Note

Purification of Debranching Enzyme from Mature Rice Seeds*

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Previously, we have observed high activity in the debranching enzyme of immature rice seeds, from which the highly purified state of the enzyme could be obtained.¹⁾ Therefore, immature rice seeds are considered to be a good source of the debranching enzyme; however, they are not always available when needed. On the other hand, a large quantity of the inactive debranching enzyme, which could be activated and solubilized by some reductants or endopeptidases, has been observed in ungerminated, mature seeds, in spite of the very low actual activity of debranching enzyme in the seeds.²⁾ In this paper we describe a purification of the debranching enzyme from polished rice seeds.

The debranching enzyme activity was expressed in terms of the pullulanase activity,¹⁾ 1 unit of which was defined as the amount of pullulanase needed to increase the reducing power³⁾ to an equivalent of 1 μ mol of maltotriose per min. The protein, determined by the method of Lowry *et al.*,⁴⁾ was calibrated for bovine serum albumin.

Polished rice seeds were ground by a Wiley mill through a 1 mm sieve. All purification procedures were performed at 0~4°C. Five kg of the rice flour were mixed with 25 liters of 10 mM sodium dithionite solution, swelled by standing for 1 hr, and homogenized in a mortar. After 24 hr standing with occasional agitation, the solution was decanted and filtered by suction. A supernatant solution obtained from the residue by centrifugation was combined with the former solution ("the crude extracts," 22 liters).

In order to adsorb the enzyme in the crude extracts without any pre-treatment on the DEAE-cellulose, an aqueous solution of sodium dithionite without a buffer substance was used for the extraction. One hundred g DEAE-cellulose which had been pre-equilibrated with 0.01 M sodium phosphate buffer (pH 6.2) was added to the crude extracts. After several hr agitation, the cellulose was filtered, washed with 0.01 M sodium phosphate buffer (pH 6.2) to remove any unadsorbed proteins, and the resultant solution was suctioned. The enzyme protein was eluted

from the cellulose with 0.1 M sodium phosphate buffer (pH 6.2) ("DEAE-cellulose eluate," 8.52 liters). This adsorption procedure was effective not only for the purification of the enzyme but also for decreasing the volume of the sample solution, and consequently, for facilitating the centrifugation of the salted-out precipitates.

The precipitates produced by the addition of 1.4 kg ammonium sulfate were discarded, and 1.2 kg ammonium sulfate were added to the supernatant. The resultant precipitates were separated by centrifugation and dissolved in 0.1 M sodium phosphate buffer (pH 7.2) ("the ammonium sulfate ppt.," 117 ml). The enzyme solution was dialyzed against 0.01 M sodium phosphate buffer (pH 7.2) ("the dialyzed solution," 125 ml). The dialyzed solution was concentrated by an Amicon cell equipped with a Diaflo PM 30 membrane, and the solvent was replaced by 0.01 M sodium phosphate buffer (pH 7.2) containing 0.25 M sodium chloride ("the concentrate," 26.6 ml).

The solution was applied to a Sephadex G-100 column (2.6 $\times$ 96 cm), and the enzyme was eluted with 0.01 M sodium phosphate buffer (pH 7.2) containing 0.25 M sodium chloride at an upward-flow rate of 12 ml per hr. The fractions of 4 ml, numbers 69~87, in which high pullulanase activity was observed, were collected and then concentrated by the Amicon cell ("Sephadex G-100 eluate," 25 ml). The specific activity of the "Sephadex G-100 eluate" was 21.4 pullulanase units/mg protein, which was as high as that of the purified debranching enzyme of milky stage rice seeds previously obtained;¹⁾ however, a vague protein band besides the clear main band was observed on the disc-electrophoretic gel, showing that further purification of the enzyme is required.

The enzyme solution was dialyzed against 0.01 M sodium acetate solution (200 ml $\times$ 3 times), and then applied to a CM-cellulose column (2.0 $\times$ 5 cm) which had been pre-equilibrated with 0.01 M sodium acetate buffer (pH 5.0). After washing the column with 100 ml of the same buffer, the enzyme protein was eluted with 0.1 M sodium acetate buffer (pH 5.0). The fractions of 5 ml, numbers 8~11, showing high pullulanase activity were collected, and an equal volume of 0.1 M sodium acetate solution was added ("CM-cellulose eluate," 40 ml). The solution was concentrated by the Amicon cell and an immercible ultrafiltration apparatus (Japan Millipore Ltd.) ("the purified enzyme solution," 2 ml).

The purified enzyme had no amylase, maltase, and Q-enzyme activities and homogeneous in gel-filtration, disc-electrophoresis, and isoelectricfocusing. The molecular weight (*ca.* 70,000) determined by gel-filtration and the optimum pH of 5.6 were identical to those of the milky stage enzyme.¹⁾ Substrate specificities of the enzyme were also similar to those of the milky stage enzyme. The enzyme rapidly hydrolyzed pullulan and scarcely hydrolyzed phytoglycogen, similar to other higher plant debranching enzymes. Kinetic data similar to those of the milky stage enzyme⁵⁾ was also obtained. In addition, the

* Studies of Rice Debranching Enzyme. Part VII. For Part VI, see *Agric. Biol. Chem.*, 45, 747 (1981).

TABLE I. PURIFICATION PROCESS OF DEBRANCHING ENZYME ACTIVATED BY SODIUM DITHIONITE

Preparation	Total activity (unit)	Recovery (%)	Specific activity (unit/mg protein)
Crude extracts ^a	26,000	100	1.15
DEAE-cellulose eluate	11,100	42.7	5.20
Ammonium sulfate ppt.	10,100	38.8	15.0
Dialyzed solution	10,400	40.0	22.2
Concentrate	9,470	36.4	23.0
Sephadex G-100 eluate	4,470	17.2	21.4
CM-cellulose eluate	1,205	4.63	29.8

^a Polished-rice flour (5 kg) was used.

FIG. 1. Ouchterlony Double Diffusion Analysis of the Enzyme.

Immunodiffusion was carried out with a 1.5% agarose plate containing 0.06 M veronal buffer (pH 8.6) on a glass slide at 37°C for 24 hr. After removing the unprecipitable proteins with physiological saline solution for 3 days, the proteins were located visually with Amido Black 10B. (A) Rabbit antiserum against the purified milky-stage enzyme, (a) milky-stage enzyme (antigen), and (b) sodium dithionite-activated enzyme.

two enzymes showed immunological identity (Fig. 1).

In conclusion, a highly purified debranching enzyme was easily obtained from polished rice seeds, and its characteristics were similar to those of the milky stage rice enzyme isolated in a previous investigation.^{1,5)}

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