Carbohydrate Research, 90 (1981) 153–157
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

Note

Inactive debranching-enzyme in rice seeds, and its activation

JIRO YAMADA
Department of Agricultural Chemistry, Faculty of Agriculture, Hokkaido University, Sapporo 060 (Japan)

(Received June 23rd, 1980; accepted for publication, August 5th, 1980)

During the germination of seeds, many kinds of active enzymes appear: some of these are synthesised *de novo*, whereas others are produced by the activation of zymogen proteins. In barley, for example, alpha-amylase is synthesised in germinating seeds, whereas a zymogen form of beta-amylase is present in ungerminated seeds[1]. Debranching enzymes that hydrolyse the (1→6)-α-D linkages in starch have been obtained from germinated cereals[2–5]. Debranching enzyme (amylopectin-1,6-glucosidase), which is present[6] in a precursor form in peas, is released by protease action during germination, and this enzyme activity is not directly controlled by the amount of growth substances in the pea cotyledons[7]. On the other hand, the activity of the debranching enzyme (R-enzyme) in rice increases significantly during germination[8]. It is improbable that this latter enzyme is produced by the action of protease on pre-existing, inactive forms of the enzyme, but the enzyme was induced by incubation in gibberellic acid. Stimulation of debranching-enzyme (limit dextrinase) production by gibberellic acid occurs in malt[9].

The occurrence of an inactive debranching-enzyme (pullulanase) in rice seeds and its activation *in vitro* is now reported.

Incubation of rice flour with 1,4-dithiothreitol (dithiothreitol) generated high pullulanase activity (Fig. 1). Maximum activity occurred after 24-h incubation with ≥6mM thiol. All of the thiols in Table I at 10mM activated the debranching enzyme, suggesting that the process involved cleavage of the disulfide bonds. This view was confirmed by the effect of other reductants (see Table I). Sodium sulfite and sodium dithionite were very effective, but sodium borohydride was moderately effective in spite of the pH (~10) which was unfavourable for enzyme stability.

The debranching enzyme was also activated to similar extents by proteases (Table II), *e.g.*, papain, α-chymotrypsin, and trypsin.

Fig. 2 shows the rate of formation of pullulanase activity in rice flour by sodium dithionite and papain at 4° and 30°. Endogenous activation of the debranching enzyme occurred, as reflected by incubation with 100mM sodium phosphate buffer (pH 7.2) at 30°. Significantly, the debranching enzyme was activated more rapidly

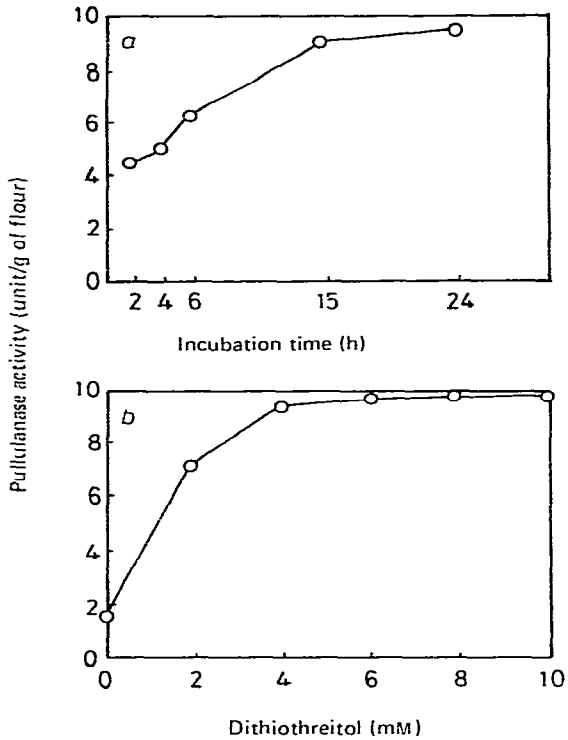

Fig. 1. Activation of debranching enzyme by dithiothreitol at 4° (see Experimental); (*a*) 5mM, (*b*) various concentrations during 24-h incubation.

TABLE I

ACTIVATION OF DEBRANCHING ENZYME BY REDUCTANT[a]

Reductant	*Pullulanase activity (unit/g of flour)*	*Reductant*	*Pullulanase activity (unit/g of flour)*
Control (100mM buffer)	1.76	Sodium sulfite	9.42
Cysteine	9.85	Sodium dithionite	9.77
Dithiothreitol	9.55	Sodium borohydride[b]	5.91
Mercaptoethanol	9.16	Sodium nitrite	0.17
Glutathione	7.38	Ascorbic acid	0

[a]Hulled-rice flour (1 g) was added to 100mM sodium phosphate buffer containing 10mM reductant (pH 7.2, 5 mL), and incubated at 4° for 24 h. After centrifugation, the resulting supernatant solution was assayed for pullulanase activity. [b]Sodium phosphate buffer (100mM, pH 7.2) was added after activation with an aqueous solution of sodium borohydride.

by papain than by the endogenous system at 30°. Furthermore, direct activation by papain was suggested by this increase of pullulanase activity without a lag phase. The effect of increase of temperature on the rate of activation by dithionite was much less than that for the endogenous system and papain.

TABLE II

ACTIVATION OF DEBRANCHING ENZYME BY PROTEASE[a]

Papain (mg/mL)	*Pullulanase activity (unit/g of flour)*	*α-Chymotrypsin (mg/mL)*	*Pullulanase activity (unit/g of flour)*	*Trypsin (mg/mL)*	*Pullulanase activity (unit/g of flour)*
0.125	8.00	0.65	6.90	12.5	6.75
0.025	8.25	0.13	8.25	2.5	7.40
0.005	5.25	0.026	6.50	0.5	6.10
0.001	5.00	0.0052	6.30	0.1	5.55

[a]Papain (Sigma, 30 units/mg of protein), α-chymotrypsin (Sigma, Type II, 40 units/mg), and trypsin (Merck, 0.2 Anson unit/g). Hulled-rice flour (1 g) was added to 100mM sodium phosphate buffer containing one of the proteases (pH 7.2, 5 mL), and incubated at 30° for 15 h. After centrifugation, the resulting supernatant solution was assayed for pullulanase activity.

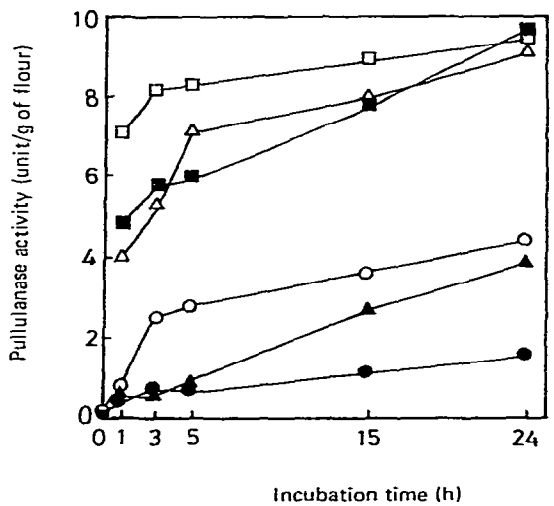

Fig. 2. Effect of temperature on activation of debranching enzyme; incubation (see Experimental) at 4° (●, ■, ▲) and 30° (○, □, △) with 100mM sodium phosphate buffer (pH 7.2, 5 mL; ●, ○) alone, or containing sodium dithionite (10mM; ■, □) or papain (0.025 mg/mL; ▲, △).

In further experiments, rice flour was incubated successively with papain at 30° and then with sodium dithionite at 4° (Table III). If the first-activation period was short (resulting in lower activity), the activity consequent on the second activation was high, but the total activation was approximately constant. Similar results were obtained regardless of the order in which the activators were used. Very low activity was observed when the buffer solution was used without an activator in the second activation. Also, the activity obtained after simultaneous incubation of the rice flour with papain and dithionite for 15 h at 30° (9.1 units/g of flour) was similar to that (8.8 units/g of flour) obtained when dithionite only was used.

TABLE III

ACTIVATION OF DEBRANCHING ENZYME BY PAPAIN AND DITHIONITE[a]

First activation			*Second activation*			*Total activation*
Activator	*Incubation (h)*	*Pullulanase activity (unit/mL)*	*Activator*	*Incubation (h)*	*Pullulanase activity (unit/mL)*	*Total pullulanase activity (unit)*
Papain (0.025 mg/mL) at 30°	0.5	0.64	Dithionite (10mM) at 4°	24	0.94	1.58
	1	0.84		24	0.74	1.58
	15	1.59		24	0.25	1.84
	0.5	0.64	Buffer at 4°	24	0.34	0.98
	1	0.91		24	0.19	1.10
	15	1.62		24	0.10	1.72
Dithionite (10mM) at 4°	1	1.10	Papain (0.025 mg/mL) at 30°	15	0.89	1.99
	3	1.28		15	0.87	2.15
	24	1.94		15	0.31	2.25
	1	1.10	Buffer at 30°	15	0.34	1.44
	3	1.26		15	0.32	1.58
	24	1.94		15	0.22	2.16

[a]After incubation of a mixture of hulled-rice flour (1 g) and 100mM sodium phosphate buffer containing the first activator (pH 7.2) (total vol., 5 mL), the mixture was centrifuged and the resulting supernatant solution assayed for pullulanase activity. The precipitate was washed six times with 100mM sodium phosphate buffer (pH 7.2, 5 mL each), and the second activator, dissolved in the same buffer, was added to the precipitate until the total volume reached 5 mL. After incubation, the mixture was centrifuged and the resulting supernatant solution assayed for pullulanase activity.

During incubation of the rice flour (1 g) with pullulan (0.1%) in a McIlvaine buffer (pH 5.6, 5 mL) at 25° for 10 min, a slight increase of the reducing sugar (0.15 μmol, corresponding to 0.75 unit/g of flour) was observed. This value was negligibly small compared to the total activity (~10 units/g of flour) obtained after activation with dithiothreitol. Thus, it is concluded that the insoluble, inactive debranching-enzyme in rice flour is activated and solubilised by reductants that are capable of severing the disulfide linkages, and by proteases, and that the active debranching-enzyme in germinated rice seeds is not synthesised *de novo*.

EXPERIMENTAL

Materials. — Hulled, non-glutinous rice seeds (*Oryza sativa* L. cv. "Yukara") were ground in a Wiley mill through a 1-mm sieve, and then ground thoroughly in a china mortar to extract the enzyme completely.

Enzyme activity. — The debranching-enzyme activity was measured at 25° in a digest (1 mL) consisting of suitable amounts of the enzyme (0.2 mL), pullulan (0.2%, 0.5 mL), and a McIlvaine buffer (pH 5.6, 0.3 mL). The enzyme activity was expressed in terms of the pullulanase activity, and 1 unit of pullulanase was defined as the amount of debranching enzyme which increased the reducing power[10] to equal 1 μmol of maltotriose per min[11].

Enzyme activation. — Rice flour (1 g) was added to 100mM sodium phosphate buffer containing one of the activating agents (pH 7.2, 5 mL), and stored at 4° with reductant or 30° with protease. After centrifugation (10,000*g*, 10 min), the resulting supernatant solution, which was diluted if necessary, was assayed for pullulanase activity.

REFERENCES

1 G. Harris, in A. H. Cook (Ed.), *Barley and Malt: Biology, Biochemistry and Technology*, Academic Press, New York and London, 1962, pp. 583–694.
2 D. J. Manners and D. Yellowlees, *Staerke*, 23 (1971) 228–234.
3 D. G. Hardie, D. J. Manners, and D. Yellowlees, *Carbohydr. Res.*, 50 (1976) 75–85.
4 I. Maeda, Z. Nikuni, H. Taniguchi, and M. Nakamura, *Carbohydr. Res.*, 61 (1978) 309–320.
5 J. Yamada, H. Tanba, and M. Izawa, *J. Fac. Agric. Hokkaido Univ.*, 60 (1980) 10–22.
6 Y. Shain and A. M. Mayer, *Physiol. Plant.*, 21 (1968) 765–776.
7 L. Vlodawsky, E. Harel, and A. M. Mayer, *Physiol. Plant.*, 25 (1971) 363–368.
8 E. P. Palmiano and B. O. Juliano, *Plant Physiol.*, 49 (1972) 751–756.
9 D. J. Manners, G. H. Palmer, G. Wilson, and D. Yellowlees, *Biochem. J.*, 125 (1971) 30P–31P.
10 M. Somogyi, *J. Biol. Chem.*, 195 (1952) 19–23.
11 J. Yamada and M. Izawa, *Agric. Biol. Chem.*, 43 (1979) 37–44.