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Partial purification and characterisation of a 2,4,5-trichlorophenol detoxifying *O*-glucosyltransferase from wheat

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Dedicated to the memory of Professor Jeffrey B. Harborne

Abstract

An enzyme preparation with UDP–glucose-dependent *O*-glucosyltransferase (OGT; EC 2.4.1.-) activity toward 2,4,5-trichlorophenol has been purified 215-fold from wheat shoots. The OGT co-purified with the major extractable glucosylating activity toward the flavonol quercetin and was characterised as a monomeric 53 kDa protein. Among the xenobiotic phenols tested, the purified enzyme preparation showed at least a 10-fold preference for 2,4,5-trichlorophenol. When assayed with flavonoids, the OGT was active toward flavonols and coumestrol, showing a clear preference for 3-hydroxy flavone when incubated with a range of monohydroxylated flavonoids. It was concluded that the major 2,4,5-trichlorophenol-detoxifying OGT in wheat shoots is most probably a flavonol-3-*O*-glucosytransferase.

Keywords: *Triticum aestivum*; Gramineae; Bronze 1; Chlorinated phenols; Detoxification; Flavonoids; Glucosylation; Xenobiotics

1. Introduction

The type 1 UDP-glycosyltransferases are an ancient and evolutionarily diverse group of enzymes which conjugate a range of synthetic and natural products with sugars in both prokaryotes and eukaryotes (Vogt and Jones, 2000). Plants contain a large family of type 1 uridine diphosphoglucose (UDPG) dependent *O*-glucosyltransferases (OGTs; EC 2.4.1.-) which are active in conjugating natural products containing a free hydroxyl or carboxyl group (Vogt and Jones, 2000). Plant OGTs also conjugate xenobiotics containing these functional groups, though little is known concerning the identity of the respective isoenzymes (Cole and Edwards, 2000). Although OGT activities toward synthetic phenols have been identified in a diverse range of plant species (Pflugmacher and Sandermann, 1998), the respective enzymes have only been purified and characterised in a restricted number of cases (reviewed by Cole and Edwards, 2000). We have recently reported a range of OGT activities toward phenols of both synthetic and natural origin in wheat (Brazier et al., 2002). In these studies, 2,4,5-trichlorophenol, a xenobiotic encountered by plants as both a pesticide metabolite and pollutant (Sandermann et al., 1991), and the flavonol quercetin were identified as preferred substrates. Activities toward both compounds were enhanced in wheat shoots following applications of herbicide safeners, suggesting the enzyme(s) catalysing these reactions were related to one another (Brazier et al., 2002). However, it was determined that the activity toward quercetin was inhibited in the presence of manganese ions, while the conjugation of 2,4,5-trichlorophenol was unaffected by this treatment, suggesting that distinct OGTs were active toward these two compounds. Subsequently, we have determined that the inhibition of quercetin conjugation by manganese salts was due to the metal ions complexing with the flavonol rather than any specific effect on the OGTs present and this has prompted a re-examination of the identity of the OGTs active in conjugating quercetin and 2,4,5-trichlorophenol in wheat.

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2. Results

2.1. Purification of an OGT from wheat which conjugates both 2,4,5-trichlorophenol and quercetin

High levels of OGT activity toward both 2,4,5-trichlorophenol and quercetin were determined in extracts from wheat shoots (Brazier et al., 2002) and these were used as the starting material for enzyme purification. Purification consisted of $(NH_4)_2SO_4$ precipitation of the crude protein extract between 40 and 60% saturation, followed by hydrophobic interaction chromatography on Phenyl-Sepharose, Q Sepharose anion exchange chromatography, gel filtration and finally resolution on a MonoQ column (Fig. 1). During the early stages of purification, the activities toward quercetin and trichlorophenol co-purified as broad peaks (Fig. 1A and B). When applied to a calibrated gel filtration column, both co-eluted with an estimated molecular mass of 53 kDa (Fig. 1C). Finally, Mono Q anion exchange chromatography resolved the OGT activities into several minor and one dominant peak, the latter eluting with 125 mM NaCl and representing the majority of activity toward both quercetin and trichlorophenol (Fig. 1D). Overall the purification obtained for the major peak in the final fraction represented a 215-fold enrichment in activity toward 2,4,5-trichlorophenol and a 249-fold enrichment toward quercetin (Table 1).

When analysed by SDS–PAGE, the active fractions from the final Mono Q separation (Fig. 1D) contained a 53 kDa polypeptide whose abundance mirrored that of both OGT activities (Fig. 2A). Since the mass of this polypeptide corresponded closely to the mass determined for the OGT by gel filtration (Fig. 1C), it was concluded that this protein was the enzyme of interest. The purified fractions were pooled and analysed by two-dimensional gel electrophoresis to identify any additional 53 kDa polypeptides with differing p*I*s. The proteins were resolved first on the basis of p*I* on an IEF gel (pH 4.0–7.0), and then separated in the second dimension on a 12% SDS–polyacrylamide gel (Fig. 2B). Using silver staining, a total of six polypeptides could be resolved with masses in the range 40–60 kDa. The 53 kDa putative OGT migrated as a single spot with a p*I* of pH 5.0. From this it was concluded that the OGT activities associated with this fraction were most likely derived from a single enzyme.

2.2. Activities of purified OGT toward chlorinated phenols and flavonoids

The purified preparation was assayed for OGT activity toward natural products and xenobiotics using

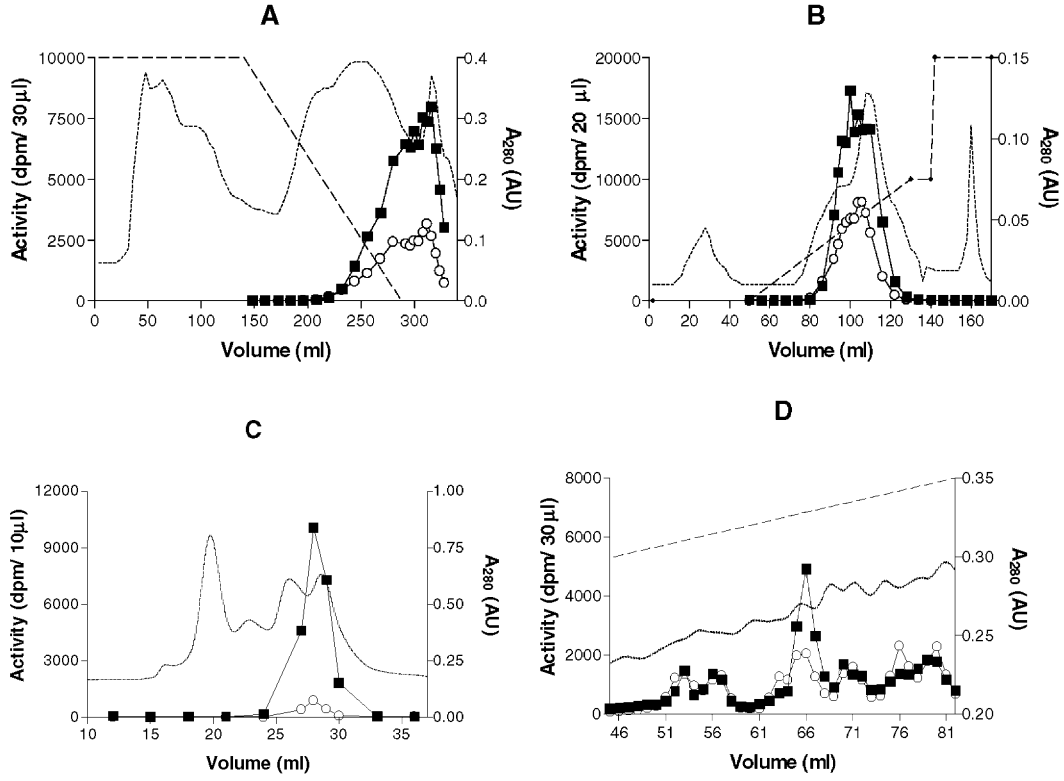

Fig. 1. Sequential purification of OGT activity toward 2,4,5-trichlorophenol (■) and quercetin (○) by chromatography on Phenyl-sepharose (A), Q Sepharose (B), gel filtration (C) and Mono Q (D). The elution of UV absorbing material (A280) is shown (···) together with changes in the concentrations of binary solvents (- - -). Enzyme activities shown correspond to radioactivity product formation (dpm) per 10–30 µl of the fraction assayed (as indicated).

Table 1
Purification of OGT activity toward 2,4,5-trichlorophenol and quercetin

Purification step	Substrates	Total protein (mg)	Total activity (pmol product min^{-1})	Specific activity (pmol product min^{-1} mg^{-1} protein)	Purification (*n*-fold)	Recovery (%)
Crude	2,4,5-Trichlorophenol	4610	30,725	6.7	1	100
	Quercetin		12,647	2.7	1	100
40–60% $(NH_4)_2SO_4$	2,4,5-Trichlorophenol	2428	20,578	8.5	1.3	67
	Quercetin		6973	2.9	1.1	55
Phenyl Sepharose	2,4,5-Trichlorophenol	168	4398	26	3.9	14.3
	Quercetin		1232	7	2.7	9.7
Q Sepharose	2,4,5-Trichlorophenol	27	1396	53	7.9	4.5
	Quercetin		382	14	5.3	3.0
Superdex 200	2,4,5-Trichlorophenol	3.7	743	204	30.5	2.4
	Quercetin		64	18	6.4	0.5
Mono Q	2,4,5-Trichlorophenol	0.04	52	1432	215	0.2
	Quercetin		26	716	249	0.2

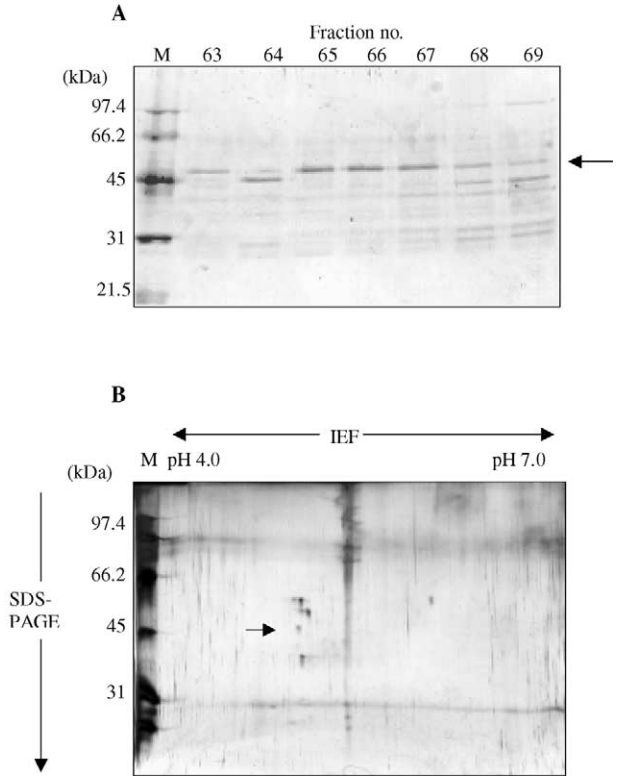

Fig. 2. Analysis of purified OGT by electrophoresis. (A) Polypeptide composition of the 1 ml fractions (63–69) of the major OGT active peak eluted from the final step of purification on Mono Q (Fig. 1D) as determined by analysis of 0.2 μg protein in each case by SDS–PAGE and staining with Coomassie Blue. The 53 kDa polypeptide co-eluting with the OGT activity is arrowed, with the molecular mass markers shown (M). (B) Silver stained polypeptides (12 μg total protein loaded) in the pooled fractions of the purified OGT resolved by 2D-gel electrophoresis, using isoelectric focussing (pH 4–7) in the first dimension and SDS–PAGE in the second dimension. The putative 53 kDa OGT is arrowed.

UDP–[^{14}C-glucose] as sugar donor (Table 2). In this study other UDP–[^{14}C]-labelled sugars were not tested as alternative co-substrates. It was found to be important to analyse the OGT promptly following purification as the associated conjugating activity was found to be labile, with a 40% loss in activity toward both

Table 2
Activities of the purified wheat OGT toward xenobiotic and natural product substrates

Substrate	Specific activity in pmol product (min^{-1} mg^{-1} protein)[a]
Xenobiotics	
2,4,5-Trichlorophenol	1199±64
2,3,6-Trichlorophenol	113±0
2,4,6-Trichlorophenol	59±0
Pentachlorophenol	46±3
4-Nitrophenol	274±6
2,4-Dinitrophenol	29±1
Phenol	29±2
Flavonoids	
Quercetin	399±10
Luteolin	135±1
Apigenin	105±3
Genistein	89±6
Coumesterol	389±14
3-Hydroxyflavone	509±41
5-Hydroxyflavone	33±3
6-Hydroxyflavone	287±15
7-Hydroxyflavone	58±5
2′-Hydroxyflavone	183±14
4′-Hydroxyflavone	48±1
2′,4′-Dihydroxyflavone	99±9

[a] Specific activity determined in duplication with values given as the mean±variation in replicates.

2,4,5-trichlorophenol and quercetin observed after storage of the enzyme for 16 h at 4 °C. Using the radioassay developed and optimised for wheat OGTs previously (Brazier et al., 2002), 2,4,5-trichlorophenol was conjugated 10-fold more readily than the other chlorinated phenols tested. The OGT was also active toward 4-nitrophenol. Of the flavonoids tested, the OGT was most active toward the flavonol quercetin with appreciable activity also seen with coumestrol (Table 2). When assayed with 2,4,5-trichlorophenol, apparent K_Ms of 12.4 and 10.6 μM were determined with acceptor and UDPG donor, respectively while with quercetin K_Ms of 10.1 μM (acceptor) and 61.8 μM (UDPG) were determined. Overall, the enzyme showed a similar preference for the conjugation of the xenobiotic and flavonol, with a K_{cat}/K_M of 3900 M^{-1} s^{-1} determined for 2,4,5-trichlorophenol and 2180 M^{-1} s^{-1} for quercetin. To establish the probable site of *O*-glucosylation on the flavonoid rings, a range of monohydroxyflavones were tested as glucose acceptors. These assays demonstrated a clear substrate preference in the order 3-hydroxy→6-hydroxy→2′-hydroxy-flavones.

3. Discussion

Our results demonstrate that wheat shoot extracts contain a 53 kDa OGT which associated with most of the conjugating activity toward 2,4,5-trichlorophenol and that this enzyme preparation also glucosylates flavonols, notably at the 3-hydroxy position. The 3-*O*-glucosylation of flavonoids is classically associated with the *bronze 1* gene in maize, inactivation of which leads to the aberrant accumulation of anthocyanin pigment derivatives in kernels (Fedoroff et al., 1984). OGTs resembling *bronze 1* have been cloned and expressed from *Antirrhinum majus* (Martin et al., 1991), *Gentiana triflora* (Tanaka et al., 1996), *Vitis vinifera* (Ford et al., 1998) and *Petunia hybrida* (Yamazaki et al., 2002). While the enzyme from *P. hybrida* catalysed the glucosylation of either flavonol or anthocyanin substrates equally efficiently (Yamazaki et al., 2002), the enzymes from *V. vinifera* and *G. triflora* showed a marked preference for anthocyanins (Tanaka et al., 1996; Ford et al., 1998). However, the possibility that these enzymes might also glucosylate xenobiotics was not reported. Attempts to categorically identify the wheat enzyme as a Bronze 1 homologue using a proteomics approach proved unsuccessful. Tryptic digestion of the purified wheat OGT released 29 peptide fragments which could just be resolved by MALDI-TOF MS. However, no corresponding sequences were obtained above the probability score after interrogation of the wheat EST or the NCBI plant protein sequence databases. Notably, no *bronze 1*-like sequences have yet been reported in wheat.

The 53 kDa enzyme was distinct from other OGTs previously identified in wheat. A 43 kDa enzyme active in conjugating pentachlorophenol was identified in wheat suspension cultures (Schmitt et al., 1985), while OGTs active toward cyclic hydroxamates with molecular masses of 47–49 kDa and 47 kDa enzymes were reported in young seedlings (Sue et al., 2000). Unfortunately, in both these earlier studies, the cross-reactivity of the respective enzymes toward natural and synthetic phenolic substrates were not reported. In soybean cell cultures, there is precedence for OGTs catalysing the glucosylation of both chlorinated xenobiotics and flavonols. Thus, a 44.6 kDa OGT active toward the herbicide metabolite 6-hydroxybentazone also conjugated quercetin (Leah et al., 1992), while a partially purified 50 kDa enzyme which detoxified 2,2,-*bis*-(4-chlorophenyl)-acetic acid also conjugated the flavonol (Wetzel and Sandermann, 1994). However, when a 47 kDa OGT active in conjugating pentachlorophenol was purified to homogeneity from soybean cultures, the resulting enzyme showed negligible activities toward quercetin and other natural products (Sandermann et al., 1991). Interestingly, in other respects the pentachlorophenol-detoxifying OGT from soybean had a similar substrate preference for synthetic phenols as the wheat enzyme. Thus, both enzymes were most active toward 2,4,5-trichlorophenol, while 4-nitrophenol, pentachlorophenol and 2,4,6-trichlorophenol were all poorer substrates.

Collectively, these results suggest that different plant species have recruited distinct OGTs to detoxify xenobiotics, rather than utilise the same enzymes in each case. Presumably this has resulted from the great diversification of the type 1 OGT in plants both in terms of their gene sequences and functions in natural product metabolism (Vogt and Jones, 2000; Ross et al., 2001). The increasing availability of homogeneous preparations of recombinant OGTs cloned from plants will help characterise the activities of single enzymes toward synthetic and natural phenolic compounds. For example, the hydroquinone-conjugating OGT from *Rauvolfa serpentina,* arbutin synthase, has recently been cloned and the recombinant enzyme assayed for activities toward a diverse range of natural products and xenobiotics (Hefner et al., 2002). In addition to hydroquinone, the enzyme showed appreciable activity toward several chlorinated phenols and flavonoids including quercetin, demonstrating that in some instances OGTs may act on very diverse substrates. It will now be of interest to determine the selectivity of other type 1 plant OGTs toward natural and synthetic products, concentrating initially upon the xenobiotic conjugating activities of the bronze 1 enzyme.

4. Experimental

4.1. Enzyme assays

OGT activity was determined by incubating protein preparations (65 μl) with 5 μl phenolic acceptor (1 mM)

and 10 μl UDP–[U-^{14}C-glucose] (833 Bq, 7.6 GBq mmol^{-1}) in 0.2 M Tris–HCl pH 8.0 containing 2 mM dithiothreitol (OTT) for 20 min at 30 °C. Radioactive conjugates were then partitioned into EtoAc and quantified by liquid scintillation counting as described previously (Brazier et al., 2002). During OGT purification, when assaying less than 50 μg of plant protein per assay, 50 μg of bovine serum albumin was added to each incubation to ensure efficient partitioning of reaction components into the organic phase. Protein content was determined using a dye binding reagent (BioRad) with γ-globulin as the reference protein. For the determination of kinetic constants, concentrations of co-substrate were kept at saturating concentrations (phenolic acceptor = 66 μM, UDP–glucose = 150 μM) while the concentration of the other substrate was varied.

4.2. Purification of OGTs

All purification steps were carried out at 4 °C unless otherwise stated. Frozen wheat shoot tissue was homogenised in liquid nitrogen using a pestle and mortar. Protein was extracted with 3 v/w 0.2 M Tris–HCl, pH 8.0 containing 10 mM ascorbic acid, 10 mM sodium metabisulphite, 5 mM DTT and 5% (w/w) polyvinylpyrrolidone and the resulting extract filtered through two layers of miracloth (Calbiochem, Nottingham, UK). After centrifugation (8500*g*, 15 min, 4 °C), the supernatant was fractionated with $(NH_4)_2SO_4$ and the protein which precipitated between 40 and 60% saturation collected by centrifugation (17,000*g*, 30 min, 4 °C). Protein pellets were taken up in 50 ml of 25 mM imidazole, pH 7.5, 2 mM DTT (buffer A) containing 1 M $(NH_4)_2SO_4$ and centrifuged (17,000*g*, 20 min, 4 °C). The resulting supernatant was applied to a pre-equilibrated column (60 ml) of phenyl sepharose. After washing with loading buffer at 4 ml min^{-1}, bound protein was eluted from the column by steadily decreasing the $(NH_4)_2SO_4$ concentration to 0 M over 32.5 min followed by a further 10 min wash with buffer A. Fractions (8 ml) containing OGT activity were pooled and desalted in 20 mM Bis–Tris–HCl, pH 6.0, 2 mM DTT (buffer B) using Sephadex G-25 gel filtration chromatography (PD-10 columns, Pharmacia). The sample was then applied to a Q Sepharose column (20 ml) and washed with 45 ml of buffer B at 2 ml min^{-1}, with bound proteins eluted using a linearly increasing concentration of 0–500 mM NaCl in a total volume of 80 ml. After collection, active fractions (2 ml) were pooled, and treated with $(NH_4)_2SO_4$ (80% saturation) and the precipitated protein resuspended in 0.2 M Tris–HCl, pH 7.5, 2 mM DTT prior to application at 0.5 ml min^{-1} on to a Superdex 200 column (24 ml), which had been pre-calibrated with proteins of known molecular mass (Kordic et al., 2002). Active fractions (0.5 ml) were pooled and applied to a Mono Q (1 ml) column and unbound protein removed by washing with 50 mM Tris–HCl, pH 7.5, 2 mM DTT. The bound protein was then eluted with a linear gradient of 80–157 mM NaCl at 1 ml min^{-1} and fractions (1 ml) collected.

4.3. Electrophoresis of proteins

Proteins were analysed by SDS–PAGE on 12% acrylamide gels and the polypeptides visualised using silver staining (Kordic et al., 2002). For two-dimensional gel analysis, the protein samples were treated with 9 M urea, 2 M thiourea, 4% (w/v) Chaps, 1% (w/v) DTT and 2% (v/v) ampholytes (pH 3–10, Amersham-Pharmacia) and applied on a 7 cm wide Immobiline Dry Strip (pH 4–7) as detailed by the manufacturer (Amersham-Pharmacia). After electrophoresis, the gel strip was equilibrated in 50 mM Tris–HCl, pH 8.8, containing 6 M urea, glycerol (30% v/v), SDS (10% w/v) and DTT (0.1% w/v) with a trace of bromophenol blue prior to electrophoresis in the second dimension using a 12% SDS–PAGE gel. Polypeptides were then visualised in 2D-gels using silver staining.

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