NOTE ON THE PRESENCE OF DEBRANCHING ENZYMES IN IMMATURE WHEAT KERNELS[1]

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Cereal Chem. 55(4): 529-533

Debranching enzymes hydrolyzing α(1,6)-D-glucosidic interchain linkages in amylopectin and in dextrins have been detected in many cereal seeds such as sorghum, maize, oats, rye, and wheat (1). The levels in ungerminated seeds, except oats, are low but increase during germination (1–8). The reason that the enzyme occurs in ungerminated cereals is unknown but could be related to incipient sprouting of a small amount of the total cereal crop or to synthesis of the enzyme during kernel development.

Isoelectric focusing on polyacrylamide slabs demonstrates the presence of two debranching enzymes in immature wheat kernels from early stages of development.

MATERIALS AND METHODS

Wheat Cultivars

Wheat cultivars were grown in the Canada Department of Agriculture experimental plots at Glenlea, Man., during the summer of 1975 and were sampled at various growing stages following anthesis. The intact heads were excised, frozen, and stored until analysis. Cultivars examined had widely varying quality and genetic characteristics. They consisted of Neepawa, Park, Cypress, and Manitou, which are hard red spring (HRS) wheats of excellent breadmaking quality; Glenlea, an HRS utility wheat; RL 4137, a sprout-resistant HRS wheat; Wascana and Wakooma, durum wheats; Idaed, a soft white spring wheat; and Pitic 62 a semidwarf soft red spring wheat.

Chemicals and Equipment

Pullulan was purchased from Hayashibora Laboratories, Okayama, Japan. The LKB 2117 Multiphor electrofocusing apparatus and ampholine were purchased from LKB Produkter, Bromma, Sweden. β-Limit dextrin was prepared from waxy maize starch as described previously (9,10).

Extraction of Enzyme

Kernels or dissected parts of kernels were extracted by grinding in a mortar and pestle with cold 0.05*M* acetate buffer, pH 5.5, and a small amount of sand followed by centrifugation at 25,000 × *g* for 15 min. For isoelectric focusing, 50 kernels were extracted with 4 ml of buffer, and for viscometric assay of activity, 100 kernels were extracted with 16 ml of buffer.

[1]Paper No. 391 of the Canadian Grain Commission, Grain Research Laboratory, 1404 - 303 Main Street, Winnipeg, Man. R3C 3G9.

0009-0352/78/000062$03.00/0

Isoelectric Focusing and Detection of Enzyme

Isoelectric focusing was done on polyacrylamide slabs using an LKB multiphor apparatus with a pH 4–6 or 3.5–11.0 ampholine gradient. Gel preparation, sample application, and electrofocusing conditions were as MacGregor (11) described. Following electrofocusing, enzymes were detected by the method of Doane (12) as modified by MacGregor et al (13).

RESULTS

The presence of debranching enzymes in immature wheat first became evident during a study on α-amylases using polyacrylamide slab isoelectric focusing. Detection of α-amylases by this technique consisted of incubating the isoelectric focused slab against a β-limit dextrin in agar or polyacrylamide slab (14,15). Addition of a solution of iodine and potassium iodide to the β-limit dextrin slab then indicated the location of α-amylase isozymes as colorless bands against a pink background.

Two bands atypical of amylases were present. For example, isoelectric focusing between pH 4 and 6 of the immature HRS wheat, RL 4137, at 11 and 14 days after flowering gave the typical pattern shown in Fig. 1. The three prominent main α-amylase components were designated α-1, α-2, and α-3, following previous terminology (15). In addition, the faint presence of two additional bands was detected; these bands had pI values of 4.32 and 4.40 as determined by a pH electrode on the surface of the gel and were designated as d-1 and d-2. Band d-2 did not become clear, but took on a purplish color that increased in intensity with increasing incubation time. Band d-1 became clear, but always had a purplish tinge. These results suggested that d-1 and d-2 might be debranching enzymes in which the cleavage of α(1,6)-D-glucosidic bands of amylopectin β-limit dextrin allowed a greater incorporation of iodine.

Isoelectric focusing of extracts of dissected tissues at six developmental stages of ripening indicated that only the endosperm contained bands d-1 and d-2. Zymograms of these bands also were much clearer in the endosperm, because they were not overshadowed by the large amount of pericarp and green layer α-amylase (10). Heat treatment at 60° C for 15 min of endosperm wheat extracts did not affect the intensity of the bands on electrofocusing. At 70° C, however, activity was completely lost. After heat treating at 60° C, band d-1 no longer became colorless on incubation with β-limit dextrin, but became purplish in the same way as band d-2. This indicated that another heat-labile amylase, possibly β-amylase, was acting in concert with band d-1. Thus, cleavage of the α(1,6)-D-glucosidic linkages in β-limit dextrin by the debranching enzymes allowed further degradation by the heat-labile amylase.

An extract of immature RL 4137 at 20 days after flowering was heat treated at 60° C to remove the heat-labile amylase associated with enzyme d-1 and electrofocused. Sections of gel containing d-1 and d-2 were excised, macerated in the presence of a small amount of water, allowed to sit for 3 hr, and centrifuged. The eluted bands d-1 and d-2 next were incubated with a solution of 1% β-limit dextrin in 0.05*M* acetate, pH 5.5, for varying periods of time, followed by addition of a solution of iodine and potassium iodide. Scanning of the wavelength over the range 400–700 nm indicated that a slight shift in absorption maximum from lower to higher wavelength occurred. In addition, an increase in

iodine-staining power was reflected by an increase in the absorption maximum. Incubation of bands d-1 and d-2 with 1% pullulan in 0.05*M* acetate buffer, pH 5.5, containing 0.1% bovine serum albumin released reducing sugars slowly, as determined by the neocuproin-HCl method (16). Both of the above findings indicated that enzymes d-1 and d-2 were debranching enzymes catalyzing the hydrolyses of α(1,6)-D-glucosidic linkage.

The effect of chemicals on components d-1 and d-2 was assessed by adding them to electrofocused gels before detection of isozymes on β-limit dextrin slabs. *p*-Chloromercuribenzoate and mercuric chloride at a concentration of $10^{-4}M$ were without effect, indicating that sulfhydryl groups were not essential for catalytic activity; the heat-labile amylase component associated with component d-1 was inactivated, however, by this treatment. Ethylenediaminetetraacetic acid, $10^{-2}M$, and 20% sodium hexametaphosphate were also without effect, indicating that the enzyme is unlike wheat α-amylase, which requires calcium ion for stability and activity (15,17). Addition of sweet potato β-amylase had a large

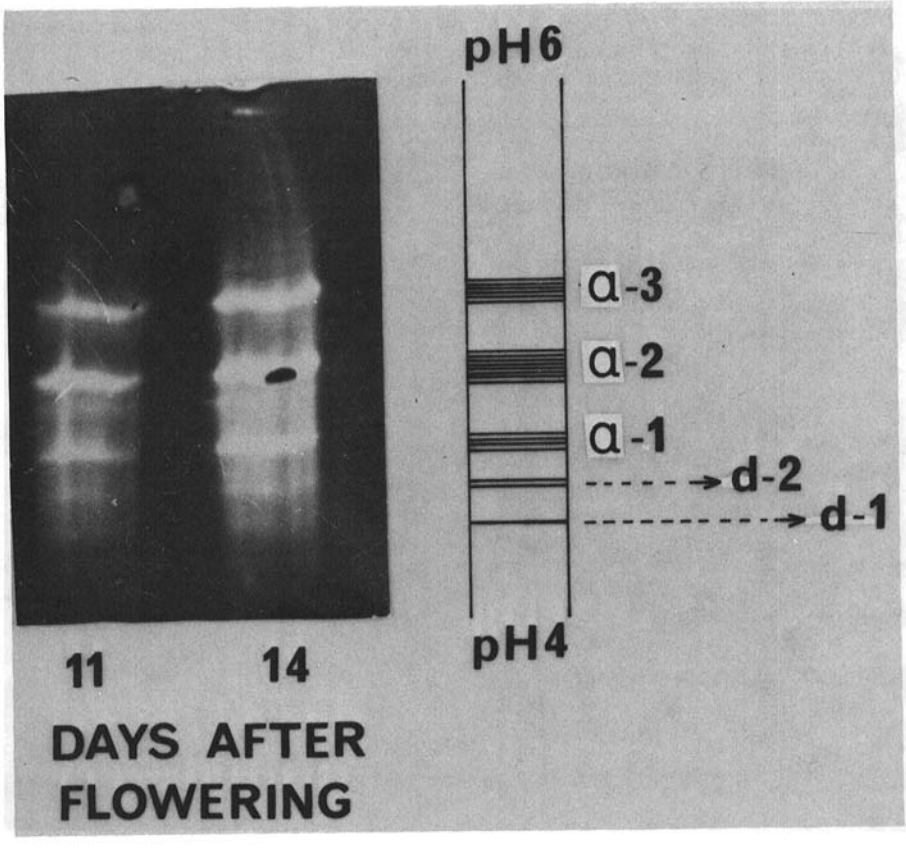

Fig. 1. Polyacrylamide slab isoelectric focusing between pH 4 and 6 of carbohydrate-degrading enzymes in immature RL 4137 wheat kernels 11 and 14 days after flowering. Enzymes were detected by incubation against a β-limit dextrin slab.

synergistic effect on the breakdown of β-limit dextrin by the debranching enzymes. As shown in Fig. 2, colorless bands formed rapidly in zymograms in the presence of β-amylase.

Isoelectric focusing confirmed the presence of both debranching enzymes in all ten wheats analyzed. The individual pI values were identical in all cultivars. The enzymes formed just after anthesis and remained until full maturity, at which time the levels seemed to decrease slightly. The cultivars Park and RL 4137 also were assayed viscometrically for debranching activity throughout kernel development using pullulan as the substrate (18). Because of the extremely low specific viscosities, a nonlinear relationship between reciprocal specific viscosity and enzyme concentration was obtained. The qualitative results, however, confirmed the results obtained with isoelectric focusing.

DISCUSSION

This is the first report that debranching enzymes exist at early stages in the development of wheat kernels. Debranching enzymes are present in the ungerminated kernels of many cereals such as barley, maize, rye, and oats, and such enzymes probably are also synthesized at early stages of kernel development. Unlike immature wheat α-amylase, which exists in the pericarp

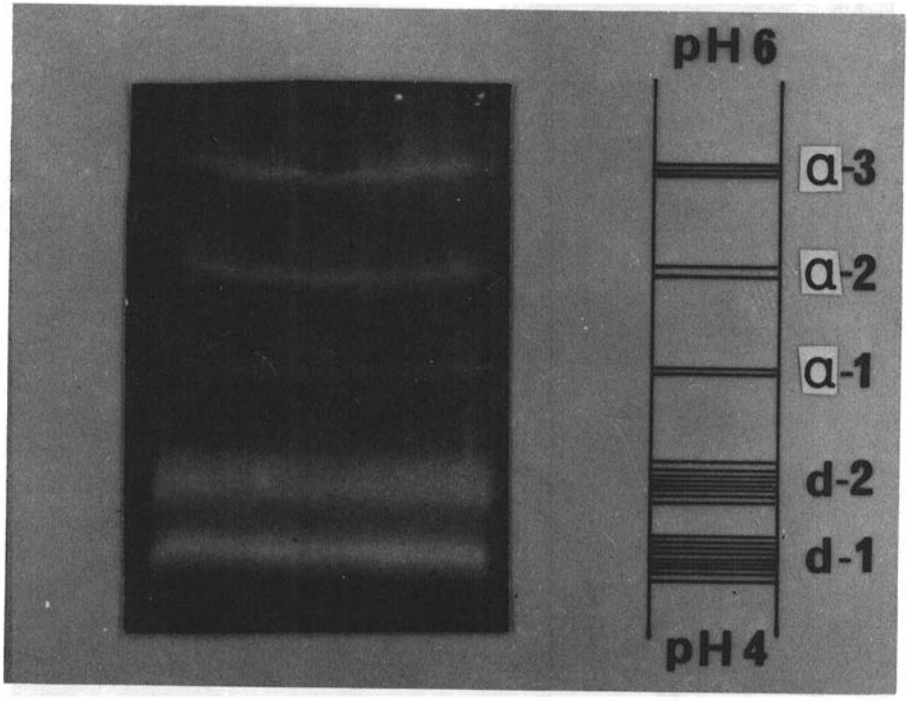

Fig. 2. Polyacrylamide slab isoelectric focusing between pH 4 and 6 of carbohydrate-degrading enzymes in the endosperm of immature RL 4137 wheat kernels 20 days after flowering. Enzymes were detected by incubation against a β-limit dextrin slab containing excess sweet potato β-amylase.

and seed coat layers, the debranching enzymes are located in the endosperm. Because the main starch component of the wheat kernel of the mature plant is also synthesized in this tissue, the precise role that the debranching enzymes play in this process is of considerable interest. The enzymes would certainly fit into Erlander's (19) hypothesis that starch is produced from a phytoglycogen precursor via the participation of a debranching enzyme. In any event, the preliminary properties described for these enzymes provide a basis for a comprehensive study of the role of enzymes in the carbohydrate metabolism of the developing wheat kernel.

Acknowledgment

We wish to thank E. Ngai for his competent technical assistance.

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[Received January 11, 1978. Accepted April 11, 1978]