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Development of Limit Dextrinase in Germinated Barley (*Hordeum vulgare* L.)

Evidence of Proteolytic Activation

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Barley (*Hordeum vulgare* L.) that had been malted for 5 d developed only a small amount of bound (inactive) limit dextrinase, and very little free (active) enzyme was detected. Continuation of malting for up to 10 d only slightly increased the amount of both bound and free forms. Grain grown under conditions of ample moisture (wet grown) for 5 d produced a much higher amount of bound enzyme but a similarly low amount of free enzyme compared to malting conditions. After 10 d of growth there was a decrease in the amount of bound enzyme and a large increase in the amount of free enzyme, such that almost all of the enzyme was present in the free form. A more detailed study of limit dextrinase development in wet-grown grains revealed that a bound form was rapidly produced soon after germination. Five to 6 d after germination the amount of bound enzyme decreased rapidly and a very low amount was found in grains 9 d after germination. Meanwhile, a free form appeared slightly later and its initial rate of development was slow. At about 5 d after germination, precisely when the bound enzyme began to decrease, the free form increased rapidly, so that by 9 d after germination nearly all the enzyme was in the free form. The release of bound limit dextrinase in vitro occurred by proteolytic modification through the action of cysteine proteinases that were kept active or activated by the presence of reduced thiols in the extraction medium. The presence of cysteine proteinases was confirmed by inhibition studies using the inhibitors iodoacetamide, *N*-ethylmaleimide, antipain, and leupeptin. In addition, most of the bound form of limit dextrinase was soluble in 0.2 M sodium acetate buffer (pH 5.0) following extraction at 30°C for 16 h and centrifugation at 3000*g*.

Limit dextrinase, amylopectin 6-gluconohydrolase (EC 3.2.1.41) also known as pullulanase, hydrolyzes α-1-6-glucosidic links in amylopectin. In conjunction with other plant carbohydrases, starch in the barley (*Hordeum vulgare* L.) endosperm can be converted into Glc to provide the plant with an energy source. From a commercial point of view, particularly that of the distilling industry, if limit dextrinase could be increased in malted barley, this could lead to an increase in the hydrolysis of wort dextrins to fermentable sugars and, consequently, to an increase in the yield of alcohol, a conclusion that is supported by the work of Enevoldsen (1970), Lee and Pyler (1984), and Sissons et al. (1991).

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Very small amounts of limit dextrinase are reported to be present in barley at the time of grain ripening (Manners and Yellowlees, 1973; Lenoir et al., 1984; McClearly, 1992), but it has been shown to be synthesized following germination (Hardie, 1975) under the influence of GA_3 (Lee and Pyler, 1984). In contrast, limit dextrinase in rice (*Oryza sativa*) (Yamada, 1981; McCleary, 1992) and peas (*Pisum sativum*) (Vlodawsky et al., 1971) is present in the mature grain and pulse, albeit in a bound form that is converted to a free form by proteolytic enzymes after germination. In malted barley almost all limit dextrinase appears to be present in a bound form (Serre and Laurière, 1989), but it can be made active by extraction in the presence of reduced thiols (McCleary, 1992). Little is known about the proportions of bound-to-free-limit dextrinase during seedling growth in barley or about the mechanism of its release from a bound form in vitro under the influence of reducing agents. It has been suggested (Lee and Pyler, 1984) that the enzyme in malted barley occurs partially bound to some insoluble material in the grain, because the total flour was found to be more active than an extract from it.

Two hypotheses are proposed for the release of limit dextrinase from a bound form in vitro and are summarized in Figure 1. In the first hypothesis, it is proposed that reducing agents such as L-Cys act upon limit dextrinase per se by cleaving disulfide bonds, as suggested by Yamada (1981). This implies that limit dextrinase itself is a sulfydryl enzyme requiring free sulfydryl groups for activity. In the second hypothesis, it is suggested that a sulfydryl proteinase(s) is activated by reducing agents through cleavage of disulfide bonds or is kept active through the prevention of disulfide bond formation. It is this enzyme(s) that releases and/or activates limit dextrinase by proteolytic modification.

Because the terms free and bound are already widely used in the literature concerning limit dextrinase, they have been retained in this report. In this instance they are intended to be synonymous with the terms active and inactive and apply only to soluble forms, because study was carried out on soluble extracts from malted or wet-grown grains. Because the amount of bound enzyme is calculated by the difference from the total enzyme activity, it is acknowledged that it is not possible to conclude that the bound enzyme is completely inactive. It may be merely less active. It also follows that the

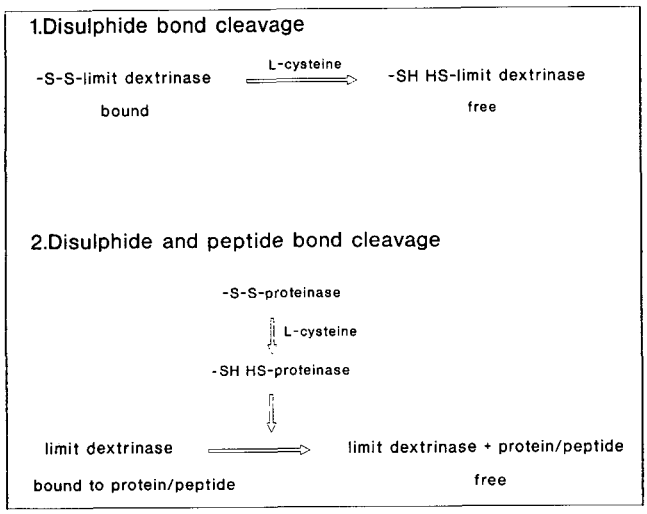

Figure 1. Two hypotheses for the release of limit dextrinase from a bound form. SH, Sulfhydryl.

activity apportioned to the free enzyme may be made up of fully active and partially active components.

The main objectives of this study, therefore, were to measure amounts of bound and free limit dextrinase in barley that had either been malted or wet grown and to determine the mechanism whereby reduced thiols enhance limit dextrinase in vitro.

Malted grains are defined as postgerminative grains subjected to controlled moisture conditions such that the potentialities of the embryo for digesting the endosperm are harnessed and then curtailed so that seedling growth is suppressed. Wet-grown grains are defined as postgerminative grains grown under conditions of ample water supply such that the endosperm is readily digested to promote rapid seedling growth.

Germinative energy has been recorded for all barleys. It is a measurement of the percentage of grains capable of germinating at the time and under the conditions of the test. It is generally taken as the percentage of grains that have germinated after 72 h at 18°C in 4 mL of water.

MATERIALS AND METHODS

Plant Material

The barley (*Hordeum vulgare* L.) cultivars used in this study were Camargue (grown in Scotland [1990 harvest]), Magie, Triumph, Golden Promise, and Tyne (a gift from Moray Firth Maltings [1991 harvest]), and Harrington (obtained as a malt flour from Canada [1991 harvest]).

Conditions of Malted and Wet-Grown Grains

Barley cv Camargue (germinative energy = 94%) was steeped in water at 16°C in the steeping cabinet of a Seeger micro-malting plant according to the following schedule: 8 h steep, 16 h air rest, 24 h steep. This process permitted the coleorhiza of most grains to rupture the seed coat, denoting that the grains had germinated. Thereafter, half the grains were transferred to the germination cabinet of a Seeger micro-malting plant and malted at 16°C under usual malting conditions, whereby grains were kept moist in a humid atmosphere but out of direct contact with water to ensure minimum development of shoot. The remaining grains were placed on damp paper towels in perforated trays that were covered by plastic bags to prevent moisture loss and grown in an incubator at 16°C. These grains were supplied with ample moisture by dipping trays in water every second day. At 5 and 10 d after germination, samples of malted and wet-grown grains were immediately plunged into liquid nitrogen and transferred to a deep freeze at −40°C to be subsequently freeze dried.

Four cultivars of barley, Magie, Triumph, Golden Promise, and Tyne (germinative energies = 98, 95, 99, and 99%, respectively), were steeped in distilled water in large beakers in an incubator at 18°C according to the following schedule: 6 h steep, 12 h air rest, 4 h steep. Thereafter, they were spread evenly on top of damp paper towels in perforated trays that were covered by plastic bags to prevent moisture loss and placed in an incubator in 18°C for 7 d. Grains were supplied with ample moisture by dipping trays in water every second day. Grains were removed on day 7, plunged into liquid nitrogen, and transferred to a deep freeze at −40°C to be subsequently freeze dried.

Barley cv Tyne (germinative energy = 99%) was steeped in distilled water in large beakers in an incubator at 18°C according to the following schedule: 6 h steep, 12 h air rest, 4 h steep. After steeping, only germinated grains were placed, coleorhiza end downward, onto dampened paper towels (25 grains per towel), spaced evenly at the middle along the length of the towel. A second damp towel was placed on top, and towels were rolled up and put into small beakers containing 3 mL of water. Beakers and towels were enclosed in plastic bags to prevent moisture loss, and no further water was added. Thirty-six replicates were prepared and at days 1 to 9 after steeping, four replicates were removed and pooled. Grains were plunged into liquid nitrogen, transferred to a deep freeze at −40°C, and subsequently freeze dried.

After freeze drying the roots and shoots were removed by sieving. Grains were kept intact until required for analysis, when they were ground for precisely 1 min to a fine powder in an electric coffee grinder (Braum, Barcelona, Spain) at room temperature.

Measurement of Limit Dextrinase

Limit dextrinase was measured spectrophotometrically utilizing the dyed substrate, Red Pullulan (Megazyme, North Rocks, New South Wales, Australia). This method is specific for limit dextrinase, which will hydrolyze α-1-6 linkages in water-soluble Red Pullulan, releasing low mol wt products. Addition of absolute alcohol to stop the reaction precipitated the remaining unhydrolyzed large mol wt substrate, whereas the released small mol wt dyed products remained soluble in the alcoholic supernatant and could be measured spectrophotometrically. Enzyme activity as measured by increase in A_{510} was proportional to enzyme concentration from A 0.1 to 2.0.

Details of the modified procedure are as follows. Replicates of 1 g of ground grains were weighed into capped bottles

and extracted in 3 mL of 0.2 M sodium acetate buffer (pH 5.0) with or without L-Cys (0.35%) for 16 h at 30°C with constant shaking. The supernatant obtained after centrifugation at 3000*g* was used as the source of limit dextrinase. The assay was conducted with aliquots of 0.5 mL of supernatant incubated with 0.25 mL of Red Pullulan (2 g/100 mL of 0.5 M KCl) for 20 min in test tubes placed in a water bath at 40°C. The reaction was stopped upon addition of 1.25 mL of absolute alcohol. Tubes were left for 10 min at room temperature and then centrifuged at 3000*g* at 5°C, and supernatants were read immediately at 510 nm. Blanks were prepared by adding alcohol to samples before the addition of Red Pullulan. Enzyme activities are expressed as increased A_{510} in 20 min g^{-1} of sample or per 25 grains. Expression of activities on a grain basis in one experiment was considered to give a more accurate account of enzyme development because it eliminated the effect of decrease in grain weight as seedling growth increased.

A measurement of the free (active) enzyme was obtained by extraction of grains in buffer alone and the total activity by extraction in buffer containing L-Cys. The amount of bound (inactive) enzyme was calculated by difference.

Dialysis Treatment

Replicates of aliquots (2.5 or 5 ml) of extract supernatants containing limit dextrinase were dialyzed in tubing (18/22 inches; Medical, International, London, England) against 3 L of 0.2 M sodium acetate buffer (pH 5.0) with or without L-Cys (0.35%). The dialysis media were changed once after 20 h. A dilute solution of methylene blue was used to test the presence or absence of L-Cys in the dialysis bags.

Chemical Inhibitors

Iodoacetamide and *N*-ethylmaleimide (Sigma) inhibit β-amylase, a known sulfydryl enzyme (Spradlin and Thoma, 1970). Antipain and leupeptin (Sigma) are specific inhibitors of sulfydryl proteinases such as Cys endopeptidases (Guerin et al., 1992), and they inhibit the release of β-amylase from a bound form (Sopanen and Laurière, 1989).

RESULTS

Development of Limit Dextrinase in Vivo

Malted versus Wet-Grown Grains

Malting grains from cv Carmargue for 5 d resulted in the production of very little free limit dextrinase so that total enzyme activity, measured in the presence of L-Cys, was almost entirely due to the presence of a bound form (Fig. 2). The extension of malting up to 10 d did not increase amounts of either free or bound enzyme.

Grains that had been wet grown for 5 d produced only low amounts of free enzyme similar to that found after malting. On the other hand, there was an almost 2-fold increase in the amount of total enzyme compared to malting, due entirely to an increase in the amount of bound enzyme produced. Wet growth of grains for up to 10 d resulted in a considerably large increase in the amount of free enzyme and a small decrease in the amount of bound enzyme.

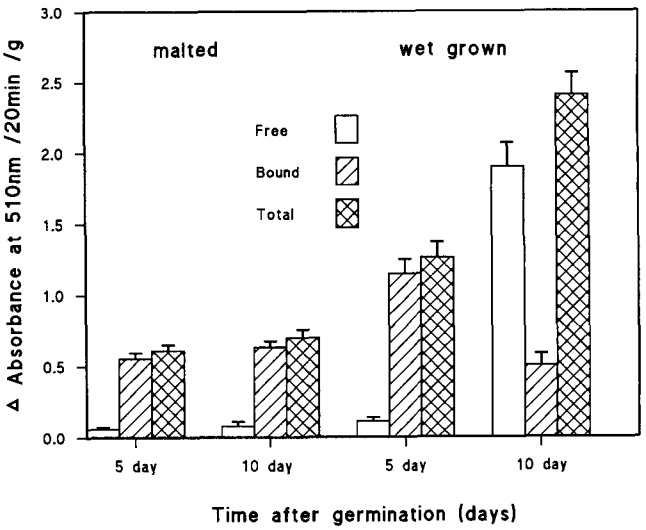

Figure 2. Amounts of free, bound, and total limit dextrinase in malted and wet-grown cv Carmargue. Grains were either malted for 5 or 10 d in a Seeger micro-maltings plant at 16°C or wet grown for 5 or 10 d on damp paper towels in an incubator at 16°C. Limit dextrinase was extracted in 0.2 M sodium acetate buffer (pH 5.0) with or without L-Cys (0.35%) for 16 h at 30°C and measured in the supernatant after centrifugation at 3000*g* for 10 min.

Amounts of Bound, Free, and Total Limit Dextrinase in Wet-Grown Grains from 0 to 9 d after Germination

When grains from the cv Tyne were wet grown, conditions greatly encouraged root and shoot growth so that by day 9 the length of shoots was, on average, 9 cm. When enzyme production was investigated on a daily basis, a bound form of limit dextrinase developed about 2 d after germination, increasing rapidly until 5 to 6 d, when its production decreased sharply (Fig. 3). A free form also appeared about 2 d after germination, but its activity only increased slowly during the early stages of seedling growth. At about 5 d after germination, precisely when the bound enzyme began to decrease, the activity of free enzyme increased rapidly so that by day 9 almost all of the enzyme was in the free form. The total amount of enzyme was also observed to decrease rapidly after 6 d.

Influence of Stage of Development on the Production of Free Limit Dextrinase

When grains were wet grown for 7 d after germination, three of the cultivars developed a greater amount of free enzyme than bound enzyme (Table I). The cv Tyne was the exception; it possessed a greater proportion of bound-to-free enzyme, and the level of bound enzyme was also significantly higher in the cv Tyne than in the other three cultivars. The low amount of free enzyme in the cv Tyne did not appear to be due to any lack of total enzyme because it possessed the second highest amount, but rather to most of the enzyme remaining in the bound form. Although no direct measurements were made of the shoot length, it was, nevertheless, observed that length of shoots increased in the following

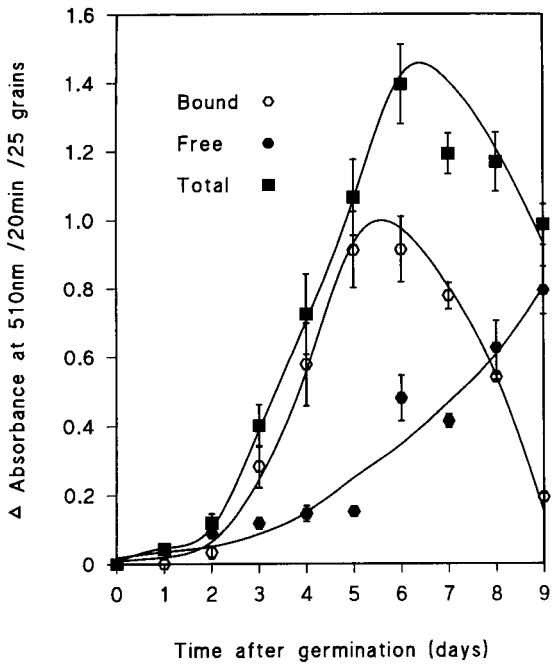

Figure 3. Development of free, bound, and total limit dextrinase in wet-grown cv Tyne. Grains were wet grown for 9 d on damp paper towels in an incubator at 18°C. Limit dextrinase was extracted in 0.2 M sodium acetate buffer (pH 5.0) with or without L-Cys (0.35%) for 16 h at 30°C and measured in the supernatant after centrifugation at 3000*g* for 10 min.

order: Tyne ≪ Golden Promise < Magie < Triumph. By far the least shoot growth occurred in cv Tyne. These results tend to confirm observations from the previous experiments, suggesting that the free enzyme is produced at a much later stage of seedling growth and most probably arising through modification of the bound enzyme.

Table I. *Activities of free, bound, and total limit dextrinase in four cultivars of barley wet grown for 7 d*

Limit dextrinase was extracted from freeze-dried grains in 0.2 M sodium acetate buffer (pH 5.0) with or without L-Cys (0.35%) for 16 h at 30°C. After centrifugation at 3000*g* for 10 min, aliquots of supernatants were assayed for limit dextrinase activity as described in "Materials and Methods." Free enzyme was measured after extraction in buffer alone, total enzyme by extraction in buffer containing L-Cys, and bound enzyme was calculated by difference. SE between varieties for free, bound, and total = 0.043, 0.037, and 0.045, respectively. Statistical significance of difference between varieties for free, bound, and total = $P < 0.001$, $P < 0.001$, $P < 0.001$. No. of replicates = 4.

Variety	Limit Dextrinase Activity			
	Free	Bound	Total	Free
	ΔA_{510} 20 min^{-1} g^{-1}			%
Magie	1.138	0.511	1.650	69
Triumph	1.127	0.676	1.803	62
Golden Promise	1.000	0.549	1.550	64
Tyne	0.816	0.953	1.770	46

Properties of Limit Dextrinase in Vitro

Solubility of Bound Limit Dextrinase

In all three experiments A, B, and C, addition of L-Cys (0.35%) to the supernatant of a 16-h buffer extract that had been centrifuged for 10 min at 3000*g* caused an increase in limit dextrinase activity during the subsequent 24 h (Table II). This increase in activity, however, was not sufficiently large enough to equal that in supernatants from grains that had been initially extracted with buffer containing L-Cys (0.35%). Supernatants devoid of L-Cys showed much less limit dextrinase activity that increased only slightly during the corresponding 24 h. It would seem, therefore, that, although a substantial amount of the bound enzyme was soluble, a small amount of activity could not be accounted for and may be attributed to insoluble material that remained behind in the discarded precipitate. After removal of the soluble form by extraction in buffer, several attempts to obtain the insoluble form by reextraction of the precipitate with buffer containing L-Cys failed.

Stability of Limit Dextrinase in an Oxidizing Atmosphere

The presence of L-Cys during the extraction of limit dextrinase from grains of the cv Triumph caused an enhanced activity of limit dextrinase, the difference between treatments with and without L-Cys being highly significant (Table III). It was observed after dialysis that activities of all extracts were lower because the dialysis bags had increased in volume by more than 2-fold and this was found to have occurred

Table II. *Solubility of bound limit dextrinase from wet-grown and malted barley*

Limit dextrinase was extracted from grains in 0.2 M sodium acetate buffer (pH 5.0) with or without L-Cys (0.35%) for 16 h at 30°C. After centrifugation at 3000*g* for 10 min, aliquots of supernatants were removed. To reduce by half the replicates of supernatants of buffer extracts, L-Cys was added to give a final concentration of 0.35%. Aliquots of all treatments were assayed for limit dextrinase activity as described in "Materials and Methods" after a further 2, 6, and 24 h. A, 5-d wet-grown cv Carmargue; B, 5-d malted cv Carmargue; C, 9-d malted cv Harrington. No. of replicates = 4.

Extraction Medium	Limit Dextrinase Activity		
	16 + 2 h	16 + 6 h	16 + 24 h
	ΔA_{510} 20 min^{-1} g^{-1}		
Experiment A			
Buffer	0.070	0.110	0.125
Buffer	0.246[a]	0.650[a]	0.678[a]
Buffer + L-Cys	1.140	1.286	1.171
Experiment B			
Buffer	0.012	0.038	0.045
Buffer	0.040[a]	0.298[a]	0.440[a]
Buffer + L-Cys	0.506	0.674	0.610
Experiment C			
Buffer	0.056	0.070	0.144
Buffer	0.373[a]	0.533[a]	0.861[a]
Buffer + L-Cys	0.976	1.006	1.189

[a] L-Cys added to supernatant.

Table III. *Effect of dialysis on limit dextrinase activity in 9-d wet-grown barley cv Triumph*

Grains were extracted in 0.2 M sodium acetate buffer (pH 5.0) with or without L-Cys (0.35%) for 16 h at 30°C. After centrifugation at 3000g for 10 min, aliquots of supernatants were dialyzed against 3 L of 0.2 M sodium acetate buffer (pH 5.0) with or without L-Cys (0.35%) such that L-Cys was removed from one of the treatments, and the other two were kept unchanged. Limit dextrinase was assayed after 20 and 44 h of dialysis. SE between treatments for undialyzed, dialyzed 20 h, and dialyzed 44 h = 0.028, 0.029, and 0.037, respectively. Statistical significance of difference between means for undialyzed, dialyzed 20 h, and dialyzed 44 h = $P < 0.001$, $P < 0.001$, $P < 0.001$. No. of replicates = 8.

Extraction Medium	Limit Dextrinase Activity			
	Undialyzed		Dialyzed	
			20 h	44 h
	ΔA_{510} 20 min^{-1} g^{-1}			
Buffer + L-Cys	1.799	Buffer + L-Cys→	1.681	1.362
Buffer + L-Cys	1.760	—Buffer→	1.632	1.584
Buffer	1.048	—Buffer→	0.891	0.840

equally with all treatments. This dilution was taken into account when expressing activities after dialysis by multiplying all *A* readings by a factor of 2.5. When L-Cys was removed by dialyzing against buffer only, thus permitting a reversion to oxidizing conditions, no loss in limit dextrinase activity was observed. This suggests that limit dextrinase was stable in oxidizing conditions. The difference between treatments originally extracted in the presence and absence of L-Cys remained highly significant, both after 20 and 44 h of dialysis.

To test for the presence of oxidizing or reducing conditions, aliquots from dialysis bags were treated with 2 drops of a dilute solution of methylene blue. This solution remained blue in the absence of L-Cys (oxidizing conditions) and became colorless in the presence of L-Cys (reducing conditions). Thus, the complete removal of L-Cys by dialysis and subsequent reversion to oxidizing conditions was confirmed. Furthermore, it was consistently observed that in the absence of L-Cys extracts turned brown, indicating the possible oxidation of polyphenols, whereas extracts containing L-Cys remained pale. When L-Cys was removed by dialysis, these extracts were duly observed to turn brown, indicating the reversion to oxidizing conditions and possible oxidation of polyphenols.

Table IV. *Effect of iodoacetamide and N-ethylmaleimide on the release of bound limit dextrinase from 9-d wet-grown cv Triumph*

Grains were extracted in 0.2 M sodium acetate buffer (pH 5.0) with or without L-Cys (0.35%) in the presence or absence of iodoacetamide (0.40%) or *N*-ethylmaleimide (0.40%) for 16 at 30°C. Limit dextrinase activity was assayed in aliquots of supernatants after centrifugation at 3000g as previously described. SE = 0.063. Statistical significance of effects for L-Cys, $P < 0.001$; iodoacetamide, $P < 0.001$; *N*-ethylmaleimide, $P < 0.001$; interaction, $P < 0.001$. No. of replicates = 6.

Extraction Medium	Limit dextrinase activity		
	No inhibitor	Inhibitor present	
		Iodoacetamide	*N*-Ethylmaleimide
	ΔA_{510} 20 min^{-1} g^{-1}		
Buffer + L-Cys	1.949	1.238	1.331
Buffer	1.298	1.307	1.222

Table V. *Effect of iodoacetamide on limit dextrinase activity after its release in 9-d wet-grown cv Triumph*

Grains were extracted in 0.2 M sodium acetate buffer (pH 5.0) for 16 h at 30°C with or without L-Cys (0.35%) in the presence or absence of iodoacetamide (0.40%). Iodoacetamide (0.40%) was added to half the replicates of treatments containing L-Cys after 16 h extraction. Limit dextrinase was measured in aliquots of supernatants centrifuged at 3000g of two treatments at 16 h of extraction and all treatments at 16 + 24 h of extraction. SE for 16-h extraction = 0.128. SE for 16 + 24-h extraction = 0.073. Statistical significance of effects for 16 and 16 + 24 h of extractions = $P < 0.001$ and $P < 0.001$, respectively. No. of replicates = 3.

Extraction Medium	Limit Dextrinase Activity		
	Extraction times		
	16 h		16 + 24 h
	ΔA_{510} 20 min^{-1} g^{-1}		
Buffer + L-Cys			2.280
Buffer + L-Cys	2.327	—Iodoacetamide→	2.395
Buffer + L-Cys + iodoacetamide	1.165		1.022
Buffer			1.252

Inhibition of Release of Bound Limit Dextrinase by Iodoacetamide and N-Ethylmaleimide

Both iodoacetamide and *N*-ethylmaleimide form irreversible complexes with sulfydryl compounds and are inhibitors of Cys proteinases. These inhibitors would reveal whether L-Cys was required to maintain limit dextrinase per se in an active form, or whether it was required to keep a Cys proteinase(s) active so that bound limit dextrinase could be released by proteolytic modification.

L-Cys significantly increased the activity of limit dextrinase in extracts from 9-d wet-grown cv Triumph (Table IV). The addition of iodoacetamide or *N*-ethylmaleimide completely prevented this increased activity, the effects of both inhibitors being highly significant. There was also a highly significant interaction in that the inhibitors had no effect on the free enzyme. When iodoacetamide was added initially to the extraction buffer, again, it was shown to prevent the increase of limit dextrinase activity in the presence of L-Cys, and the effect was highly significant (Table V). However, when iodoacetamide was added after the bound enzyme had been released, it had no effect on limit dextrinase activity. Nor did iodoacetamide have any effect on the activity of the free enzyme. These results strongly suggest that limit dextrinase is not a sulfhydryl enzyme and that limit dextrinase release is mediated by a sulfhydryl-requiring process.

Table VI. *Effect of antipain on the release of bound limit dextrinase from 9-d and 7-d wet-grown cv Triumph*

Grains were extracted in 0.2 M sodium acetate buffer (pH 5.0) for 16 h at 30°C with or without L-Cys (0.35%) and in the presence or absence of antipain (0.004%). Limit dextrinase activity was assayed in aliquots of supernatants after centrifugation at 3000*g* as previously described. SE for 9 d = 0.106. SE for 7 d = 0.044. Statistical significance of effects for both 9 and 7 d: L-Cys, $P < 0.001$; antipain, $P < 0.001$; interaction, $P < 0.001$. No. of replicates for 9 and 7 d = 6.

Extraction Medium	Limit Dextrinase Activity			
	9 d		7 d	
	No inhibitor	Antipain	No inhibitor	Antipain
	ΔA_{510} 20 min^{-1} g^{-1}			
Buffer + L-Cys	2.039	1.152	1.676	0.613
Buffer	1.110	0.980	0.183	0.170

Inhibition of Release of Bound Limit Dextrinase by Antipain and Leupeptin

To provide further evidence of the possible role of a Cys proteinase(s) in the release of bound limit dextrinase, antipain and leupeptin, specific inhibitors of Cys proteinases, were added during limit dextrinase extraction. The increased activity of limit dextrinase in the presence of L-Cys was completely inhibited by antipain in the 9-d wet-grown grains and to a slightly lesser extent with the 7-d wet-grown grains of the cv Triumph, which was highly significant in both instances (Table VI). There was also a highly significant interaction in that antipain had no inhibitory effect on the free enzyme, its effect being entirely due to the inhibition of the release of the bound form.

Leupeptin at a concentration of 0.001% only partially prevented, but at concentrations of 0.002 and 0.004% fully prevented the enhanced activity of limit dextrinase in the presence of L-Cys, with a significant effect (Table VII). There was also a highly significant interaction due to the fact that leupeptin had no inhibitory effect on the activity of the free enzyme.

Table VII. *Effect of leupeptin on the release of bound limit dextrinase from 9-d wet-grown cv Triumph*

Grains were extracted in 0.2 M sodium acetate buffer (pH 5.0) with or without L-Cys (0.35%) in the presence or absence of leupeptin (0.001, 0.002, and 0.004%). Limit dextrinase activity was assayed in aliquots of supernatants centrifuged at 3000*g* as previously described. SE = 0.146. Statistical significance of effects, $P < 0.001$. No. of replicates = 2.

Extraction Medium	Limit dextrinase activity			
	No inhibitor	Inhibitor present		
		0.001% Leupeptin	0.002% Leupeptin	0.004% Leupeptin
	ΔA_{510} 20 min^{-1} g^{-1}			
Buffer + L-Cys	1.847	1.631	1.104	1.199
Buffer	1.002			0.934

Irreversible and Reversible Inhibition

The possibility was considered that the inhibitors exerted their effect merely by inactivating L-Cys. Although this is an unlikely explanation in the case of antipain and leupeptin because both are peptides and inhibit Cys proteinases specifically by mimicking substrates, it is a plausible explanation in the case of iodoacetamide and *N*-ethylmaleimide because these inhibitors can irreversibly bind to all sulfhydryl compounds including L-Cys. It was decided that dialyzing extracts containing inhibitors from 7-d wet-grown cv Triumph might clarify this issue.

In both experiments A and B when aliquots of supernatants containing limit dextrinase were dialyzed against buffer, it was confirmed that the greatest activity of limit dextrinase was found in the presence of L-Cys but in the absence of inhibitors (Table VIII). Also, there was very little loss in activity of limit dextrinase when L-Cys was subsequently removed by dialysis. When supernatants containing limit dextrinase extracted in buffer alone were subsequently dialyzed in the presence of L-Cys, their activity increased by 138 and 228% in experiments A and B, respectively. On the other hand, no significant increase in activity was observed when limit dextrinase was extracted in buffer with iodoacetamide and subsequently dialyzed in the presence of L-Cys. This suggested that iodoacetamide had irreversibly bound to a Cys proteinase(s) and that it was not L-Cys that was limiting. In contrast, there were larger and more significant increases in experiment B when supernatants containing limit dextrinase extracted in the presence of antipain were subsequently dialyzed in buffer containing L-Cys. This suggested that antipain, a reversible inhibitor, had been removed during

Table VIII. *Effect of dialysis on limit dextrinase activity in extracts containing iodoacetamide or antipain from 7-d wet-grown cv Triumph*

Aliquots of supernatants from grains that were extracted for 16 h at 30°C in 0.2 M sodium acetate buffer (pH 5.0) with or without L-Cys (0.35%) and in the presence or absence of iodoacetamide (0.40%) or antipain (0.004%) were dialyzed for 20 h in the same buffer in the presence or absence of L-Cys (0.35%). No. of replicates = 6.

Extraction Medium	Limit Dextrinase Activity		
	Dialysis medium		Activity increase
	Buffer	Buffer + L-Cys	
	ΔA_{510} 20 min^{-1} g^{-1}		%
Experiment A			
Buffer + L-Cys	0.646	0.708	10
Buffer + L-Cys + iodoacetamide	0.048	0.076	68
Buffer	0.148	0.363	138
Buffer + iodoacetamide	0.068	0.089	31
Experiment B			
Buffer + L-Cys	0.476	0.492	8
Buffer + L-Cys + antipain	0.172	0.399	132
Buffer	0.065	0.213	228
Buffer + antipain	0.065	0.212	226

dialysis, leaving the Cys proteinase(s) free to be acted on by L-Cys.

DISCUSSION

When barley, cv Triumph, was malted for 5 d, a process specifically designed to suppress seedling growth, very low amounts of limit dextrinase were produced in the grain (Fig. 2). Moreover, the enzyme was present only in a bound form. Furthermore, when malting was continued for up to 10 d, the production of limit dextrinase did not increase. On the other hand, when the same cultivar was grown under conditions of ample water supply such that vigorous seedling growth took place, much higher amounts of limit dextrinase were produced after 5 d, albeit in a bound form. After 10 d there was considerable increase in the amount of total enzyme produced, which now existed mainly in the free form, and the amount of bound enzyme decreased. These results suggest that at some time between days 5 and 10 the bound enzyme is rapidly converted into the free enzyme.

When limit dextrinase production was investigated more closely on a daily basis with the cv Tyne, a bound form of the enzyme increased rapidly soon after germination, and the appearance of a free form increased slowly. It was noted, moreover, that a rapid increase in the amount of free enzyme occurred precisely at a time when the bound enzyme was rapidly decreasing, thus suggesting that the bound form is converted to the free form similar to limit dextrinase in rice (Yamada, 1981) and peas (Vlodawsky et al., 1971). Yamada (1981) showed that the conversion of a bound to a free form was sufficient to explain the increase in limit dextrinase activity after germination, whereas Iwaki and Fuwa (1981) and Palmiano and Juliano (1972) did not find that the increase in limit dextrinase activity was due to the action of proteolytic enzymes on a preexisting form.

The question of de novo synthesis of limit dextrinase in rice is controversial. In peas the conversion to the free form in vivo has been suggested to occur by the action of proteolytic enzymes (Shain and Mayer, 1968). Results of this study suggest that the mechanism whereby the bound form in germinated barley is released in vitro is also by proteolysis, more specifically by the action of Cys proteinases. These have already been implicated in the release of bound β-amylase: when Sopanen and Laurière (1989) extracted β-amylase in the presence of the Cys proteinase inhibitors, antipain or leupeptin, they failed to obtain a release of the bound form. Limit dextrinase per se was not inhibited by sulfhydral compounds, implying that free sulfhydral groups are not required for its activity and that it is not a sulfhydral enzyme. After L-Cys was withdrawn by dialysis (Table IV), the resulting oxidizing atmosphere did not destroy activity; this also reinforced the suggestion that limit dextrinase is not a sulfhydryl enzyme. Furthermore, limit dextrinase in mature wheat kernels was not inhibited by *p*-chloromercuribenzoate and mercuric chloride at a concentration of 10^{-4} M, indicating, that the sulfhydral groups of this enzyme in another grain were not essential for catalytic activity (Kruger and Marchylo, 1978). Thus, the requirement of L-Cys would appear to be solely for the purpose of keeping active or activating a Cys proteinase(s). After limit dextrinase had become proteolytically modified, any inactivation of Cys proteinase(s) by reversion to oxidizing conditions upon dialysis, or the subsequent addition of iodoacetamide, did not reduce limit dextrinase activity, presumably because the proteinase(s) was no longer necessary. The inhibiting effects of the sulfhydral compounds iodoacetamide, *N*-ethylmaleimide, and, more specifically, the Cys proteinase inhibitors antipain and leupeptin greatly support the notion that the activation of limit dextrinase results from the action of a Cys proteinase(s).

The development of limit dextrinase in different barley cultivars was reported in early work (Longstaff and Bryce, 1991). The two cultivars Triumph and Golden Promise showed the development of the bound form of the enzyme before the appearance of the free form, in agreement with observations in this study. In the case of the cv Golden Promise, however, very little free enzyme was produced even after a period of 10 d of seedling growth. Moisture supplied to grains was much less in the previous experiment, and less seedling growth took place. This may have limited the production of Cys proteinases in the case of cv Golden Promise. Stage of seedling growth as defined by the length of shoot correlated reasonably well with conversion of bound enzyme to free enzyme (Table I). Dell'Aquila (1992) reported a close relationship between rate of protein synthesis and the level of hydration in germinating wheat embryos, and, moreover, protein synthesis in embryo tissue was reduced when the embryo was subjected to osmotic stress.

Kilning at 60°C compared to freeze drying barley that had been wet grown for 9 d decreased the amount of bound enzyme but had little effect on the free enzyme (Longstaff and Bryce, 1991). The explanation put forward at that time was that the "releasing factor" was probably more heat labile than limit dextrinase. Because malt Cys proteinases are generally considered to be relatively heat labile (Bamforth and Quain, 1989) this previous observation of the effect of kilning supports the theory of proteolytic modification of limit dextrinase. Moreover, recent work by Daussant et al., (1987), in which limit dextrinase was assayed using an immunochemical procedure, has implied that this enzyme is more heat resistant than was previously acknowledged.

The fact that β-amylase also exists in a bound and free form makes comparison between it and limit dextrinase inevitable. In the case of β-amylase there is a bound form that is insoluble through its attachment to protein on the surface of starch granules (Hara-Nishimura *et al.*, 1986; MacGregor et al., 1971). However, because β-amylase is itself a sulfhydral enzyme, it has been suggested that aggregates formed by interchain disulfide bonds also account for the origin of some bound β-amylase, and it is this form that can be extracted in the presence of strong reducing agents because of the ability of these agents to break disulfide linkages (Shewry *et al.*, 1988). Thus, reduced thiols in the absence of Cys proteinases can keep β-amylase active because they act on β-amylase per se. Reduced thiols do not appear to act on limit dextrinase per se (Tables IV–VII). Furthermore, there is now convincing evidence for several soluble forms of protein-bound β-amylase (Shinke and Mugibayashi, 1971). Sopanen and Laurière (1989) and Guerin et al. (1992) have reported that the activity of soluble, also referred to as "free" β-amylase, can be greatly increased with a combination of

thiols and endopeptidases but only slightly with thiols alone, and the latter authors have suggested that this is due to proteolytic modification. Consequently, the form of limit dextrinase that has been depicted in this study as bound yet soluble may be similar to the free β-amylase reported by Guerin et al. (1992). Results from this study suggest that only a small amount, one-third or less of limit dextrinase, is insoluble (Table II). Attempts to reextract the precipitate with L-Cys and measure the insoluble enzyme were not successful. This failure might be explained if the Cys proteinase(s) was itself soluble, because it would have been inadvertently removed during the initial extraction of soluble limit dextrinase and also in subsequent water washings of the precipitate. Alternatively, it might be argued that all of the bound limit dextrinase is soluble and the subsequent addition of L-Cys to a buffer extract failed to result in maximum limit dextrinase activity because some of the Cys proteinase(s) had suffered irreversible inactivation.

Recently, Li et al. (1992) isolated a debranching enzyme from the leaves of sugar beet that showed a high activity with pullulan as substrate, low activity with soluble starch and amylopectin, and no activity with native starch granules. Furthermore, the purified enzyme appeared to be activated by DTT and inhibited by *N*-ethylmaleimide. This enzyme, contrary to limit dextrinase in germinated barley, may contain sulfhydral groups that are necessary for maximum catalysis.

Hardie (1975) reported that limit dextrinase was synthesized de novo in barley after germination. Although this study lends support to his finding, it does not confirm it. An alternative explanation is also possible for the increase in bound limit dextrinase reported here. Cys proteinases are known to be synthesized de novo after germination in most seeds including barley (Mikola and Enari, 1970; Mikola, 1983; Ari et al., 1988). The inability to detect any bound limit dextrinase (or only very low amounts) at the time of grain ripening could be due to the absence of Cys proteinases at this stage. The notion that limit dextrinase might not be required in an active form immediately after germination is readily conceivable because raw starch is not hydrolyzed by it and amylopectin is hydrolyzed only slowly. The best substrates for limit dextrinase are the small mol wt dextrins remaining after the hydrolysis of starch by α- and β-amylases. These dextrins have been well characterized by Enevoldsen and Bathgate (1969).

In conclusion, this study has provided evidence of the conversion of a bound form of limit dextrinase to a free form during the stage of seedling growth. In vitro this conversion is dependent on the action of a Cys proteinase(s) that requires the presence of L-Cys or other reduced thiols in the extract medium to maintain its activity.

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