

NOTE

ENZYMES

Bound and Free Forms of Barley Limit Dextrinase¹

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Limit dextrinase (LD) is a cereal starch-degrading enzyme that hydrolyzes the α -1,6 bonds in amylopectin, debranching the molecule to linear dextrans. There is evidence for the existence of a soluble inhibitor of LD activity (Macri et al 1993) that binds a portion of the extractable enzyme, referred to as latent. The unbound portion retains activity and is referred to as free. There also exists a small quantity of enzyme activity (EA) associated with the grist that can be released with reducing agents that is referred to as bound (Sissons 1992). To obtain maximum activity during in vitro assays, the use of a sulfhydryl reducing agent in the extract buffer and a long extraction time are necessary, which suggests that the release of latent LD is a slow process requiring reducing conditions (Longstaff and Bryce 1993).

The purpose of this study was to investigate the release of the bound forms of LD and measure changes in the quantity and activity of LD during germination.

MATERIALS AND METHODS

Barley Samples and Germination

Barley samples (*Hordeum vulgare* L. cv. Schooner) from a 1990 crop grown at the Waite Agricultural Research Institute, South Australia, were used. Seeds were surface-sterilized for 4 min in 3% hypochlorite solution, washed in sterile water, blotted dry, and placed into sterile petri dishes containing two sheets of filter paper and 4 ml of sterile, distilled water. The seeds were germinated in the dark at 15°C for periods of up to seven days and then freeze-dried. Before milling, the roots and shoots were removed.

Preparation of Free and Bound LD

Extracts were prepared from barley seed and seven-day germinated barley. Samples were ground in a Udy mill (0.5-mm screen), and the flour was extracted in a sodium acetate buffer (100 mM, pH 5.0) for 5 hr at 30°C. The extract was centrifuged (1,500 \times g, 15 min), and the supernatant and three washes of the pellet were combined. (This fraction contained both the free and latent forms.) Release of the LD in the pellet was achieved by resuspending in acetate buffer plus 2-mercaptoethanol (1 mM and 10 mM) or acetate plus papain (1 mg/ml) (P4762 from papaya latex and 2-mercaptoethanol, Sigma Chemical Co., St. Louis, MO) or acetate plus cysteine (20 mM) for 19 hr at 30°C. The resulting suspension was centrifuged, and the supernatant was assayed for activity.

Release of Latent LD

A flour prepared from seven-day germinated barley seeds was extracted with acetate buffer for 5 hr at 30°C and centrifuged. Portions of the supernatant were treated with cysteine solutions to give final concentrations of 0, 10, 20, 60, and 100 mM. These solutions were incubated at 30°C for either 10 min, or 1, 5, or 19 hr, then assayed for EA.

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Development of LD During Germination

Barley seeds were germinated as described previously and harvested at intervals up to seven days. The seeds were freeze-dried and ground in a Udy mill after the roots and shoots were removed. Extracts of free and bound LD were prepared.

Assay of LD

Enzyme protein was measured specifically using an enzyme-linked immunosorbent assay (ELISA) (Sissons et al 1992) to quantify immunological activity (IA). EA was measured using a dyed red-pullulan substrate (McCleary 1992).

RESULTS AND DISCUSSION

Release of Bound Forms of LD

Incubation of the soluble LD fraction from a seven-day barley extract with cysteine for 19 hr had no significant effect upon IA, but it increased EA (Table I). The increase in EA was accelerated and enhanced by cysteine. A concentration of 20 mM was equally as effective as a concentration of 100 mM (data not shown). The increase in EA due to cysteine could be due to: 1) a conformational change in the structure of LD that promotes its activity; purified LD isolated from the leaves of sugar beet was activated by dithiothreitol, suggesting the enzyme may contain sulfhydryl groups necessary for catalysis (Li et al 1992); 2) the activation by cysteine of cysteine proteinases (Longstaff and Bryce 1993) that dissociates the LD from another soluble component, resulting in an increase in EA (Macri et al 1993). Even without cysteine, some release occurs, suggesting this process is time-dependent and possibly temperature-dependent. The absence of any change in IA after cysteine treatment of the extract could be explained, because the relatively small inhibitor would not be likely to alter the IA of the LD.

To effectively release LD from the pellet fraction, a reducing agent or papain was required (Table II). Cysteine gave the best solubilization; 2-mercaptoethanol was more effective at 10 mM than it was at 1 mM. Papain had very little effect.

After a 16-hr extraction of seven-day germinated barley seeds, the EA in the supernatant, three washings of pellet, and the resuspended pellet was 5.5, 1.1, 0.5, 0.3, and 0.3 ($A_{590}/20$ min/ml), respectively. Incubation of these fractions with papain and 2-mercaptoethanol for 20 hr at 30°C increased activity to 26.4, 3.7, 0.9, 0.5, and 5.5, respectively. The difference between the activities in the supernatant and the three washings of pellet before and after treatment represents the amount of latent LD. Based

TABLE I
Release of Latent Limit Dextrinase from Barley^a

Incubation (hr)	Enzyme Activity (mU/ml)		Immunological Activity (μ g/g)	
	Cysteine		Cysteine	
	—	+	—	+
0.2	17	21	78	80
1	21	32	ND ^b	ND
5	24	66	ND ^b	ND
19	35	76	80	82

^aData are means of two observations.

^bNot determined.

on this data, the proportions of free, latent, and bound LD in this sample of germinated barley were 19.9, 65.8, and 14.3%, respectively.

TABLE II
Release of Bound Limit Dextrinase from Barley

	Immunological Activity ($\mu\text{g/g}$) ^a
Buffer (B)	1.3 \pm 0.2
B + 1 mM 2-ME ^b	4.9 \pm 0.7
B + 10 mM 2-ME	26.9 \pm 3.1
B + 1 mM 2-ME + Papain	8.2 \pm 1.5
B + 10 mM 2-ME + Papain	24.4 \pm 1.8
B + 20 mM Cysteine	32.6 \pm 3.8

^aData are the means of four observations with standard deviations ($n = 4$).

^b2-ME = 2-mercaptoethanol.

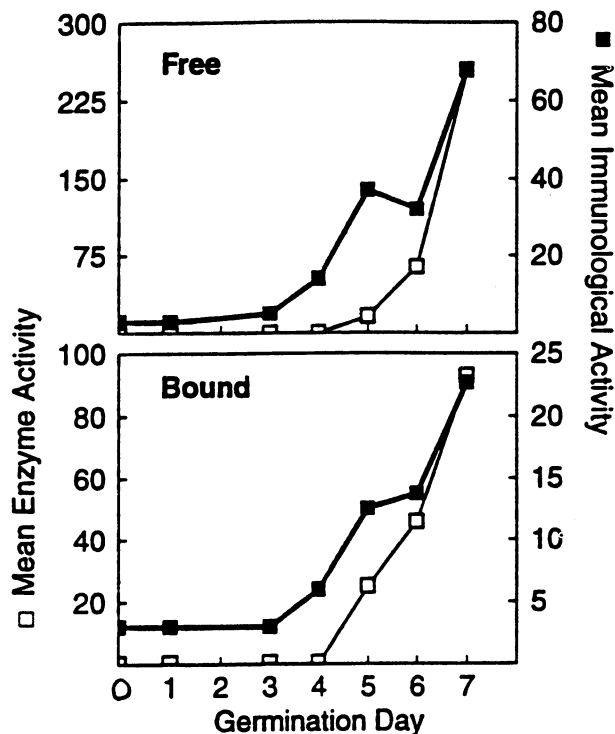


Fig. 1. Development of free and bound limit dextrinase during germination. Immunological activity ($\mu\text{g/g}$) (■); enzyme activity (mU/ml) (□). Data are means \pm standard deviation (too small to be seen) of three replicates.

Development of LD During Germination

A small amount of LD protein is present in ungerminated barley (Sissons et al 1993), but this has no measurable EA (Fig. 1). This could be due to the lower sensitivity of the red-pullulan assay when compared to the ELISA (Sissons et al 1992), or perhaps the LD present in barley is bound to an enzyme inhibitor (Macri et al 1993), so that the complex has no activity, but is detectable by the ELISA. An increase in the amount of enzyme protein in both fractions did not occur until day 3, whereas EA increased after day 4. The pattern of increase was similar but the free enzyme increased to a greater extent. Longstaff and Bryce (1991) reported the bound enzyme appeared before the free. In their study, bound was described as the arithmetic difference in activities between an extract prepared with (total) and without (free) cysteine in the extraction buffer. This method of determining bound LD would measure both soluble and insoluble forms.

CONCLUSIONS

A significant portion of LD extracted from germinated barley is in a latent form. The IA data shows that LD is extracted, but it is not activated or released. This activation process depends upon the extraction conditions. Further study is required to elucidate the mechanism involved.

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