In vitro phase II metabolism of xanthohumol by human UDP-glucuronosyltransferases and sulfotransferases

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Xanthohumol (XN) is the principal prenylated flavonoid of the hop plant and has recently gained considerable interest due to its potential cancer-chemopreventive effects. However, the metabolism of XN has not yet been investigated in detail. Therefore, we studied the *in vitro* phase II metabolism of XN using nine human recombinant UDP-glucuronosyltransferases (UGT) and five sulfotransferases (SULT). The identification of the metabolites formed was elucidated using HPLC with diode array detection as well as HPLC/API-ES MS. XN was efficiently glucuronidated by UGT 1A8, 1A9, and 1A10; further important UGTs were UGT 1A1, 1A7, and 2B7. With respect to the sulfation reaction, SULT 1A1*2, 1A2, and 1E1 were the most active SULT forms. UGT 1A3, 1A4, and 1A6 as well as SULT 1A3 and 2A1 were of minor importance for the conjugation of XN. Three mono-glucuronides as well as three mono-sulfates were identified. Considering the tissue distribution of the tested UGT and SULT enzyme forms, these findings suggest a prominent role for the glucuronidation and sulfation of XN in the liver as well as in the gastrointestinal tract.

Keywords: Phase II metabolism / Sulfotransferase (SULT) / UDP-Glucuronosyltransferase (UGT) / Xanthohumol

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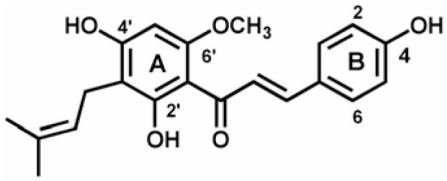
1 Introduction

Xanthohumol (XN) (3′-[3,3-dimethyl allyl]-2,4,4′-trihydroxy-6′-methoxychalcone) (Fig. 1) is the major prenylated flavonoid of the female inflorescences ("hops" or "hop cones") of the hop plant (*Humulus lupulus* L.), an ingredient of beer. XN constitutes 82 – 89% of the total amount of prenylated flavonoids of different European hop varieties [1]. Hop cones are known not only as brewing materials but also as a tranquilizer in folk medicines [2]. Recently, XN has gained considerable attention due to its various biological effects. Studies have shown that XN is an effective antiproliferative agent in human cancer cell lines and prevents carcinogen-induced preneoplastic lesions in mouse mammary gland organ culture at nanomolar concentrations. Besides the potential cancer-chemopreventive activity, XN exhibits antimutagenic and antioxidative effects and protects against arteriosclerosis and osteoporosis *in vitro* [3 – 6]. However, up to now the metabolic fate of XN has not been analyzed in detail. So far Yilmazer *et al.* [7, 8] have investigated the phase I and II metabolism *in vitro* using rat and human liver microsmes Nokandeeh *et al.* and Avula *et*



xanthohumol

Figure 1. Chemical structure of XN.

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Abbreviations: DAD, diode array detector; **MGluc**, mono-glucuronide; **MSulf**, mono-sulfate; **PAPS**, adenosine-3′-phosphate-5′-phosphosulfate; **SULT**, sulfotransferase; t_R, retention time; **UDPGA**, uridine-5′-diphosphate-β,D-glucuronic acid ester; **UGT**, UDP-glucuronosyltransferase; **XN**, xanthohumol

al. [9, 10] have studied the bioavailability and metabolism of XN after oral as well as intravenous administration to rats. Hydroxylation, *O*-methylation, *O*-acetylation, epoxidation, cyclization involving the prenyl side chain, and glucuronidation have been observed *in vitro* and *in vivo* in rats [7 – 9, 11]. XN was excreted primarily in feces with more than 89% in the unchanged form [9]. However, so far it has not been investigated if XN also is a substrate for cytosolic sulfotransferases (SULT). Moreover, the specific enzyme isoforms responsible for XN-conjugation and their location – *i.e.*, the hepatocyte or the enterocyte – are presently unknown. The goal of the present study is to investigate the phase II metabolism of XN *in vitro* using a series of human recombinant UDP-glucuronosyltransferases (UGT) and SULT. The formed metabolites were identified using HPLC with diode array detection (DAD) as well as HPLC/API-ES MS.

2 Materials and methods

2.1 Chemicals

XN was isolated from an ethanolic extract of hops [9]; the purity was >99% according to HPLC/MS analysis. Microsomal recombinant human UGTs 1A1, 1A3, 1A6, 1A7, 1A10, 2B7, control baculosomes, and cytosolic recombinant human SULT, all expressed in baculovirus-infected Sf9 insect cells, were obtained from PanVera (Madison, WI, USA). Microsomal recombinant human UGT isoforms (UGT 1A4, 1A8, and 1A9), expressed in human lymphoblastoid cells, were purchased from Gentest (Woburn, MA, USA). Adenosine-3′-phosphate-5′-phosphosulfate (PAPS) was provided by W. Meinl and H.-R. Glatt (German Institute of Human Nutrition, Potsdam, Germany). Uridine-5′-diphosphate-β,D-glucuronic acid ester (UDPGA), tetrabutylammonium phosphate monobasic reagent, alamethicin, DMSO, and DTT were obtained from Sigma-Aldrich Chemical (Deisenhofen, Germany). All other chemicals were of the highest grade available.

2.2 Enzymatic formation of glucuronic acid conjugates

Incubations were carried out according to manufacturer's recommendation as follows. Reaction mixtures contained 1 mM UDPGA, 50 µM XN (from 1 mM stock solutions in DMSO), 20 mM $MgCl_2$, 1 mM DTT, and 25 µg/mL alamethicin in 50 mM Tris-HCl buffer (pH = 7.4) and were preincubated for 5 min at 37°C. Reactions were initiated by the addition of 0.5 mg/mL UGT. Total reaction volumes were 100 µL. Reaction termination was achieved after 2 h at 37°C by adding 25 µL 20% TCA. Controls were carried out by omitting UDPGA or by using control baculosomes. Neutralization was accomplished by adding 25 µL of 1 M NaOH. Injection volumes of 100 µL were analyzed directly by HPLC/DAD and HPLC/MS after centrifugation at 10 000 *g* for 5 min.

Quantitation of the formed glucuronosyl metabolites was carried out on the basis of the peak area in the HPLC chromatogram at 371 nm (absorbance maximum of XN) assuming similar ε values of the aglycone and its conjugates. The UV spectra of the XN-mono-glucuronides (MGlucs) MGluc 1, MGluc 2, and MGluc 3 showed maxima at 359, 368, and 365 nm, respectively.

2.3 Enzymatic formation of sulfate conjugates

Incubations with SULT were performed according to Ireson *et al.* [12]. Reaction mixtures with 25 µM XN (from 1 mM stock solutions in DMSO), 10 mM $MgCl_2$, and 20 mM DTT in 100 mM potassium phosphate buffer (pH = 7.3) were preincubated for 5 min at 37°C. Reactions were initiated by the addition of 72 µM PAPS and 0.25 mg/mL SULT. Controls contained no PAPS or protein. Total reaction volumes were 100 µL. Reaction termination and sample clean-up were carried out in the same way as described for the glucuronidation assay.

Quantitation was done in the same way as described for the glucuronides. The UV spectra of the XN-mono-sulfates (MSulf) MSulf 1, MSulf 2, and MSulf 3 showed maxima at 366, 355, and 354 nm, respectively.

2.4 HPLC analysis

Samples were analyzed by LC with UV detection at 371 nm using a Prontosil (150 nm × 4.0 mm id, particle size 3 µm) RP column (Bischoff, Leonberg, Germany). The solvent system consisted of 0.1% formic acid in water (pH = 3) (A) and ACN (B). For the separation of the sulfate conjugates formed, 5 mM tetrabutylammonium phosphate monobasic reagent was added to the solvent system A to improve the peak form by HPLC/DAD analysis. Elution was effected using a linear gradient from 29 to 100% B in 35 min, 100% B for 8 min, and from 100 to 29% B in 7 min. The flow rate was 0.8 mL/min and the eluate was recorded with a DAD at 371 nm. Observed peaks were scanned between 190 and 600 nm.

2.5 LC/MS analysis

HPLC/MS analysis was performed on an HP 1100 series HPLC (Agilent Technologies, Waldbronn, Germany) equipped with an autoinjector, quaternary HPLC pump, column heater, UV detector, and HP Chem Station for data

collection and handling. The HPLC was interfaced to an HP series 1100 mass selective detector equipped with an API-ES chamber.

For the analysis of the metabolites formed, conditions in the negative mode were as follows: capillary voltage 3.5 kV; fragmentor voltage 100 V; nebulizing pressure 60 psi; drying gas temperature 350°C; drying gas flow 11 L/min. Data were collected using both the scan mode and SIM mode. Spectra were scanned over a mass range of *m/z* 100 – 700 at 1.08 s *per* cycle. For LC, the same conditions as described above were used.

3 Results

3.1 Enzymatic formation of glucuronic acid conjugates

Enzymatic synthesis of XN-glucuronides was characterized using purified human recombinant enzymes. Overall yields, calculated by the peak area of the formed conjugates in comparison to the total peak area of the nonconjugated and conjugated form, for the nine tested UGTs are shown in Table 1.

Table 1. Overall yields of MGluc formation after incubation of the nine tested human recombinant UGTs with 50 μM XN and 50 μg protein for 120 min. The amounts of the three MGlucs detected (MGluc 1 at t_R = 12.4 min; MGluc 2 at t_R = 16.8 min; MGluc 3 at t_R = 17.8 min) are expressed as percent of the total glucuronides formed. Analysis was done in duplicate and mean values are shown.

UGT form	Overall yield, %	% of total amount		
		MGluc 1	MGluc 2	MGluc 3
1A1	44.3	16.1	77.0	6.9
1A3	15.5	7.3	92.7	0.0
1A4	4.8	8.4	68.9	22.7
1A6	2.9	13.0	87.0	0.0
1A7	36.6	7.8	92.2	0.0
1A8	92.8	9.6	90.2	0.2
1A9	73.6	13.8	85.9	0.3
1A10	87.8	18.4	79.7	1.9
2B7	44.1	15.1	83.9	1.0

XN is most efficiently conjugated by UGT 1A8, 1A9, and 1A10. The overall yields decrease from 1A8 > 1A10 > 1A9 >> 1A1 > 2B7 > 1A7 >> 1A3 >> 1A4 > 1A6 under the conditions used in the assay. Three MGlucs were clearly detected by HPLC analysis and were identified by HPLC/MS in the negative mode (loss of the glucuronic acid moiety of 176 amu). In Fig. 2, a corresponding HPLC chromatogram of XN after incubation with UGT 1A10 is shown. The

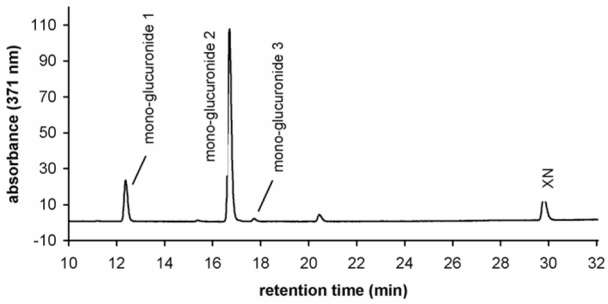

Figure 2. Representative HPLC chromatogram of the incubation of XN with UGT 1A10 based on detection at 371 nm.

Table 2. Overall yields of MSulfs formed after incubation of the five tested human recombinant SULTs with 25 μM XN and 25 μg protein for 120 min. Amounts of the three MSulfs (MSulf 1 at t_R = 31.2 min; MSulf 2 at t_R = 32.3 min; MSulf 3 at t_R = 35.9 min) are expressed as percent of the total sulfates formed. Analysis was done in duplicate and mean values are shown.

SULT form	Overall yield, %	% of total amount		
		MSulf 1	MSulf 2	MSulf 3
1A1*2	30.1	11.2	67.3	21.5
1A2	38.8	0.0	37.9	62.1
1E1	46.0	81.1	13.0	5.9
1A3	3.4	0.0	100	0.0
2A1	4.2	79.9	20.1	0.0

API-ES mass spectrum, representative of all three XN MGlucs, showed a deprotonated quasi molecular ion $[M-H]^-$ at *m/z* 529 and a characteristic fragment ion at *m/z* 353 (deprotonated XN aglycone) due to the loss of the glucuronic acid moiety. With all UGT enzyme forms, the MGluc with the retention time (t_R) at 16.8 min (MGluc 2) is formed to the highest extent. In Table 1, the yields of the single MGlucs formed are shown in detail. However, it was not possible to identify the position of the glucuronidation with the single quadrupol HPLC/MS system used in this study.

3.2 Enzymatic formation of sulfate conjugates

Enzymatic synthesis of XN-sulfates was characterized using purified cDNA-expressed human SULTs. The overall yields for the five tested SULTs and the amount of each MSulf formed are shown in Table 2.

SULT 1A1*2 (a variant allele of SULT 1A1), 1A2, and 1E1 show the highest activities. The overall yields decrease from SULT 1E1 > 1A2 > 1A1*2 >> 2A1 > 1A3 under the conditions used in the assay. Interestingly, none of the three hydroxyl groups in the molecule is conjugated predominantly. The MSulf with the t_R at 31.2 min (MSulf 1) was predominantly formed by SULT 1E1 and 2A1, the MSulf at

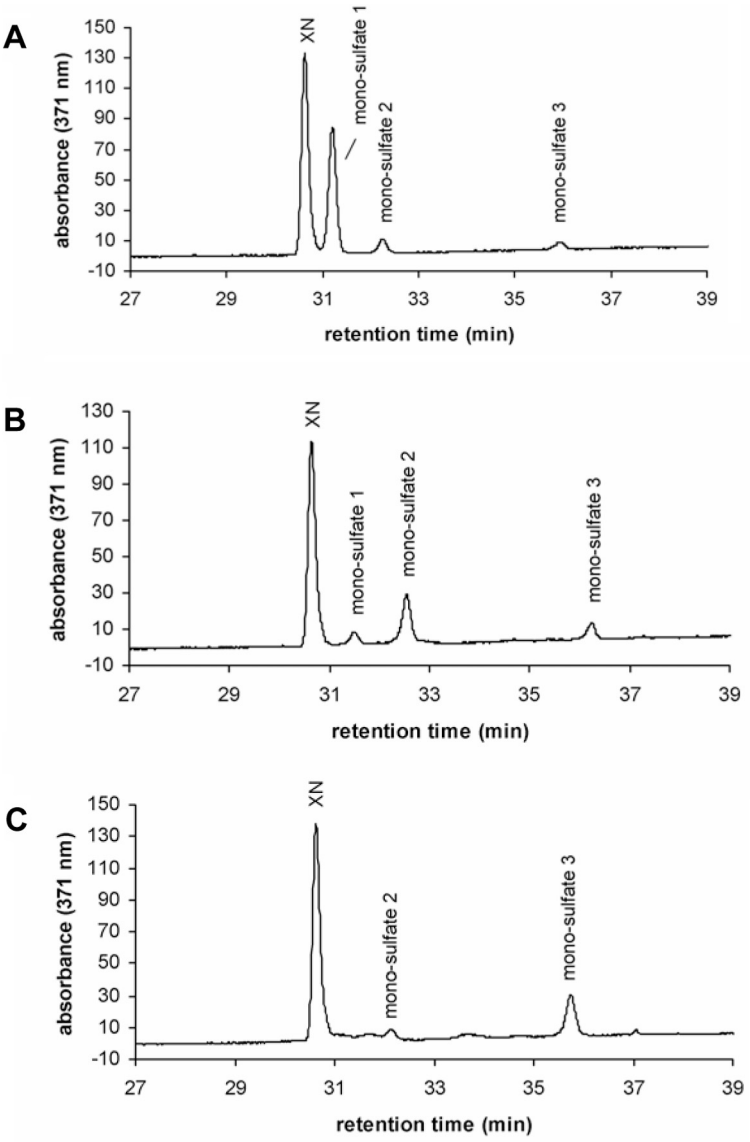

Figure 3. Representative HPLC chromatogram of the incubation of XN with SULT 1E1 (A), SULT 1A1*2 (B), and 1A2 (C) based on detection at 371 nm.

32.3 min (MSulf 2) by SULT 1A1*2 and 1A3, whereas the third MSulf at 35.9 min (MSulf 3) was found in the highest yield after incubation with SULT 1A2. In Fig. 3, corresponding HPLC chromatograms of XN and the sulfates formed are depicted after incubation with SULT 1E1, 1A1*2, and 2A1 in order to point out the importance of the three MSulfs generated. The API-ES mass spectrum, representative of all three XN MSulfs, shows a deprotonated quasi molecular ion $[M-H]^-$ at *m/z* 433 and a characteristic fragment ion at *m/z* 353 (deprotonated XN aglycone) due to the loss of the SO_3 moiety (*m/z* 80).

4 Discussion

In order to study the phase II metabolism of XN we focused our attention on glucuronic acid and sulfo-conjugation *in vitro* using human recombinant UGT and SULT enzymes. Glucuronidation and sulfation are important metabolic reactions in which many xenobiotics and endobiotics are converted into more hydrophilic compounds which are readily eliminated from the body [13]. Glucuronidation reactions are catalyzed by UGTs that are located on the luminal side of the endoplasmatic reticulum and the nuclear envelope of hepatocytes and cells in other organs. Currently, 15 human UGT isoforms have been identified, nine of these have been tested in this study [14]. In mammalian organisms, two classes of SULTs can be distinguished. One class metabolizes macromolecular endogenous structures and comprises mainly membrane-bound forms localized in the Golgi apparatus. The other class of enzymes is soluble, usually cytosolic localized and metabolizes xenobiotics and small endogenous compounds. Of this class, ten SULT genes encoding 11 enzymes have been identified in humans

so far [15, 16]. Five of these enzyme forms have been used in our study. We could show that XN is efficiently glucuronidated by UGT 1A8, 1A9, and 1A10; further important UGTs are UGT 1A1, 1A7, and 2B7. With respect to the sulfation reaction, SULT 1A1*2, 1A2, and 1E1 are the most active isoforms. UGT 1A3, 1A4, and 1A6 as well as SULT 1A3 and 2A1 are of minor importance regarding the phase II metabolism of XN. As depicted in Tables. 1 and 2, XN is conjugated with glucuronic acid and sulfate at each of the three possible hydroxyl groups. However, we were not able to identify the position for conjugation with the analytical methods used. It has been shown by NMR analysis that the conjugation with glucuronic acid is favored at C-4′ *in vitro* and *in vivo* in rats [7, 9]. One might conclude that the metabolite at t_R = 16.8 min is 4′-XN-glucuronide. This suggests that the presence of a prenyl group in the A-ring does not exclude UGT-catalyzed glucuronidation of XN as might be expected due to limited access of the substrate to the ligand-binding domain as a result of steric hindrance. Furthermore, Yilmazer *et al.* were able to identify and characterize three additional MGlucs using HPLC/MS-MS as well as NMR. They could show that the metabolite second most formed is 4-XN-glucuronide; in our study this is presumably the metabolite observed at t_R = 12.4 min. The position of conjugation of the other two conjugates formed by incubation with microsomes to a minor extent has not been identified due to insufficient amounts. Nonenzymatic oxidation or isomerization to the flavanone isomer isoXN and further glucuronidation at different hydroxyl groups and/or conjugation of XN at C-2′ were discussed by this group [7]. We were able to identify only one further MGluc. Since we used pure enzymes and baculsomes expressing no UGT as a control, nonenzymatic formation of XN-metabolites seems to be unlikely. Therefore, we assign the third conjugate to be the 2′-glucuronide. For the sulfation no conclusion can be drawn as to which hydroxyl group in the molecule is predominantly sulfated by the different isoenzymes tested.

In general, XN is conjugated by the tested SULTs to a lesser degree than by the UGTs. Up to date no XN-sulfate has been described whether *in vitro* nor in rat feces [8]. This would imply that conjugation with sulfate is of minor importance for the phase II metabolism of XN in rats and maybe also in humans. However, it has been shown for isoflavones, which are also members of the flavonoid family and found most abundantly in soy, that glucuronide and sulfate conjugates are predominantly found in urine and plasma and occur in very low amounts in feces [17, 18]. Consequently, sulfate conjugates of XN might be detectable in urine *in vivo*. Further studies are needed to clarify the importance of the sulfate conjugation reaction not only for XN but also for its metabolites.

Considering the tissue distribution of the tested UGTs and SULTs [14, 16] it is noteworthy that XN is prone to glucuronidation and sulfation in the liver as well as the gastrointestinal tract. For instance, UGT 1A8 and 1A10 can be detected in all tissues of the gastrointestinal tract except for the liver, *e.g.*, in the esophagus, stomach, the ileum, jejunum, intestine, and colon. UGT 1A9 is expressed in the liver, the kidney, and the colon. SULT 1A1 can be detected in the liver and to a minor extent in numerous other tissues including colon, placenta, endometrium, brain, *etc.* SULT 1E1 is expressed in the liver, the jejunum, the endometrium, *etc*. Therefore, the gastrointestinal tract as well as the liver play major roles in the phase II metabolism of XN.

It seems to be important to characterize the biological activities of the major phase II metabolites of XN. Up to date it is not known whether these conjugates have altered activities (*i. e.*, increased, decreased, or equivalent) relative to the parent compound. For isoflavones it has been shown that hydroxylation enhances, whereas conjugation with sulfate reduces the antioxidant capacity *in vitro* [19, 20]. For glucuronides of quercetin and catechins it depends on the position of the glucuronosyl group whether they show higher, lower, or the same antioxidant activities compared to the corresponding aglycones [21, 22]. Thus, it is not possible to predict the antioxidant efficacy and other biological properties of phase II conjugates relative to the aglycone XN.

In conclusion, this is the first study investigating the enzymatic basis of glucuronic acid and sulfate conjugation of XN. The phase II metabolites were characterized using purified human recombinant UGT and SULT isoenzymes. Considering the tissue distribution of the tested UGTs and SULTs, the findings suggest a prominent role for glucuronidation and sulfation of XN in the liver as well as in the gastrointestinal tract. The fact that the biological activities of flavonoids investigated so far are dramatically altered with the chemical structure implies that the conjugation of XN leads to significant changes of its biological activities.

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