality (*7*), and in our experiments, it was linked to high β-glucanase activity and/or high β-glucan degradability (**Figure 2**). These last two traits, therefore, appear to be essential features of good malting barleys. Further insights in the role of masked β-glucans and their freeing enzyme(s) are, however, needed, and genotype differences in β-glucan degradability are to be evaluated over several field trials to confirm their heritability.

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