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Comparative characterisation of recombinant invertebrate and vertebrate peptide *O*-Xylosyltransferases

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Abstract Chondroitin and heparan sulphates have key functions in animal development and their synthesis is initiated by the action of UDP-α-D-xylose:proteoglycan core protein β-D-xylosyltransferase (EC 2.4.2.26). cDNAs encoding this enzyme have been previously cloned from mammalian species; this in turn facilitated identification of corresponding *Caenorhabditis elegans* (*sqv-6*) and *Drosophila melanogaster* (*oxt*) genes. In the present study, we report the expression in *Pichia pastoris* and subsequent assay using either MALDI-TOF MS or RP-HPLC of recombinant forms of the *Caenorhabditis* xylosyltransferase SQV-6 and the human xylosyltransferase I, in addition to extending our previous studies on the xylosyltransferase from *Drosophila*. The enzyme activities were tested with a number of peptide substrates based on portions of the human bikunin, human perlecan and *Drosophila* syndecan core peptides. Whereas a variant of the latter, containing two Ser-Gly motifs was only modified on one of these motifs, the perlecan peptide with three Ser-Gly motifs could be multiply modified in vitro. Using this substrate, we could for the first time follow, by mass spectrometry, the xylosylation of a peptide with multiple xylosyltransferase acceptor motifs.

Keywords Xylosyltransferase · Glycosaminoglycan

Abbreviations

ESI	electrospray ionisation
MALDI-TOF MS	matrix-assisted laser desorption/ionisation time-of-flight spectrometry
RP-HPLC	reversed phase HPLC
OXT	*Drosophila* peptide O-xylosyltransferase
SQV-6	*Caenorhabditis s*quashed *v*ulva 6 gene product or peptide O-xylosyltransferase
XT-I	human xylosyltransferase I
XT-II	human xylosyltransferase II

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Introduction

Until recently proteoglycans were often thought of as an extracellular glue of probably no specific biological function; however, the cell-cell and cell-matrix interactions which they mediate are now considered of profound importance in the development and morphogenesis of multicellular organisms [1]. The significance of proteoglycans is shown, for instance, by the correlation of mutations in genes encoding proteoglycan biosynthesising enzymes with a disease of bone formation (hereditary multiple exostoses) in man [2] or with defects in vulval formation in nematodes (sqv mutants) [3]. Use of RNAi in *Caenorhabditis* and *Drosophila* also confirms the importance of these enzymes for embryogenesis [4,5]. Animal proteoglycans contain glycosaminoglycan chains which fall into a number of categories: chondroitin sulphate, dermatan sulphate, heparin, heparan sulphate and keratan sulphate which consist of sulphated disaccharide repeats linked to a core protein via a glycan [6]. Whereas keratan sulphates are attached through typical N- or O-glycans, the first four categories have a glycan core of GlcAβ1-3Galβ1-3Galβ1-4Xyl *O*-linked to the protein. The biosynthesis of this core tetrasaccharide is catalysed by the sequential action of a series of glycosyltransferases, the first of which is peptide *O*-xylosyltransferase [7].

Despite reports around thirty years ago on the partial purification of the chicken cartilage enzyme [8], the first peptide sequences derived from a purified form of a xylosyltransferase were only recently published [9]. This information allowed the cloning of two human cDNAs, one of which was demonstrated to encode an active xylosyltransferase [10] and showed that the encoded enzymes are members of the uniquely highly diverse glycosyltransferase family 14 (http://afmb.cnrs-mrs.fr/CAZY/index.html). Subsequently the activity of a recombinant form of the *Drosophila* enzyme was reported by this laboratory [11]. More recently, while our own studies were underway, the *Caenorhabditis* xylosyltransferase was verified to be encoded by the *sqv-6* gene; the cDNA was used to complement a defect in xylosyltransferase activity in a mutant Chinese hamster ovary cell line but no further enzymology was described [12]. In the present report, we describe a fuller characterisation of the *Caenorhabditis* and human xylosyltransferases SQV-6 and XT-I and also follow up our previous studies on the *Drosophila* enzyme by testing further peptide substrates.

Experimental procedures

Cloning and expression of xylosyltransferase constructs

Total RNA was extracted from a wild-type N2 *Caenorhabditis* elegans mixed population and from human embryonic kidney (HEK) cells using Trizol reagent (Invitrogen). Two-step RT-PCR was performed using Superscipt III reverse transcriptase and Expand polymerase mix (Roche Biochemicals). PCR was typically performed using 35 cycles of 1 min at 58–60°C, 3 min at 72°C and 1 min at 95°C, followed by a final extension step of 8 min at 72°C. PCR products were then purified after gel electrophoresis using either the QIAGEN or Pharmacia GFX PCR reaction purification kits and ligated into the pGEM-T vector (Promega).

To clone the *Caenorhabditis sqv-6* cDNA, initially two overlapping segments were generated by PCR using the primer pairs 1) CeXT5 5′-ATGTTATTCAACGGGACG-3′ and CeXT8 5′-CGTTTCACTTGACGCTC-3′ and 2) CeXT1 5′-GTCGAATCCTCTTCCATC-3′ and CeXT2 5′-ATGTGT TCTAAATCAAGGTC-3′ with Expand polymerase mix (Roche Biochemicals) prior to ligation into the pGEM-T vector. Positive clones were selected and sequenced using the BigDye kit (Applied Biosystems). The combined overlapping sequence of the CeXT5/CeXT8 and CeXT1/CeXT2 fragments was submitted to the Genbank/EMBL database under the accession number AJ496235. In order to prepare the construct for expression as a soluble protein, a clone containing the CeXT1/CeXT2 PCR fragment, lacking the first fifty codons, was then used as a template for PCR with *Pfu* polymerase (Promega) using the CeXT1 and the CeXT2/XbaI (5′-GCTCTAGATGTGTTCTAAATCAAGG TC-3′) primers. The blunt-ended PCR product was purified using the Amersham GFX DNA purification kit, cut with XbaI and ligated into pICZαA cut with PmlI (to generate a blunt end) and XbaI.

Fragments encoding soluble forms of the human xylosyltransferases I and II lacking the putative cytoplasmic, transmembrane and stem regions were generated by PCR using Expand polymerase and the primers HuXTI/1/ClaI (CCATCGATCCCTAAGTGTGACATCTCA) with HuXTI/2/XbaI (TCGTCTAGACACTCCTCGGTGCCCAGT) and HuXTII/1/ClaI (CCATCGATCCCCAAGTGCGAGATCG) with Hu XTII/2/XbaI (TCGTCTAGAGCTGGGGCCCTGCTAC). These fragments were then cloned using the pGEM-T vector, prior to excision with ClaI and XbaI and ligation into the pICZαC vector cut with the same enzymes. For the *Drosophila* enzyme, the previously-reported non-tagged soluble form [11] and a newly-constructed C-terminally His-tagged soluble form were used.

Generally, expression vector DNA (ca. 5 μg) from PCR-positive *E. coli* clones was cut with MssI prior to electroporation into *Pichia pastoris* GS115 cells. Expression of recombinant xylosyltransferase was induced by methanol as described previously for *Drosophila* xylosyltransferase [11], at either 16 or 30°C. For preparation of the medium, best results in terms of expression, as well as ease of assaying activity, were obtained when either Gibco Peptone 140 (no longer available) or granulated peptone from Merck was used. Culture supernatants were collected, concentrated ten-fold with an Ultrafree centrifugal concentration device with a cut-off of M_r 30,000, subject to buffer exchange using the same device and stored at 4°C. Alternatively, culture supernatants (10 ml) were subject to ammonium sulphate precipitation at 60% saturation at 4°C. The resultant pellet was then resuspended in 10 mM HEPES, pH 8, containing 0.02% sodium azide.

Recombinant xylosyltransferase assays

The xylosyltransferase assay conditions were as previously described for *Pichia*-expressed *Drosophila* xylosyltransferase with simultaneous quantification and identification of products by either MALDI-TOF MS or RP-HPLC [11]. For assaying the recombinant enzymes, UDP-xylose was either purchased from Carbosource Services (Athens, GA) or purified from incubations of recombinant *Cryptococcus neoformans* UDP-GlcA decarboxylase with UDP-GlcA [13] by anion-exchange HPLC (SAX Hypersil 5 μm column, 0.4 × 25 cm) at 1.5 ml/min using an ammonium-formate gradient (pH 3.2, 2–360 mM, 5–20 min). As acceptors, the following peptides (custom synthesised by ThermoHybaid, Ulm, Germany) based on portions of human bikunin, human perlecan and *Drosophila* syndecan were used: Bik, QEEEGSGGGGQR (m/z 1191);

Per, DSISGDDLGSGDLGSGDFQR (m/z 1999); Syn, DDD SIEGSGGR (m/z 1108); Syn2, DDDSIEGSGSGGR (m/z 1252). The Bik, Syn and Syn2 peptides were used at a final concentration of 1 mM, whereas, due to its lower solubility, the Per peptide was used at a final concentration of 0.4 mM. pH optima were measured using a series of 40 mM 2-amino-2-methyl-1,3-propanediol (AMPD) solutions as buffers, whereas cation dependency was measured using 10 mM solutions of the relevant chloride salts. RP-HPLC was performed using an ODS Hypersil 5 μm column (0.4 × 25 cm) and a gradient of 0–12% of 95% acetonitrile from 2–5 min, then 12–14% of 95% acetonitrile from 5–13 min at a flow rate of 1.5 ml/min; detection was performed at 214 nm. MALDI-TOF MS of either crude reaction mixtures or of purified peptide fractions was carried out using α-cyano-4-hydroxycinnamic acid as matrix on a bench-top Dynamo instrument (ThermoBioanalysis).

Native xylosyltransferase assay

The *Caenorhabditis* N2 Triton extract with a protein content of 4.5 mg/ml (as judged by use of the Sigma modified Lowry protein assay kit) was prepared as previously described [14]. 9 μg of the extract were assayed overnight in a final volume of 10 μl at 37°C (10 mM Mn(II), 40 mM MES, pH 7) using the Bik peptide (0.4 mM) and, as previously described for some assays with the recombinant *Drosophila* enzyme [11], 2 μl of a crude incubation of UDP-GlcA with the aforementioned decarboxylase as the source of UDP-Xyl. The product and substrate of the xylosyltransferase reaction were measured by MALDI-TOF MS as described above. The presence of UDP-Xyl in the decarboxylase reaction mix was proven before use by analysis with anion-exchange HPLC using a potassium-phosphate gradient [13].

MALDI-Q-TOF MS

For determination of the xylose acceptor site on the Syn and Syn2 peptides, reactions with affinity-purified His-tagged *Drosophila* xylosyltransferase were directly used. An aliquot of the HPLC-purified peptides (0.5 μL) was subjected to MALDI Q-TOF MS and tandem MS performed on a Waters Micromass Q-TOF Ultima Global (Manchester, UK) using α-cyano-4-hydroxycinnamic acid as the matrix. For MALDI Q-TOF tandem MS the respective singly charged ion was selected, collision energy was adjusted manually. Spectra were acquired and analysed using MassLynx 4.0 software (Waters Micromass, UK).

ESI-MS

Incubations of the Per peptide with UDP-Xyl and concentrated supernatants supernatant of yeast expressing *Drosophila* OXT or human xylosyltransferase I were subject to HPLC (with a gradient of 0–48% 95% acetonitrile from 2–14 min) and the fractions 11–11.5, 11.5–12 and 12–12.5 min were collected and dried. After verification by MALDI-TOF MS of the presence of, respectively di-, mono- and non-xylosylated forms of the Per peptide, half of each fraction was incubated with methylamine as previously described [11,15]. The samples were then subject to offline ESI tandem MS on a Q-TOF Ultima Global (Waters Micromass, Manchester, UK). The main instrument parameters were usually set as follows: capillary voltage 3 kV, cone voltage 80 V, collision cell pressure 6–8 × 10^{-5} mbar. Collision energy was adjusted manually to obtain the maximum of different fragment ions to assign the particular glycosylation sites. Tandem MS spectra were analyzed using the BioLynx function of MassLynx 4.0 SP4 software.

Results

Expression and properties of the worm xylosyltransferase

The previously-published studies on the characterisation of fly OXT were extended to include the *Caenorhabditis* orthologue (reading frame Y50D4C.4). Since preliminary data suggested that the *Drosophila* enzyme is active if the first eighty residues up to, but not including, the Pro-Xaa-Cys-Glu/Asp motif are deleted (data not shown), a similar construct for the worm enzyme lacking the first fifty amino acids was prepared. Thus, exons 2–6 of the *sqv-6* cDNA were cloned into the pPICZαA vector, resulting in an untagged form which includes the motif. After transformation of yeast, colonies were selected for expression studies and upon induction at 16°C (but, unlike the fly enzyme, not at 30°C), the predicted enzyme activity was indeed detected by HPLC using the Syn peptide as acceptor substrate (Figure 1, A and B). Activity was approximately equal regardless of whether *Pichia pastoris* GS115 (*his4*) or SMD1168 (*his4*, *pep4*) strains were used as hosts. The percentage conversion to product was measured using the RP-HPLC method after using Syn as the peptide substrate and 10 mM divalent cations in assays performed for up to 6 h (still within the linear range with respect to time). Bacterial expression of a His-tagged soluble form of the enzyme was also performed using the pET30a vector, resulting in a protein of ca. M_r 90,000 (theoretically M_r 92,000, including tag) detectable by Western blotting with anti-His antibodies in the soluble fraction of the bacterial lysate; no activity, though, was detected with the bacterially-expressed form (data not shown).

Performing the reaction at different temperatures (16, 23, 30, 37 and 50°C; see Figure 2A) over up to 6 h showed that the yeast-expressed worm enzyme has a lower temperature optimum than the fly enzyme [11], a result compatible

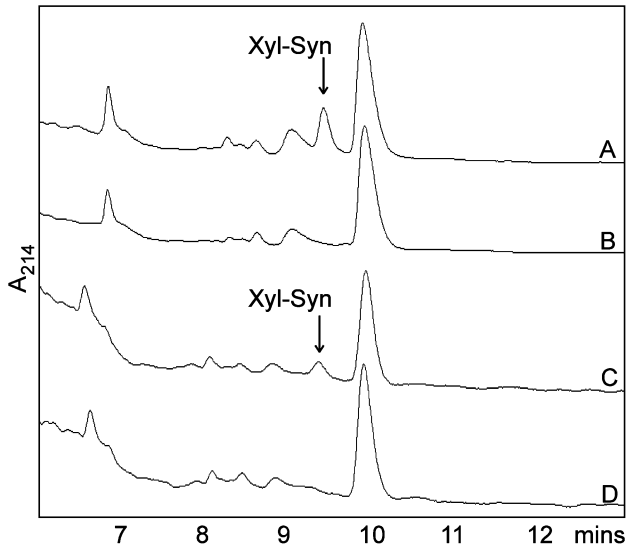

Fig. 1 *Assay of recombinant Caenorhabditis SQV-6 and human XT-I by RP-HPLC.* RP-HPLC analysis of the xylosyltransferase activity in concentrated supernatants of yeast expressing soluble forms of *Caenorhabditis* SQV-6 and human XT-I measured using the Syn peptide by incubation at, respectively, 23°C for 6 h or 37°C for 18 h (both at pH 8, 10 mM Mn^{2+}) in the presence (A, C) and absence (B, D) of UDP-xylose. The Syn substrate elutes at ∼ 10 min, whereas, as previously shown for incubations with the *Drosophila* enzyme, the xylosylated form (Xyl-Syn) elutes less than one minute earlier. The identity of products prepared from SQV-6 and XT-I eluting at this time were also verified by MALDI-TOF MS as having the expected m/z value (1240).

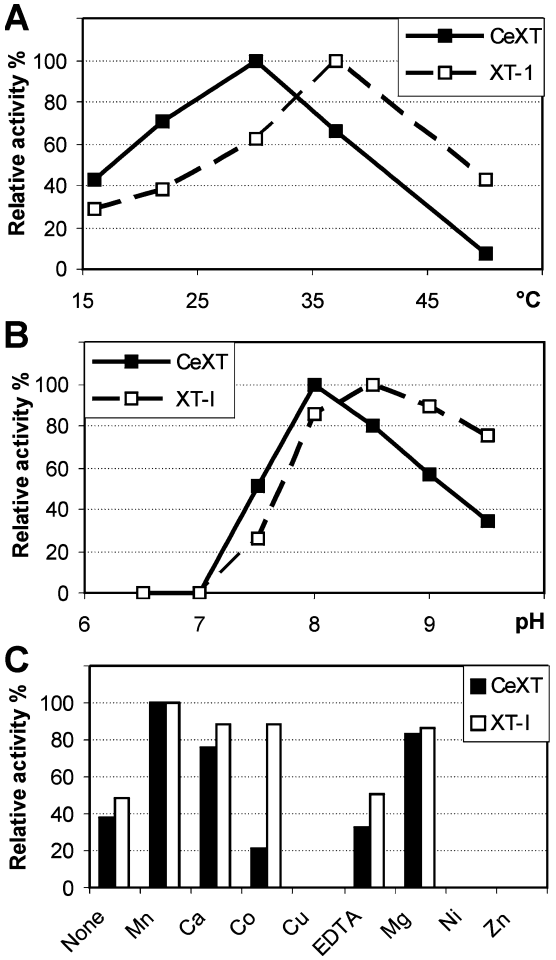

Fig. 2 *Enzymatic properties of soluble recombinant Caenorhabditis SQV-6 and human XT-I.* RP-HPLC based xylosyltransferase assays using the Syn peptide were performed with a concentrated supernatant of yeast expressing *Caenorhabditis* SQV-6 (generally assayed at 23°C) or with a resuspended 60% ammonium sulphate fraction of a supernatant of yeast expressing human XT-I (generally assayed at 37°C). Assays were performed for 4 h using either (A) different temperatures (HEPES, pH 8, 10 mM Mn^{2+}), (B) AMPD buffers of different pH (in the presence of 10 mM Mn^{2+}) or (C) different divalent cations as chloride salts (10 mM, HEPES, pH 8). Data from duplicate experiments were normalised to 100% for the condition resulting in the highest activity.

with worms being naturally soil inhabitants. For convenience, was 23°C used in all subsequent assays of SQV-6. The pH dependence of the worm enzyme showed an optimum of pH 8 using AMPD buffers (Figure 2B), as compared to 7–8 for fly OXT determined using HEPES buffers [11]; in preliminary MALDI-TOF MS-based assays, the highest activity of SQV-6 was achieved using either Tris pH 7.5 or HEPES pH 8 (data not shown). The metal ion dependence of recombinant SQV-6, with Mn (II) resulting in the highest activation of activity and Cu (II), Ni (II) and Zn (II) acting as inhibitors (Figure 2C), is also similar to that found with the recombinant fly enzyme [11] and was confirmed in independent MS-based assays. There is still activity in the presence of no cations and in the presence of EDTA; furthermore, increasing the final concentration of EDTA to 20 mM or 30 mM had no obvious negative effect on the enzyme's activity (data not shown). This activation by, but not absolute dependence on, divalent cations is similar to that observed with the native rat xylosyltransferase [16].

Expression of the human xylosyltransferases I and II

Previously the human xylosyltransferase I (XT-I), but not the xylosyltransferase II (XT-II), was found by others to be active using a radioactivity-based assay using recombinant bikunin as a protein substrate [10]. Thus, we decided to also express both these proteins in *Pichia* (lacking the stem, but including the Pro-Xaa-Cys-Glu/Asp motif, as for the SQV-6 construct) and to assay their activities by both mass spectrometry and HPLC using the Syn peptide (for HPLC data, see Figure 1, C and D). Initial enzymatic assays overnight indicated that the XT-I, but seemingly not XT-II, was expressed in an active form in *Pichia* at both 16 and 30°C. The levels of xylosylation from ten-fold concentrated supernatants were, however, rather lower than those obtained with the *Caenorhabditis* and *Drosophila* enzymes; thus, it was decided to perform ammonium sulphate precipitation in order to enrich the enzyme. Indeed, the activities of recombinant forms of fly OXT and human XT-I were detected in the 60%, but not in the

90%, pellet. The activity was sufficient to characterise XT-I further with, under the conditions used, an approximately two-fold higher amount of product at 8 h as compared to 4 h. In contrast to the worm enzyme, XT-I had a temperature optimum of 37°C (Figure 2A), the same as that previously shown for native bovine aorta and rat enzymes [16,17]; on the other hand, the human enzyme displayed an optimum of pH 8.5 similar to that of worm SQV-6 (Figure 2B), but higher than the pH optima of 6.5–7.5 for native xylosyltransferase from rat, bovine or chicken sources [16–18]. The metal ion dependence was also very similar to that of the worm and fly enzymes with no absolute requirement for divalent cations (Figure 2C); however, an approximate doubling of activity was observed in the presence of Ca (II), Co (II), Mg (II) and Mn (II).

Testing of four different xylosyltransferase acceptor substrates

In the previous study on the fly xylosyltransferase only the Syn peptide (based on part of the sequence of fly syndecan core protein) was used. This peptide was, as shown above, also efficiently utilised by the recombinant worm enzyme as well as by the human XT-I. Considering, however, the rather low K_m values of mammalian xylosyltransferases when using peptides based on part of the sequence of human bikunin [16,19], it was decided to also design the Bik peptide (modified to contain a C-terminal Arg residue, which was considered to be more compatible with MALDI-TOF MS than a form containing the usual Lys residue). Furthermore, in order to examine whether a peptide containing two Ser-Gly motifs (a common feature of many heparin sulphate attachment sites) would become dixylosylated, the Syn2 peptide was also synthesised.

The three peptides were tested with culture supernatants of yeast expressing recombinant *Caenorhabditis*, human and *Drosophila* xylosyltransferases; the Bik and Syn peptides were also tested with the *Caenorhabditis* extract. An increase in m/z of 132 was observed for all peptides upon incubation with the recombinant enzymes (Figure 3). In general, however, a certain lack of species specificity for transfer of xylose to peptide (*i.e.*, a "human" peptide or a "fly" peptide

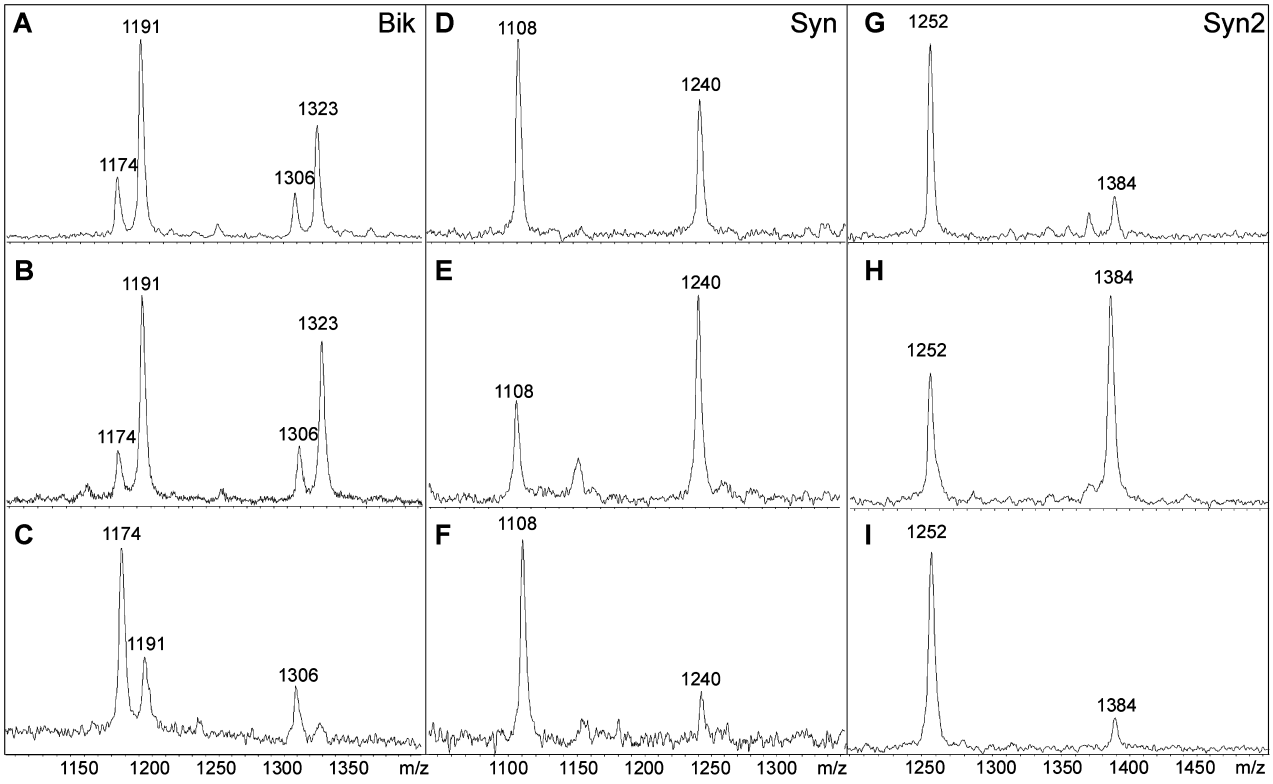

Fig. 3 *Assay of recombinant xylosyltransferase activities with peptide acceptors containing a single acceptor site.* MALDI-TOF MS-based xylosyltransferase assays were performed in the presence of UDP-xylose and of 10 mM Mn(II) ions at pH 8 (40 mM HEPES buffer) using either the Bik (A-C), Syn (D-F) or Syn2 (G-I) peptides with *Caenorhabditis* SQV-6 (A, D, G; 24 h, 23°C), *Drosophila* OXT (B, E, H; 12 h, 30°C), human XT-I (C, F, I; 24 h, 30°C) enzymes expressed at 16°C. Peaks corresponding to the xylosylated product (m/z 132 greater than the substrate) were absent from the samples with no added UDP-xylose and from incubations with empty-vector control supernatants (data not shown). The peaks in the Bik samples (A, B, C) which are m/z 17 smaller than the original peptide and its xylosylated form are due to the formation of pyroglutamate at the N-terminus of the peptide. Although the Syn2 peptide has two Ser-Gly motifs, only one of these was xylosylated.

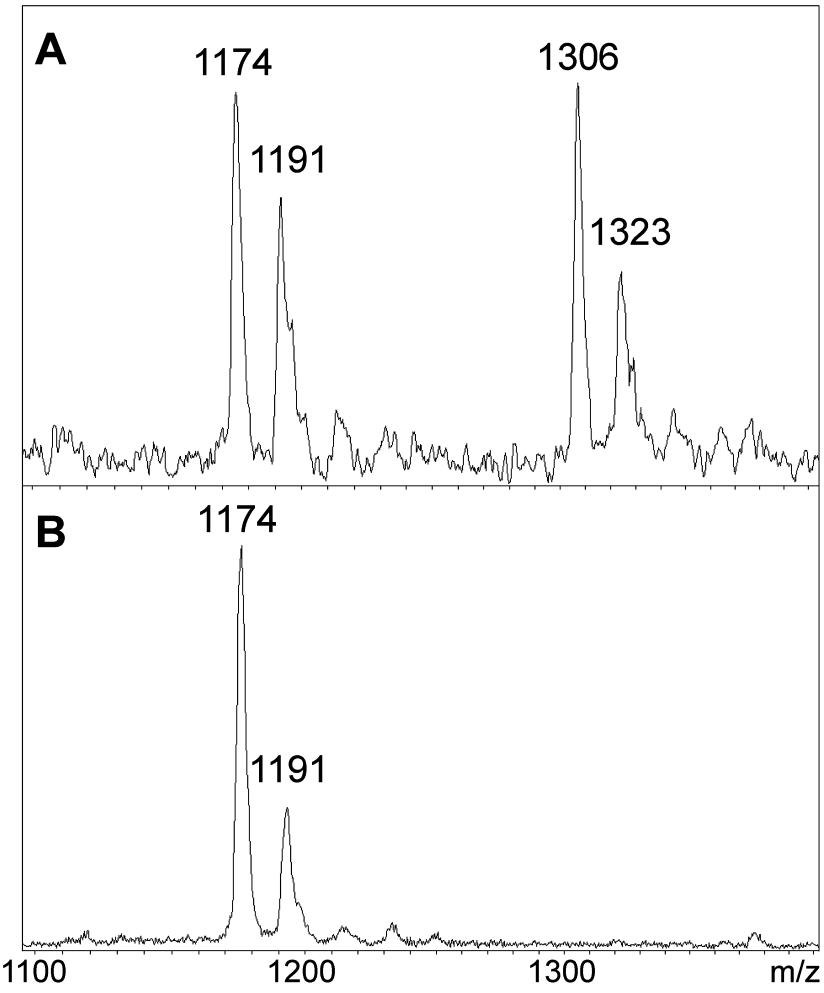

Fig. 4 *Assay of native Caenorhabditis elegans xylosyltransferase.* MALDI-TOF MS-based assays with an extract of *Caenorhabditis* using Bik peptides in the presence (A) and absence (B) of an incubation of UDP-GlcA decarboxylase with UDP-GlcA as a source of UDP-Xyl (24 h, 23°C, pH 7, Mn^{2+}).

can be used with a fly, a human or a worm xylosyltransferase) is obvious. In the case of Syn2, which contains two Ser-Gly motifs, only a single xylose was transferred. Preliminary data indicated that the K_m values of recombinant worm SQV-6 for the Bik and Syn peptides are ~300 μM and ~750 μM respectively (data not shown), as compared to the previously-determined K_m value of recombinant fly OXT for the Syn peptide of ~500 μM [11]. In the case of the worm extract, the Gln residue at the N-terminus of the Bik peptide was predominantly converted to pyroglutamate by a glutamine cyclotransferase ($\Delta m/z = -17$), an effect also seen to a variable extent with the *Pichia* supernatants when incubated overnight. Nevertheless, xylosylation was observed using the worm extract (Figure 4); this is indeed the first in vitro assay described for a native invertebrate xylosyltransferase and corresponds to an activity of 10 pmol μg^{-1} h^{-1}.

Considering that the Syn2 peptide containing two 'artificial' Ser-Gly motifs was not dixylosylated, a more natural peptide with at least two such motifs was sought. Thus, a portion of the human perlecan core peptide, corresponding to residues 62–80 (part of domain I) containing three Ser-Gly motifs and previously shown to carry glycosaminoglycan chains [20,21], was designed to contain, like the Bik peptide, a C-terminal Arg residue. The conversion of this peptide (Per) was more differential than with the other substrates (Figure 5), since the *Caenorhabditis* SQV-6 barely utilised this substrate, whereas the *Drosophila* OXT and human XT-I modified it with up to two xylose residues.

Identification of sites xylosylated by xylosyltransferases in vitro

In a previous study, the product of *Drosophila* OXT with the Syn peptide was analysed by cleavage of the peptide by endoproteinase GluC; by these means it was concluded that the second, and not the first, serine residue of the DDDSIEGSGGR peptide had been xylosylated [11]. With the Syn2 peptide used in the present study, a second Ser-Gly motif was introduced into the sequence; as described above, only a single xylose residue was transferred regardless of enzyme source. In order to show which serine had

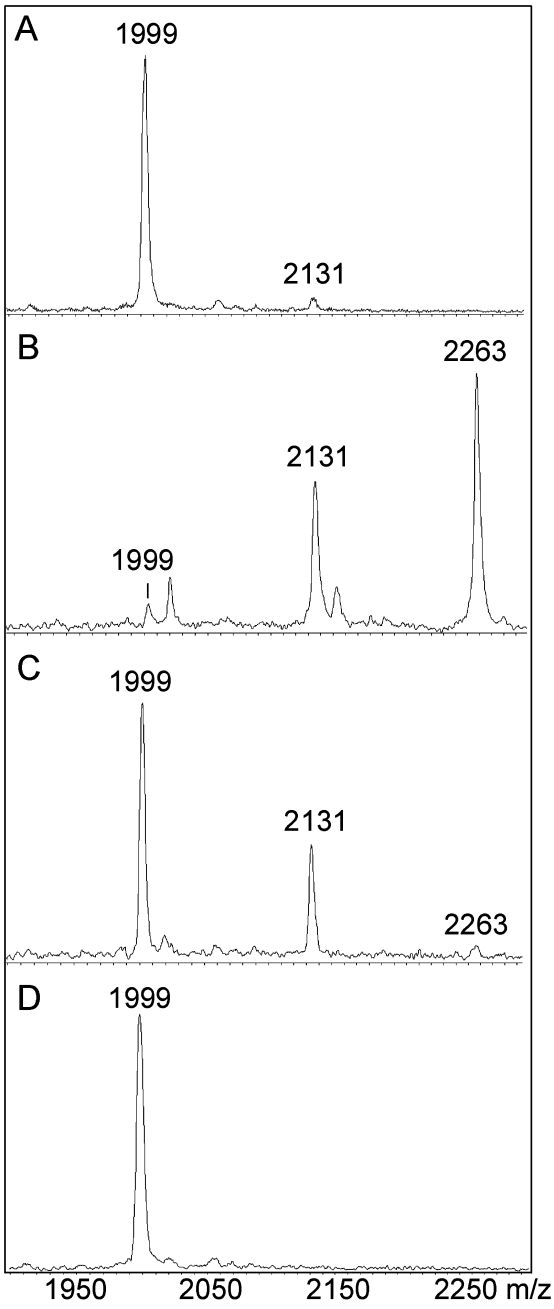

Fig. 5 *Assay of recombinant xylosyltransferases with a peptide containing multiple acceptor sites.* MALDI-TOF MS-based xylosyltransferase assays were performed in the presence of UDP-xylose for 22 h (HEPES, pH 8, Mn^{2+}) using the Per peptide with *Caenorhabditis* SQV-6 (A, 23°C), *Drosophila* OXT (B, 30°C) or human XT-I (C, 37°C). The spectrum resulting after an incubation of human XT-I with Per in the absence of UDP-Xyl is also shown (D).

been modified, Syn and Syn2 were used as acceptors with a partially-purified His-tagged form of the *Drosophila* enzyme; the products were purified by RP-HPLC and analysed by Q-TOF MS in MALDI mode. This analysis showed that only the first Ser-Gly of Syn2 had been modified, while confirming that only the Ser-Gly motif, but not the Ser-Ile, of the Syn peptide had been xylosylated (Figure 6).

Since the Syn2 peptide was not dixylosylated, it was decided to analyse the modification of the Per peptide, which could be dixylosylated when using a crude supernatant of yeast expressing *Drosophila* OXT or human XT-I as the enzyme source (see above). Since the xylose residue was rather labile under the experimental conditions used, the xylosylated peptides were first subject to β-elimination with methylamine. It was then demonstrated that, with monoxylosylated peptides, the modification occurred on either the second and third Ser-Gly motifs (with some bias towards the second motif), but not on the first motif (Figure 7B). Although there was some dixylosylated peptide in the human XT-I sample, it was only possibly to confidently analyse the dixylosylated *Drosophila* sample: in this case, both the second and third Ser-Gly motifs were demonstrably modified (Figure 7A).

Discussion

Peptide *O*-xylosyltransferase activities have been known for some forty years [7], but it is only five years ago that the first such enzyme was characterised in a recombinant form [10]. In the present study, we have extended our previous study on the *Drosophila* xylosyltransferase [11] to include a fuller characterisation of the *Caenorhabditis* enzyme and human xylosyltransferase I. Although assays of recombinant forms of both these enzymes have been published by others [10,12], their temperature, pH and cation dependence have previously not been reported. In engineering forms of these enzymes for expression in yeast, we took account of preliminary data indicating that the first eighty residues could be deleted from the fly enzyme while still retaining activity (data not shown). All expression constructs encode forms of the enzymes lacking the entire putative stem regions, which are of variable length, but including the first obviously conserved motif in the N-terminal region (Pro-Xaa-Cys-Glu/Asp) found in all xylosyltransferase sequences identified to date [7]. The significance of this motif is not clear, but recent data on human XT-I expressed in baculovirus suggests, in contrast to preliminary data with the fly enzyme, that it is not essential for activity of XT-I [22].

The results from the present study suggest that the worm SQV-6 and human XT-I have similar properties to those previously reported for the recombinant fly OXT [11]. None of these enzymes have an absolute requirement for divalent cations and none are inhibited by EDTA, but they are activated especially by Mn (II). Furthermore, the pH optima of the recombinant *Caenorhabditis* SQV-6, *Drosophila* OXT and human XT-I are around pH 8; although we deal with recombinant forms, it is interesting to note that such a pH optimum is somewhat closer to that of, *e.g.*, the ER-localised

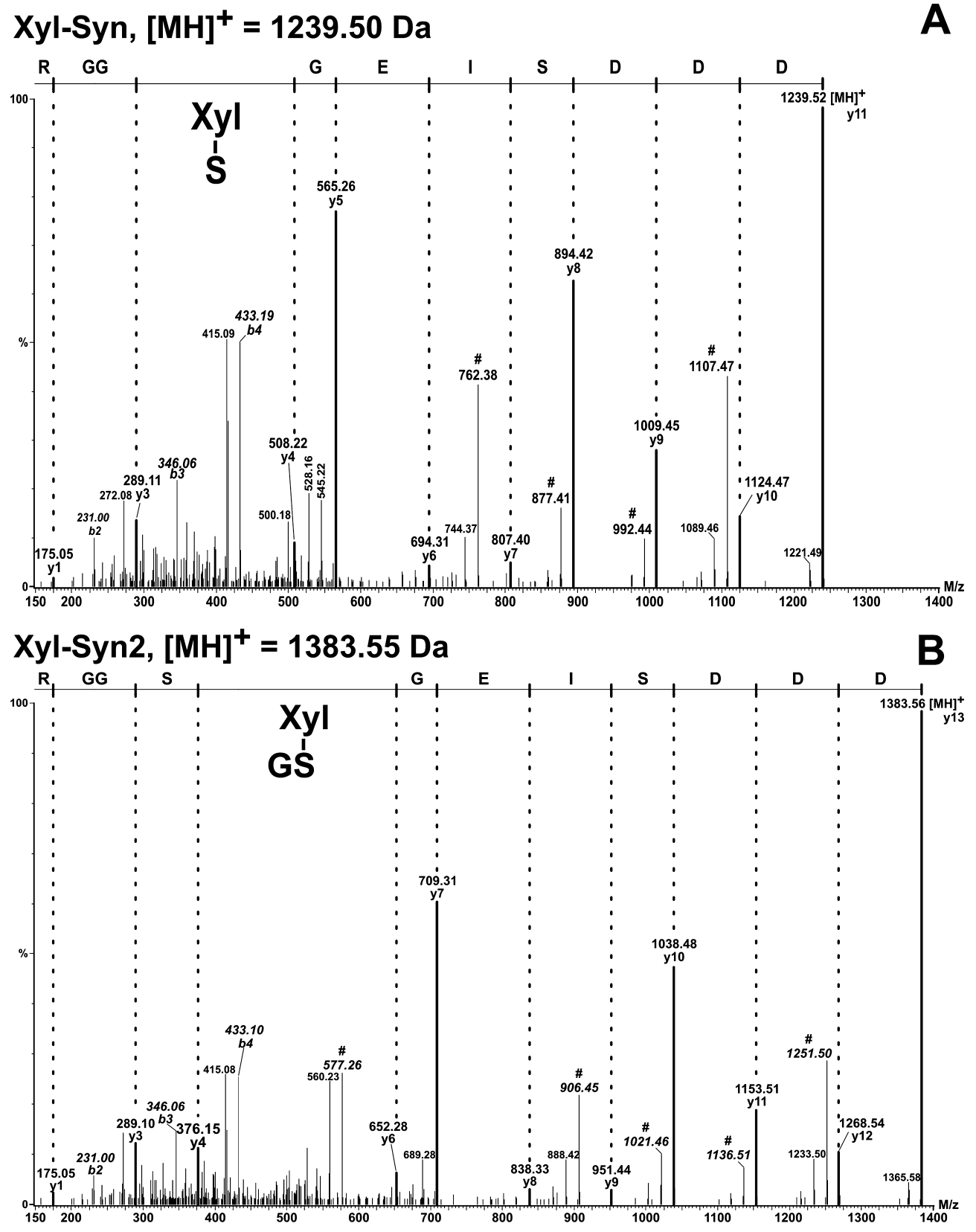

Fig. 6 *MALDI Q-TOF tandem MS analysis of the products of Drosophila OXT.* The RP-HPLC purified xylosyltransferase assay products of the His-tagged form of OXT with the (A) Syn and (B) Syn2 peptides were subject to tandem MS. The y-ion series is labelled in detail, showing that a xylose was added to Ser8 in both samples. Detected b-ions are assigned in italic letters, ions resulting from a loss of xylose are indicated with a hash sign.

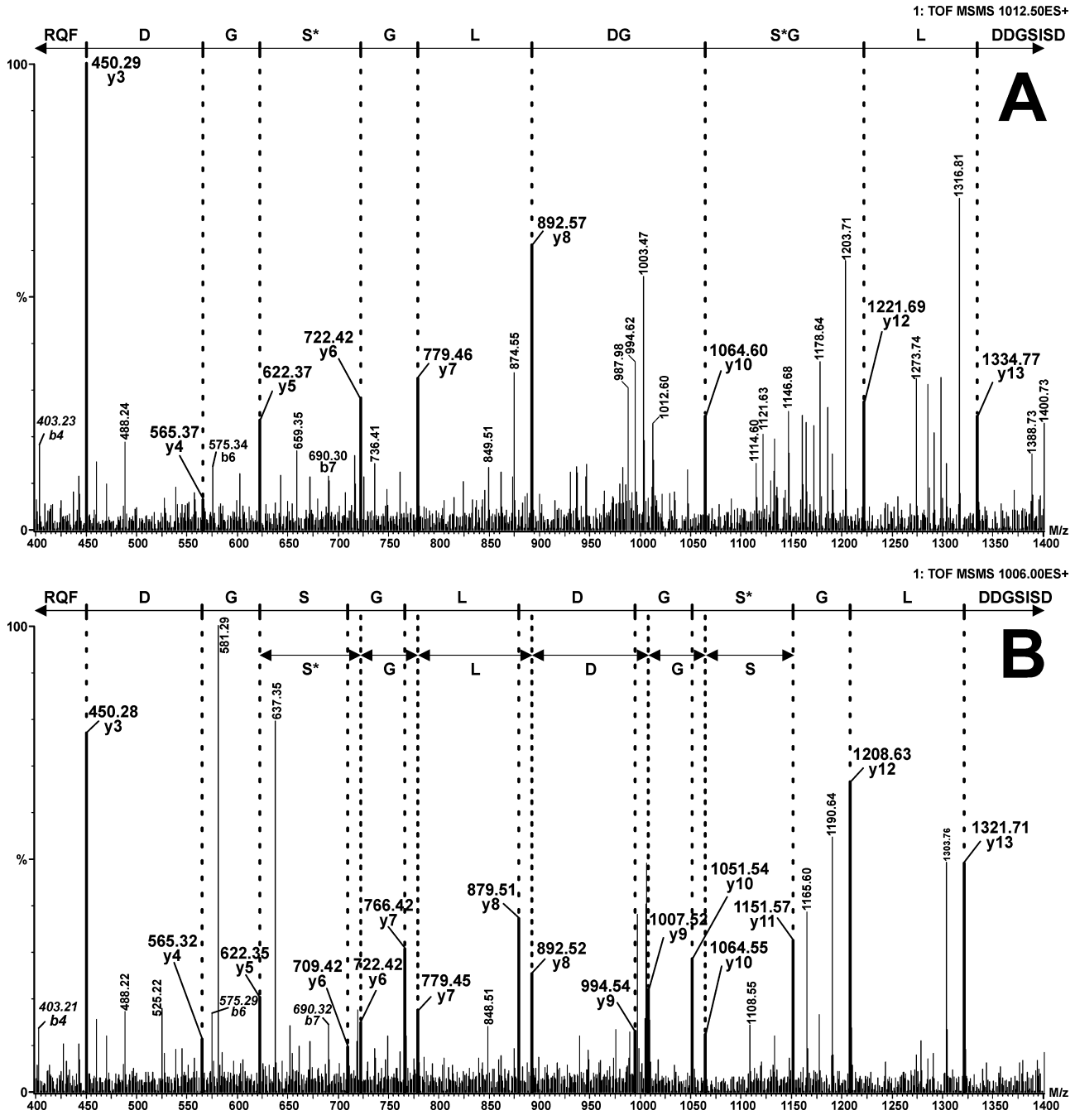

Fig. 7 *ESI Q-TOF tandem MS analysis of products of Drosophila and human xylosyltransferases.* The RP-HPLC purified xylosyltransferase assay products were subjected to β-elimination followed by tandem MS. The y-ion series of the doubly xylosylated product of *Drosophila* OXT (A) gave a clear spectra showing the sugar attachment sites Ser10 and Ser15 (designated by S*), whereas the monoxylosylated product of the human XT-I (B) exhibited two sets of y-ion series, indicating that xylose was attached either to Ser10 or to Ser15. No signals indicating xylosylation on Ser2 or Ser4 were detected. Detected b-ions are assigned in italic letters, peaks not assigned to the y- or b- ion series are labelled vertically.

UDP-Glc:glycoprotein glucosyltransferase [23], than that of a typical Golgi-localised enzyme such as the N-glycan modifying *N*-acetylglucosaminyltransferase I [24]. Whether expression in *Pichia* as soluble forms has an effect on the pH optimum of the xylosyltransferases is unknown and testing this would require a direct comparison with the same enzymes produced in another system; however, a number of *Caenorhabditis* glycozymes expressed in yeast in this laboratory, *e.g.*, the core α1, 6-fucosyltransferase [14], display the pH optima previously shown for the corresponding native mammalian enzymes.

Since one may assume that the actual active site for xylose transfer is in the region homologous to other family 14 transferases [7,11], it may be that the C-terminal region has some other function. It is interesting, though, that the C-terminal region of the worm enzyme is difficult to align with those of other xylosyltransferases, except for the Trp-Ser-Ser/Thr-Xaa-Xaa-Pro-Asp-Pro-Lys-Ser-Asp/Glu motif found towards the very end of the sequence. Based on homology, one may conclude that conserved motifs towards both the N- and C-termini are required for expression and/or activity of invertebrate xylosyltransferases: however, that the C-terminus of the fly enzyme (data not shown) and human XT-I [25] can carry His-tags means some changes in this region are tolerated. Also, the differences between the worm and other sequences in the C-terminal region could mean that the worm enzyme has some unusual properties, since if in this species there was no selective pressure to retain certain features of this region, then one could expect more sequence divergence. The only obvious differences we found, though, were shifts in the temperature and pH optima, as well as a lower relative activity with the Per peptide as substrate. That this region is, however, important, is shown by the fact that the mutant form of the *Caenorhabditis sqv-6* gene has an amber mutation of the 764th codon, introducing a stop codon, and is apparently inactive [12]. It is interesting to note that the worm enzyme lacks a 'DXD' motif in the C-terminal region, even though this is apparently necessary for the activity of the human enzyme [26]; since we assume the C-terminal region is not involved in the catalytic mechanism and that the enzyme is not absolutely dependent on cations, we would hypothesise that this is not a true 'DXD' motif with a role in cation binding, but that this motif has some other function in those xylosyltransferases that contain it.

One possible function for the large C-terminal region, specific for fly and mammalian xylosyltransferases, could in theory be phosphorylation of the xylose residue within the GlcAβ1-3Galβ1-3Galβ1-4Xylβ1-*O*Ser glycosaminoglycan core sequence. It has been previously noted that the xylose residue of many glycosaminoglycans is phosphorylated (for instance in *Drosophila*), but not in *Caenorhabditis* [27], and that this modification may already begin to occur before the action of the first galactosyltransferase [28]. Thus we also sought to determine whether the *Drosophila* xylosyltransferase is capable of performing the relevant xylose 2-kinase reaction. Enzyme incubations in the presence of ATP, however, showed no presence of a species with m/z 80 larger than the normal xylosyltransferase product (data not shown) and suggest fly OXT does not phosphorylate the xylose residue. Indeed, though, this result is consistent with other data indicating that phosphorylation of the xylose takes place after the addition of the first galactose residue to the core region [29]. This could suggest that, rather than encoding an additional enzymatic function, the C-terminal region is responsible for interaction(s) with other proteins (*e.g.*, other enzymes involved in glycosaminoglycan biosynthesis), for certain aspects of acceptor substrate recognition or for modulation of the activity, *e.g.*, by feedback inhibition mediated by products such as heparin [30]. In this context, it is noteworthy that the worm, fly and human XT-I enzymes were totally inhibited by 0.5 mg/ml heparin when using Syn as the acceptor substrate (data not shown). Thus, this leaves the somewhat lower activity towards the Per peptide as being the only major difference between the worm enzyme on one hand and the *Drosophila* and human XT-I enzymes on the other.

The recombinant fly OXT, human XT-I and worm SQV-6 enzymes are capable of using all four peptide substrates tested (Bik, Per, Syn and Syn2). In the latter case, only one xylose residue was transferred even though the peptide contained two Ser-Gly motifs. Furthermore, Q-TOF collision data would suggest that only the first of these two motifs was xylosylated. Since tandem Ser-Gly sequences are a feature of many heparin sulphate attachment sites and that such motifs enhance heparin sulphate assembly [31], the expectation was that each Ser of such sequences is capable of accepting xylose. However, obviously other factors are involved; perhaps the distance from the acidic patch, also a common feature of putative xylosylation sites [32], is too long or perhaps multiple Ser-Gly sequences in some substrates merely aid the transfer of xylose to a single site or that multiple xylosylation of such sequences only occurs in vivo.

In the case of the perlecan peptide (DSISGDDLGSGDLGSGDFQR), transfer of up to two xylose residues was detected when using *Drosophila* OXT and human XT-I; it appeared that the addition of xylose to the second or third Ser-Gly motifs was biased towards the second motif, but no strict order of xylosylation was observed. In the case of the dixylosylated product of the *Drosophila* enzyme, both the second and third, but not the first, motifs were obviously modified. Even though all three Ser-Gly motifs in this peptide are of the form D/E-X-X-S-G-D, and therefore compatible with previous xylosylation consensus sequence data [19,32], the lack of transfer to the first motif may be due to its proximity to the N-terminus of the peptide or that there is only one acidic residue preceding the first motif within the peptide used; indeed both the Bik and Syn peptides have at least three acidic residues preceding the xylosylation site. Thus, these data are an additional indication that the region N-terminal to the potential xylosylation site also plays a factor in facilitating xylosylation. Indeed, in the case of a larger peptide, there is data to indicate that the first motif of the murine perlecan domain I, which albeit has a slightly different sequence from the human domain I used as the basis for our peptide, does accept a xylose residue in a cellular context [20,21], but that alterations N-terminal to the first motif affect the type of final glycosaminoglycan chain [33], rather than the extent of xylosylation. As discussed above, even though the worm

enzyme efficiently utilised the Bik and Syn peptides, the level of incorporation into the Per peptide by SQV-6 was comparatively low. In this context it is interesting that the worm also has a perlecan homologue (UNC-52) which, however, has a domain I unlike that of mammals [34]. On the other hand, the homologous *Drosophila trol* gene product also lacks this region [35], while fly OXT efficiently uses the Per peptide as a substrate; thus it is unclear whether the low activity of SQV-6 towards the Per peptide is correlated with the *in vivo* substrates of this enzyme, some other factor, or indeed to the previously noted divergence of its C-terminal domain.

It is also noteworthy that vertebrates have two xylosyltransferase isoforms; however, only the activity of human XT-I has been previously proven. Whether the XT-II is indeed enzymatically active has been to date unclear, although preliminary data suggested a potentially low level of transfer to the Per peptide (data not shown). Certainly, in other assays, mixing supernatants of yeast expressing XT-I or OXT with supernatants from yeast transformed with XT-II did not result in any obvious additive effect (data not shown), which would be inconsistent with XT-II being a xylosylpeptide xylosyltransferase. Perhaps XT-II has a particularly stringent substrate specificity, a situation reminiscent of certain polypeptide *N*-acetylgalactosaminyltransferases [36], or requires some unknown co-factor for activity. However, the relevance of XT-II obviously requires further experimentation, whether it be at a biochemical level or by the use of knock-out systems.

In summary, during the present study we have examined properties of two invertebrate and one vertebrate peptide *O*-xylosyltransferases expressed in *Pichia pastoris*, the *Drosophila* OXT previously described by us, the *Caenorhabditis* SQV-6 protein and the human xylosyltransferase XT-I. While this work was in progress, SQV-6 was shown by others to complement the defect in the ability to transfer xylose to silk in a xylosyltransferase-defective Chinese hamster ovary cell line [12]. On the other hand, the recombinant human XT-I was previously assayed only by incorporation of radioactivity into a small protein substrate [10], although a very recent report includes mass spectrometric analysis of the products of human XT-I [37]. Our study, though, is the first in which both vertebrate and invertebrate forms of recombinant xylosyltransferases have been characterised using the same four short peptide acceptors and in which their products were analysed by both HPLC and MALDI-TOF MS. Furthermore, we have observed some bias in substrate preference, particularly towards the peptide with multiple xylosylation motifs. Such fine substrate specificity may well have profound effects on the modification of proteoglycan core proteins in vivo, which is key to many aspects of animal development.

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