Supplementary Information

Production of α-L-iduronidase in maize for the potential treatment of a human lysosomal storage disease

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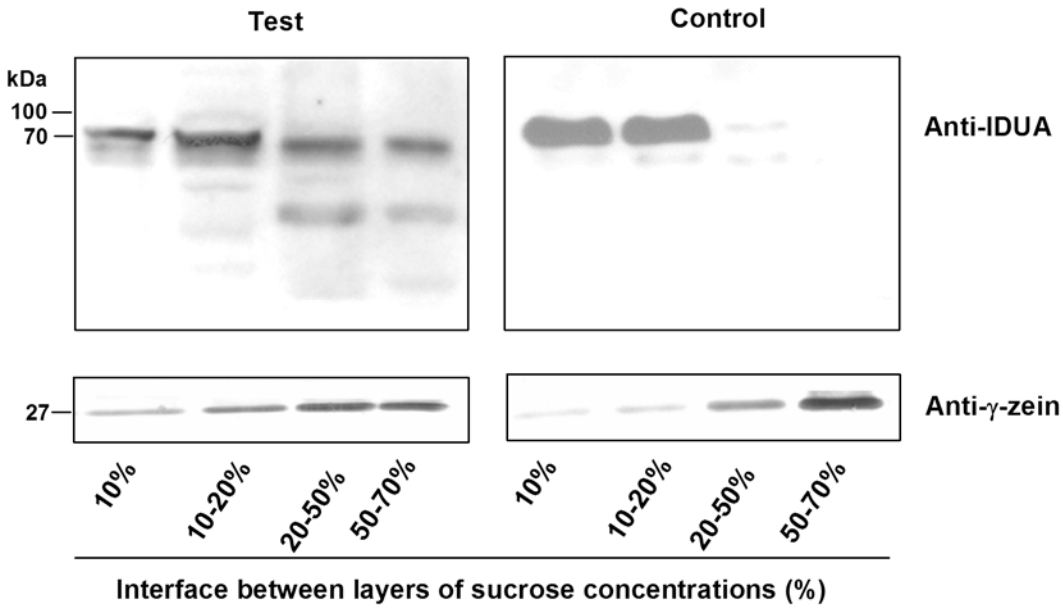
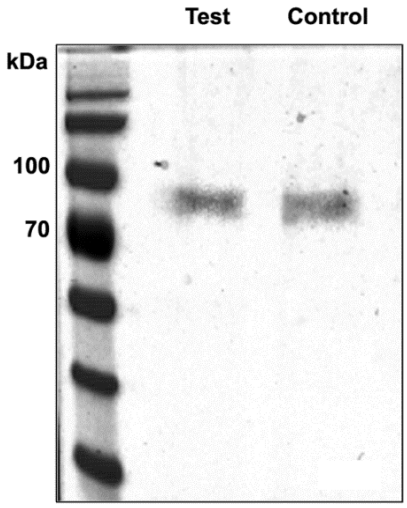
Supplementary Information contains:

Supplementary Figures



Supplementary Fig. S1. Localization of human α-L-iduronidase by western blot analysis of subcellular fractions. Homogenates of maize endosperms from transgenic lines expressing the test construct (left panel) and the control construct (right panel) were separated on discontinuous sucrose gradients and the interfaces between layers of sucrose concentrations were collected. Equal protein (20 μg) from each fraction was loaded in each lane. Protein bodies primarily sediment between the 50-70% sucrose layers. The 'control' α-L-iduronidase was predominantly distributed in the supernatant and 10%-20% interface of the sucrose gradient and not at the 50-70% sucrose interface (right panel). In contrast, the 'test' α-L-iduronidase was detected in the 50-70% sucrose gradient interface as well as in other sucrose fractions, similar to γ-zein (left panel).



Supplementary Fig. S2. Analysis of purified α-L-iduronidase from transgenic maize seeds by SDS-PAGE. Shows profiles of α-L-iduronidase (200 ng) purified from transgenic maize seeds expressing the test-construct and the control-construct.

Supplementary Table S1. Percent distribution of control and test α-L-iduronidase based on the density of gold particles in different subcellular sites

		Cytoplasm	ER-PB	PSV	Cell wall & extracellular region
Control	**zein**	14.0	59.0**	14.0	12.6
	IDUA	5.0	5.0	85.0**	5.0
Test	**zein**	8.2	70.0**	10.9	10.9
	IDUA	10.3	62.0**	14.0	13.7

Values are based on the number of gold particles corresponding to γ-zein (5 nm) or α-L-iduronidase (IDUA) (10 nm) as a percent of the total labeling of each respective particle on 3-5 images (each for the control and test sections). ** indicate significant differences ($p<0.05$ by t-test). PSV= protein storage vacuole.

Supplementary Table S2. Purification of α-L-iduronidase from maize seeds

Purification step		Total estimated α-L-iduronidase (μg)*	Total activity (units)**	Specific activity (nmol/min/mg TSP)	Purification fold	Yield ***
Crude extract	Test	224.5	143.8	0.088	1	100
	Control	120	131.2	0.023	1	100
Con A-Sepharose chromatography	Test	131	34.5	3.5	39.8	58.5
	Control	50.2	19.2	0.69	30	41.8
Anti-α-L-iduronidase affinity column	Test	21.6	103.7	4805	54602	9.6
	Control	5.3	20.4	3858	167739	4.4

* α-L-Iduronidase protein amount was estimated based on densitometry on western blot.

** The total activity in the crude extract and Con A eluant was likely underestimated because of the presence of endogenous inhibitors. When CHO-cell-culture-produced α-L-iduronidase (the commercial enzyme product, Aldurazyme^{TM}) was added to the Hi-II crude extract and incubated for 1 h, the activity was reduced to 60%.

***Yield was calculated based on estimated α-L-iduronidase protein amount rather than total activity because of endogenous inhibitors.

Supplementary Table S3. Kinetic parameters of α-L-iduronidase using 4-methylumbelliferyl-α-L-iduronide (4-MUI) as substrate

	K_m	k_{cat}
Maize-IDUA	78 ± 3	6.4 ± 0.07
CHO-IDUA	24 ± 1	3.9 ± 0.06

The data are the means of three replicate experiments ± S.E.

Supplementary Table S4. Kinetic parameters of recombinant soluble GlcNAc-1-phosphotransferase using α-L-iduronidase as substrate

	K_m (μM)	k_{cat} (min^{-1})
Maize-IDUA	0.87 ± 0.1	0.52 ± 0.18
De-P CHO-IDUA	1.7 ± 0.08	0.32 ± 0.12

The data are the means of three replicate experiments ± S.E.

Supplementary Methods

Agrobacterium-mediated maize transformation

Maize Hi-II seeds were obtained from the Maize Genetics Cooperation Stock Center[52] and maize type-II callus was initiated[53]. The α-L-iduronidase constructs (Fig. 1) were cloned into the binary vector pCambia with *Hin*d III and *Eco*RI sites and the resultant vector was transferred into *Agrobacterium* (GV3101) by electroporation. Transformation of maize was conducted essentially as described in Zhao *et al*.[54]. After the initial incubation (10 min) and 3 days of co-cultivation with GV3101, calli were transferred to PHI-C medium [CHU(N6) basal salts (Sigma) 4.0 g/l; vitamin mix 1.0 ml/l; 2,4-D 2.0 mg/l; L-proline 0.69 g/l; sucrose 30.0 g/l; MES 0.5 g/l; phytagel 2.0 g/l; silver nitrate 0.85 mg/l; carbenicillin 100 mg/l; pH 5.8] and grown in the dark at 28 °C for 5 days. The calli were then transferred to selection media (glufosinate 3.0 mg/l in PHI-C) and incubated in the dark at 28 °C for the first two weeks of selection. The calli were transferred to fresh selection media at two-week intervals. After 2 months of selection, resistant calli were regenerated following the protocols described by Armstrong[55].

Affinity purification of maize-derived α-L-iduronidase

Maize kernels were ground into fine powder in liquid nitrogen using a mortar and pestle. The powder was then extracted in buffer A (20 mM Tris, pH 7.0, 0.5 M NaCl, 0.5 mM PMSF and 0.02% sodium azide). Purification was carried out essentially as described by Clements *et al*.[56]. After centrifugation for 20 min at 3500 rpm, the supernatant was passed through three layers of Miracloth (EMD Biosciences, Inc. La Jolla, CA, USA) and

centrifuged at 45,000 rpm at 4 ºC for 1 h with Beckman Coulter's Optima L-100 K ultracentrifuge. The supernatant was loaded onto a Con A-Sepharose column (GE Healthcare Life Science, Quebec, Canada) at 10 ml/h for overnight (recycling) at 4 ºC. The column was washed with 100 ml buffer A and bound protein was eluted with 150-200 ml buffer B (15% methyl-α-D-mannopyranoside in buffer A) (recycling for 16-24 h). The eluant was concentrated with Amicon 30 kDa centrifuge filters (Millipore Corp., Bedford, MA, USA) and loaded onto a column containing Affi-Gel-bound to monoclonal anti-α-L-iduronidase. After washing the unbound protein with buffer A, α-L-iduronidase was eluted with buffer C (50 mM sodium citrate, pH 4.0, 2 M NaCl, 0.02% sodium azide) and the eluant was concentrated with Amicon 30 kDa centrifuge filters.

Cellular fractionation

Cell fractionation was performed according to Larkins and Hurkman[47]. Briefly, developing maize endosperms were homogenized with buffer: 20 mM Tris-HCl (pH 7.5), 10% (w/v) sucrose, 50 mM KCl, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM EDTA and 1 mM PMSF. After removing cellular debris by centrifugation at 600 rpm for 5 min at 4 °C, supernatants were loaded onto three step gradients of 1 ml of 20% sucrose, 1 ml of 50% sucrose and 1 ml of 70% sucrose in the above buffer, and centrifuged at 24,000 rpm in an SW40 rotor (Beckman, Palo Alto, CA, USA) for 2 h at 4 °C. The fractions at each interface of the discontinuous sucrose gradient were collected and analyzed by SDS-PAGE and western blotting.

Determination of enzyme kinetic parameters of α-L-iduronidase

Michaelis Menten kinetics for CHO-cell-derived α-L-iduronidase and maize-derived α-L-iduronidase were determined at 37 °C and pH 4.5 using a fluorometric assay. Reactions were performed in a total volume of 100 µL, in 0.1 M dimethylglutarate buffer, pH 4.5, 2 mM sodium metabisulfite and 0.7% bovine serum albumin. 4-Methylumbelliferyl-α-L-iduronide (4-MUI) was used as the substrate, at concentrations of 1-250 µM for the CHO-cell-derived α-L-iduronidase and 2.5-1000 µM for the maize-derived α-L-iduronidase, to ensure the coverage of a sufficient range encompassing the K_m. Reactions were started upon the addition of enzyme (0.035 µg per assay for CHO-cell-derived α-L-iduronidase and 0.008 µg per assay for the maize-derived α-L-iduronidase) and allowed to proceed for a fixed length of time (10 min for CHO-cell-derived α-L-iduronidase and 60 min for the maize-derived α-L-iduronidase), over which time it had been determined that the rate of the reaction remained linear. Reactions were stopped by the addition of 0.1 M glycine buffer, pH 10.7. The amount of 4-MU produced at each substrate concentration was determined by measuring the fluorescence (emission wavelength 355 nm and excitation wavelength 485 nm), and converting the value into a concentration using a standard curve which was prepared using 4-MU concentrations between 0.1 and 125 µM. Rates of catalysis were determined by dividing by the reaction time and concentration of the enzyme, and fit to a Michaelis Menten curve using GRAFIT[57]. All measurements were made in triplicate.

Anti-horseradish-peroxidase affinity chromatography for removal of the minor contaminant of Golgi-modified α-L-iduronidase

Approximately 8% of the α-L-iduronidase derived from expression of the 'test' construct contained matured (complex) N-glycans, i.e., N-glycans with xylose and/or fucose. This small fraction of the α-L-iduronidase was removed from the test sample by passing the purified α-L-iduronidase (a single band on SDS-PAGE) through an anti-horseradish-peroxidase affinity column (recycling overnight at 4ºC). The column specifically binds to xylose and/or fucose residues[58], allowing for the removal of any α-L-iduronidase containing these sugars from the sample. The polyclonal anti-horseradish-peroxidase antibodies (Sigma-Aldrich Canada, Oakville, Ontario, Canada) were cross-linked to Affi-Gel 10 (Bio-Rad Laboratories Inc., Mississauga, ON, Canada) according to the manufacturer's instructions. Following the procedure, Western blot analysis of the resultant α-L-iduronidase was conducted using an antibody specific for plant complex N-glycans[59].

Supplementary References

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