Occurrence and Characterization of a UDP-glucose:hydroxamic Acid Glucosyltransferase Isolated from Wheat (*Triticum aestivum*) Seedlings

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Cyclic hydroxamic acid glucosides are present at high concentrations immediately after germination in wheat (*Triticum aestivum* L.). Changes in the activity of UDP-Glucose:cyclic hydroxamic acid glucosyltransferase (EC 2.4.1.-) in wheat were investigated using the cyclic hydroxamic acids 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) and its 7-methoxy derivative (DIMBOA) as sugar acceptors. Glucosyltransferase activity on both substrates was detected in dry seeds, with activity increasing after imbibition, peaking in shoots and roots 36-48 hours after imbibition and decreasing thereafter. The transience of glucosyltransferase activity was concurrent with the transient occurrence of the hydroxamic acid glucosides [Nakagawa E., Amano T., Hirai N., and Iwamura H. (1995) *Phytochemistry* **38**, 1349-1354], suggesting that glucosyltransferases regulate the accumulation of hydroxamic acid glucosides in wheat seedlings. Two peaks in activity of UDP-Glucose:DIMBOA glucosyltransferase were detected using a Mono Q column, indicating the presence of at least two isozymes of this glucosyltransferase. The enzyme in the major peak was purified about 1500-fold and shown to be in a monomeric form with a molecular mass of 47 or 49 kDa. The enzyme reacted strongly with DIMBOA, less so with DIBOA. The enzyme of the minor peak on the Mono Q chromatogram, which was also a monomeric enzyme with a molecular mass of 47 kDa, showed similar substrate specificity to that of the major peak enzyme.

Introduction

Many plants accumulate various secondary metabolites in the form of glucosides. The glucosylation of these compounds changes their chemical nature and biological activity, and thus plays an important role in the regulation of the physiological functions.

The cyclic hydroxamic acids (Hxs), 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) and its 7-methoxy analogue (DIMBOA), are the major secondary metabolites in some gramineous plants including wheat, maize, and rye (Niemeyer, 1988), They are involved in the defense systems against pathogens and hervibores, based on their antimicrobial and antifeeding activities (Niemeyer, 1988). In intact tissue, they are present as 2-*O*-β-glucopyranosides (HxGlcs), an inactive form of Hx. When tissue is damaged mechanically by infection, HxGlcs are readily hydrolyzed by a glucosidase, and glucose and toxic aglycones are released. In wheat, maize, and rye, HxGlcs have been shown to occur at high level soon after germination, and are thought to function in the defense systems of nonautotrophic plants (Nakagawa *et al.,* 1995; Ebisui *et al.*, 1998; Sue *et al.*, 2000 a).

The biosynthetic pathway of Hxs branches from that of tryptophan at the point of indole (Desai *et al.*, 1996). DIBOA, the major Hx species in rye, is biosynthesized from indole through four sequential reactions by monooxygenases (cytochrome P450s) (Frey *et al.*, 1997), and DIMBOA, the predominant Hx species in wheat and maize, is considered to be synthesized from DIBOA *via* 7-hydroxy-DIBOA (Glawschnig *et al.*, 1997). Recently, we have shown that the two P450 enzymes that catalyze the first and the last reactions in the four P450 steps and HxGlc-glucosidase are highly active soon after germination (Sue *et al.*, 2000 b; Tanabe *et al.*, 1999). Glucosyltransferase is the enzyme that catalyzes the final reaction to HxGlc and is thus considered to be the key enzyme in the regulation of the Hxs levels in plants. Although

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glucosyltransferase has been partly characterized in maize and rye, the activity of this enzyme in wheat has only been measured using crude extracts. As part of our investigation into the expression mechanisms of Hxs in nonautotrophic seedlings of gramineous plants, we examined the occurrence of UDP-Glc:Hx glucosyltransferase in wheat and partially purified the enzymes from shoots.

Materials and Methods

Plant materials and preparation of cyclic hydroxamic acids (Hxs)

Wheat (*Triticum aestivum* L.) seeds were germinated and grown as described previously (Sue *et al.*, 2000b). 2,4-Dihydroxy-1,4-benzoxazin-3-one (DIBOA) and its 2-*O*-β-glucoside (DIBOA-Glc) were isolated from shoots of 2–3-day-old rye (*Secale cereale* L.) and 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) and its 2-*O*-β-glucoside (DIMBOA-Glc) were prepared from shoots of 2-day-old maize (*Zea mays* L.) as described previously (Sue *et al.*, 2000b). 2-Hydroxy-1,4-benzoxain-3-one (HBOA) and its 7-methoxy analogue (HMBOA) were chemically synthesized by the methods of Honkanen and Virtanen (1960), and their glucosides (HBOA-Glc and HMBOA-Glc) were prepared by reducing DIBOA-Glc and DIMBOA-Glc, respectively, as described previously (Sue *et al.*, 2000b).

Measurement of UDP-Glc:Hx glucosyltransferase activity in wheat seedlings

Changes in glucosyltransferase activity were measured using crude enzyme solution prepared from wheat seedlings. The shoots and roots were removed separately and frozen in liquid nitrogen. They were then ground to powder and homogenized in 5 volumes of 50 mM tris(hydroxymethly)-aminomethane-HCl buffer (Tris-HCl buffer, pH 7.5) containing 4 mM 2-mercaptoethanol (2-ME), followed by centrifugation at 12,000 *g* for 15 min. The resulting supernatant was subjected to ultrafiltration using a microcon-10 (Millipore) to remove endogenous Hxs, and was used as crude enzyme solution. Glucosyltransferase activity in dry and imbibed seeds was also measured using crude extracts. Every seed was divided into two pieces (embryo and endosperm), and an enzyme solution prepared from each part as described above. Glucosyltransferase activity was determined by incubating the crude enzyme solution in 50 mM Tris-HCl buffer (pH 7.5) containing 4 mM 2-ME, 0.5 mM UDP-Glc, and 200 μM Hx in a final volume of 500 μl at 35 °C. The reaction was terminated by the addition of 50 μl of 1N HCl, and the product (HxGlc) quantified by HPLC as previously described (Sue *et al.*, 2000b).

Purification of UDP-Glc:Hx glucosyltransferase from wheat seedlings

UDP-Glc:Hx glucosyltransferase was purified from 48-hour-old wheat shoots (20 g). All operations were carried out at 4 °C. Ammonium sulfate was added to the crude extracts, and glucosyltransferase precipitated out between 45 and 60 % of ammonium sulfate saturation. The precipitate was collected by centrifugation and resuspended in a 50 mM Tris-HCl buffer (pH 7.5) containing 4 mM 2-ME. The resulting solution was loaded onto a Blue Sepharose (Pharmacia) column (20 ml) previously equilibrated with the same buffer. After washing away unbound proteins using the same buffer, glucosyltransferase was eluted with 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM UDP-Glc and 4 mM 2-ME. After concentrating by ultrafiltration, the active fraction was subjected to gel filtration through a Superdex 200 HR10/30 column (Pharmacia), and the protein eluted using 50 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl and 4 mM 2-ME. The fractions with glucosyltransferase activity were collected and placed on a Mono Q HR5/5 column (Pharmacia) equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 4 mM 2-ME. The proteins were eluted with a linear gradient of NaCl from 110 to 260 mM at a flow rate of 1 ml/min. To estimate molecular mass, we used the following proteins as standards: ferritin (440 kDa), human IgG (160 kDa), transferrin (81 kDa), ovalbumin (43 kDa), and myoglobin (17.6 kDa). At each step of purification, UDP-Glc:Hx glucosyltransferase activity was measured using 0.2 mM DIMBOA as the substrate. We determined protein content according to the method of Bradford (1976) using bovine serum albumin as standard.

Enzyme assay

The activity of UDP-Glc:Hx glucosyltransferase was measured in 50 mM Tris-HCl buffer (pH 7.5) containing 4 mM 2-ME, 2–10 µl of enzyme solution and Hx, and 0.5 mM UDP-Glc in a final volume of 500 µl. After incubation for 5–20 min at 35 °C, the reaction was terminated by the addition of 50 µl of 1N HCl, and the product (HxGlc) quantified by HPLC as described previously (Sue *et al.*, 2000b). The volume of enzyme solution and the reaction time were adjusted so that the reactions proceeded linearly.

Electrophoresis

The polypeptide profile of active fractions at each purification step was analyzed by SDS-PAGE (10 % resolving gel) with silver nitrate staining according to the method of Laemmli (1970).

Results and Discussion

Occurrence of UDP-Glc:Hx glucosyltransferase in wheat seedlings

In view of previous findings that the concentrations of DIBOA-Glc and DIMBOA-Glc increase soon after imbibition (Nakagawa *et al.*, 1995) and decrease as plants begin autotrophic growth, we examined changes in glucosyltransferase activity in seeds and seedlings using DIBOA and DIMBOA as sugar acceptors. As shown in Fig. 1A, substantial glucosyltransferase activity was present in dry seeds (0.3–1.3 pkat/mg FW), although the Hxs were hardly detectable (Nakagawa *et al.*, 1995). Glucosyltransferase activity on DIBOA and DIMBOA in the embryo began to increase after imbibition, and at 24 h, it was three to four times as high as the activity observed in the embryo of the dry seeds (2.5 and 4.1 pkat/mg FW for DIBOA and DIMBOA, respectively). On the other hand, activity in the endosperm did not change during imbibition for either substrate (Fig. 1A). In shoots, glucosyltransferase activity increased after germination and reached a maximum 36–48 h after imbibition (11.9 and 17.6 pkat/mg FW for DIBOA and DIMBOA, respectively), thereafter decreasing to about one third of the maximum activities at 84 h (4.2 and 5.5 pkat/mg FW for DIBOA and DIMBOA, respectively) (Fig. 1B). In roots, the activities for both Hxs changed in a similar fashion

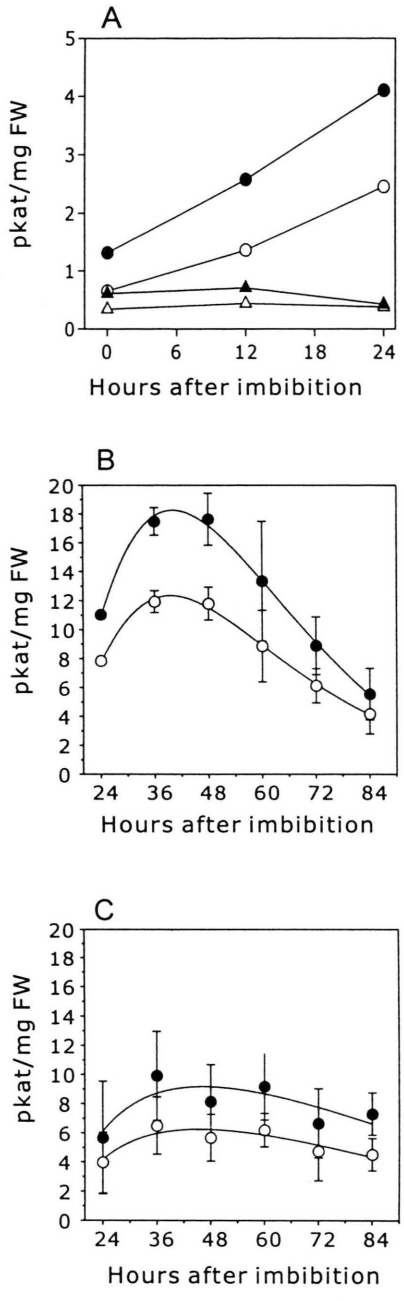

Fig. 1. Changes in UDP-Glc:Hx glucosyltransferase activity in imbibed seeds (**A**), shoots (**B**), and roots (**C**). **A**: open circular, UDP-Glc:DIBOA glucosyltransferase activity in embryo; closed circular, UDP-Glc:DIMBOA glucosyltransferase activity in embryo; open triangle, UDP-Glc:DIBOA glucosyltransferase activity in endosperm; closed triangle, UDP-Glc:DIMBOA glucosyltransferase activity in endosperm. **B, C**: open circular, UDP-Glc:DIBOA glucosyltransferase activity; closed circular, UDP-Glc:DIMBOA glucosyltransferase activity.

as the activities in shoots, the maximum activities being about half of those in shoots (6.5 and 9.9 pkat/mg FW for DIBOA and DIMBOA, respectively) (Fig. 1C). The timing of the increases and decreases in glucosyltransferase activity observed in the present study correlates well to that of the HxGlcs (Nakagawa *et al.*, 1995).

The biosynthetic pathway of Hxs branches from that of tryptophan at the point of indole (Frey *et al.*, 1997; Melanson *et al.*, 1997), and DIBOA is synthesized from indole through four consecutive reactions by cytochrome P450 enzymes *via* indolin-2-one, 3-hydroxyindolin-2-one, and HBOA (Frey *et al.*, 1997). In addition, we have recently shown that two P450 enzymes that are responsible for the production of indolin-2-one and DIBOA and the β-glucosidase that catalyses the hydrolysis of HxGlcs occur concurrently with the transient occurrence of HxGlc in wheat seedlings (Tanabe *et al.*, 1999; Sue *et al.*, 2000b). In the present study, glucosyltransferase was shown to appear at high levels in nonautotrophic wheat. These findings indicate that the whole set of enzymes involved in Hx biosynthesis after indole is highly activated in plants during this stage of growth.

Purification and characterization of UDP-Glc:Hx glucosyltransferase

We extracted UDP-Glc:Hx glucosyltransferase from shoots of 48-hour-old wheat seedlings. The glucosyltransferase was purified by ammonium sulfate precipitation, affinity chromatography on Blue-Sepharose, and gel filtration, resulting in a 151-fold increase in specific activity with a yield of 20.4 % (Table I). Upon gel filtration through a Superdex 200 column, the glucosyltransferase was eluted in a single peak at 13.5 ml (Fig. 2A), and the molecular mass of the enzyme was estimated to be about 40 kDa. The active fractions were collected and further purified by anion exchange chromatography on a Mono Q column. As shown in Fig. 2B, two peaks of activity were observed on the chromatogram; at 160–190 mM (peak 1) and 195–220 mM (peak 2) NaCl, suggesting the presence of at least two glucosyltransferase isozymes. Although the specific activity in peak 1 could not be determined due to low protein yield, the specific activity of the enzyme in the peak 2 finally increased about 1500-fold.

When the fractions corresponding to the peak 1 on the Mono Q chromatogram were analyzed by

Table I. Purification of glucosyltransferase from wheat shoots.

	Specific activity (nkat/mg protein)	Recovery (%)	Purification (fold)
Crude	0.7	100	1.0
Blue Sepharose	30.8	34.1	46.7
Superdex 200	99.6	20.4	151
Mono Q (peak 2)	982	4.7	1490

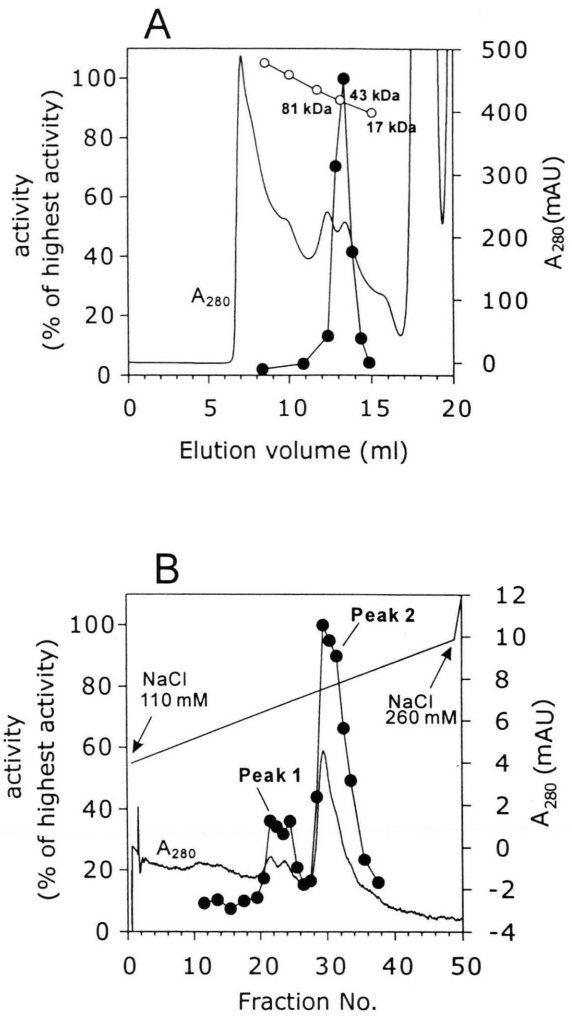

Fig. 2. Elution profile of UDP-Glc:DIMBOA glucosyltransferase activity on gel filtration through a Superdex 200 column (**A**) and on anion exchange chromatography through a Mono Q column (**B**). In **B**, each fraction volume was 1 ml.

SDS-PAGE, the intensity of a band of 47 kDa correlated with enzyme activity (data not shown), and the 47-kDa band was the most intense among detectable proteins (Fig. 4). Thus, this 47-kDa protein was considered to be responsible for the enzyme activity in peak 1.

On the other hand, two intense bands with molecular masses of 47 and 49 kDa were detected by SDS-PAGE analysis of the fraction corresponding to peak 2. The intensities of both bands correlated with the enzyme activities in the fractions obtained after anion exchange chromatography with a Mono Q column (Fig. 3). Since the 47- and 49-kDa polypeptides showed identical profiles on the SDS-PAGE gel, we could not distinguish the band representing glucosyltransferase. However, in conjunction with the gel filtration result, this finding indicates that the glucosyltransferase in peak 2 is present in a monomeric form with a molecular mass of 47 or 49 kDa. Some glucosyltransferases such as UDP-Glc:sinapic acid glucosyltransferase in *Brassica napus* show high structural specificity for the nucleotide moiety of the sugar donor (Wang and Ellis, 1998). Thus, we tested utilization of UDP-hexanolamine as a matrix for affinity chromatography to separate the two proteins, however it could not bind the protein under the conditions of the experiment. Hydroxyapatite chromatography was also unable to separate the proteins because the enzyme had no ability to bind the matrix under various conditions. Development of new purification methods is required to determine the protein responsible for glucosyltransferase enzyme activity. In addition, the possibility exists that both proteins are responsible for the glucosyltransferase activity. Since *Triticum aestivum* is a hexaploid with three genomes, it would not be surprising if wheat has several genes encoding glucosyltransferases and several enzymes with similar properties.

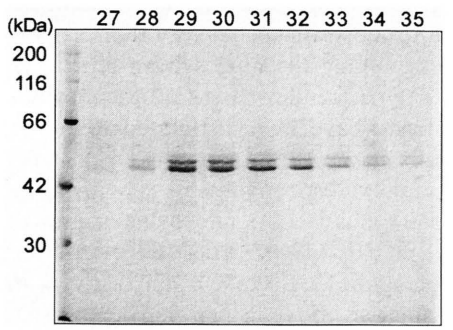

Fig. 3. SDS-PAGE analysis of the fractions from Mono Q chromatography. The numbers on lanes correspond to the fraction numbers of the Mono Q chromatography shown in Fig. 2 **B**. The gel was stained with silver nitrate.

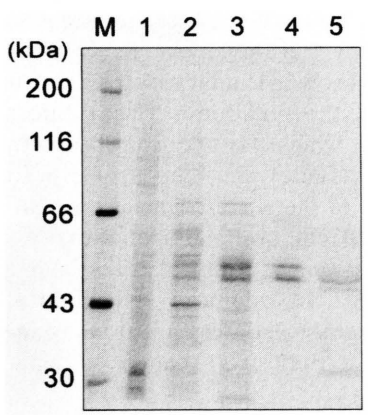

Fig. 4. SDS-PAGE analysis at various purification steps. Fractions with glucosyltransferase activity were applied to SDS-PAGE and proteins stained with silver nitrate. M, marker protein; lane 1, crude enzyme solution; lane 2, active fractions from Blue Sepharose; lane 3, active fractions from Superdex 200; lane 4, active fractions from Mono Q (peak 2); lane 5, active fractions from Mono Q (peak 1).

Characterization of the UDP-Glc:Hx glucosyltransferase was carried out using the partially purified enzyme in the fraction corresponding to peak 2 on the Mono Q chromatogram. To examine the optimum pH of glucosyltransferase, the enzyme's activity on DIBOA and DIMBOA was measured in potassium phosphate buffer (pH 5.2–7.4), Tris-HCl buffer (pH 7.4–9.2), and glycine-NaOH buffer (pH 9.2–11). Since maximum activity was observed around pH 7.5–7.7 for both sugar acceptors, we carried out the enzyme reactions at pH 7.5 in Tris-HCl buffer in subsequent experiments. The divalent cation, Cu^{2+}, inhibited enzyme activity completely at a concentration of 5 mM, while Mn^{2+} showed a 59 % decrease in activity. Other cations such as Ca^{2+} and Mg^{2+} and a chelator, EDTA, did not exhibit any influence on glucosyltransferase activity. The reducing agent, 2-ME, enhanced activity by about 40 %. The effects of Cu^{2+} and 2-ME on wheat glucosyltransferase are similar to the effects of them on maize glucosyltransferase, which is inhibited 97 % by Cu^{2+} and enhanced about 80 % by 2-ME (Bailey and Larson, 1989).

Table II. Kinetic parameters of the glucosyltransferase corresponding to peak 2 fraction obtained after anion exchange chromatography with Mono Q.

	V_{max} (nkat/mg protein)	K_m (μM)
DIBOA	446	41.3
DIMBOA	1424	40.6
HBOA	n.d.	n.d.
HMBOA	n.d.	n.d.
Esculetin	n.d.	n.d.
Salicyl alcohol	n.d.	n.d.

n.d.: not detected

The substrate specificity of Hx glucosyltransferase corresponding to peak 2 was investigated with Hxs, lactams, esculetin and salicyl alcohol. The concentration of UDP-Glc was fixed at 0.5 mM. Although the K_m for DIBOA is comparable to that for DIMBOA (41.3 and 40.6 μM for DIBOA and DIMBOA, respectively), the V_{max} for DIMBOA was about four times higher than that for DIBOA (446 and 1424 nkat/mg protein for DIBOA and DIMBOA, respectively). The enzyme did not have any activity with HBOA and HMBOA even after prolonged incubation up to 60 min, suggesting that glucosylation occurs after the formation of Hx in the biosynthetic pathway of Hxs. Other aglycones, esculetin and salicyl alcohol, were also unable to serve as sugar acceptors. The K_m value for UDP-Glc was 40 μM when DIMBOA was used as a sugar acceptor at 0.3 mM.

The substrate specificity of Hx glucosyltransferase corresponding to peak 1 was similar to that of Hx glucosyltransferase corresponding to peak 2. Although the V_{max} for the enzyme in peak 1 could not be determined, the ratio of the V_{max} for DIMBOA to that for DIBOA was 2 to 3. The K_m values for DIBOA and DIMBOA were 37 and 64 μM, respectively.

In rye, the K_m of partially purified Hx glucosyltransferase for DIBOA and DIMBOA are similar (73 and 82 μM for DIBOA and DIMBOA, respectively) whereas the V_{max} for DIMBOA is about twice that for DIBOA (Leighton *et al.*, 1994). The glucosyltransferase in rye does not accept lactams as substrates. In maize, the glucosyltransferase appears as two peaks on anion exchange chromatography (Bailey and Larson, 1989). One of the peak fractions (the enzyme eluted at higher KCl concentration) has ability to transfer glucose to both DIBOA and DIMBOA, while the other (the enzyme eluted at lower KCl concentration) was almost completely unable to transfer glucose to DIBOA. Activity for lactams was not detected in either peak fraction. On the basis of these findings, the substrate specificity of Hx glucosyltransferase in wheat was similar to the enzyme in rye and one of the enzymes in maize.

In addition to substrate specificity, other properties are similar among the glucosyltransferases in Gramineae. The molecular masses of the enzymes in rye and maize have been estimated to be 43 kDa (Leighton *et al.*, 1994) and 50 kDa (Bailey and Larson, 1989), respectively, by gel filtration. These values are close to the molecular masses of the present enzymes (47–49 kDa). Furthermore, the effects of cations on the enzyme activity in wheat are similar to those in maize. Thus, wheat, rye and maize are considered to contain glucosyltransferases that have similar properties, although the predominant Hx species are different.

The physiological significance of the presence of at least two Hx glucosyltransferases in wheat is unclear. Although the enzymes corresponding to the two peaks of activity were not completely purified, the two peaks showed similar substrate specificity. At this point, the situation in wheat differs from that of maize, which has two isozymes with different specificity (Bailey and Larson, 1989). To determine the role of the two isozymes in wheat, detailed analyses of temporal and spatial expression of the two isozymes in both wheat and maize would be necessary. In addition, cytogenetical analyses may be useful to address functions of individual isozymes, if they are encoded on separate genes.

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