

# Chapter 10

## Whole Cell-Dependent Biosynthesis of Drug Metabolites Using Genetically Engineered Budding Yeast

Shinichi Ikushiro, Miyu Nishikawa, and Toshiyuki Sakaki

**Abstract** Xenobiotic phase I and II reactions generally render a compound more water soluble and pharmacologically inactive, thereby eliminating the need for further evaluation. However, if the metabolite forms a toxic compound such as acylglucuronide, additional safety assessment may be needed. Glucuronidation is the most common pathway for detoxification and elimination of hydrophobic xenobiotics in mammals. Thus, development of an efficient in vitro synthesis of glucuronides from parent drugs often becomes critical during studies of drug metabolism undertaken in the development of a new pharmaceutical product. To produce glucuronides as drug metabolites, we have developed coexpression systems for mammalian cytochrome P450 (P450), UDP-glucuronosyltransferase (UGT), and UDP-glucose dehydrogenase in *Saccharomyces cerevisiae* cells, and combination between each of the human P450s and UGTs was achieved. Glucuronide formation in yeast cells was performed in reaction medium containing 8 % glucose, and most of the glucuronides were readily recovered from the cell medium. In addition, we have expressed human sulfotransferase (SULT) with P450s in *S. cerevisiae* cells, and successfully obtained sulfo-conjugates from the cell medium. Coexpression of P450 electron transfer and glucuronidation or sulfo-conjugate production systems allow us to obtain the phase I metabolites and phase II metabolites from the parent compound. In conclusion, our yeast expression systems of xenobiotic-metabolizing enzymes have made it possible to produce xenobiotic phase I and phase II metabolites on the milligram to gram scale.

**Keywords** Budding yeast • Cytochrome P450 • Glucuronide • P450 • Phase I metabolite • Phase II metabolites • Sulfo-conjugate • Sulfotransferase • SULT • UDP-glucuronosyltransferase • UGT • Xenobiotics

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S. Ikushiro (✉) • M. Nishikawa • T. Sakaki  
Toyama Prefectural University, Toyama, Japan  
e-mail: [ikushiro@pu-toyama.ac.jp](mailto:ikushiro@pu-toyama.ac.jp)

## 10.1 Introduction

Many endogenous and exogenous compounds such as bilirubin, steroids, drugs, and environmental pollutants are biotransformed by xenobiotic phase I and phase II enzymes. The phase I enzymes such as cytochrome P450 (P450) introduce a functional group, mainly a hydroxyl group, into the compounds. Subsequently, the phase II conjugating enzymes such as UDP-glucuronosyltransferase (UGT), sulfotransferase, and glutathione-S-transferase can catalyze the conjugation of the hydrophilic molecules glucuronic acid, sulfuric acid, and glutathione, respectively, to the resulting functional group of the compounds (Iyanagi 2007). These reactions catalyzed by phase I and II enzymes would be expected to occur sequentially and cooperatively during the xenobiotic process. These enzymes belong to gene families containing a large number of isozymes that have different substrate specificities, resulting in variety in the xenobiotic pathway. The idea that phase I and II reactions, such as hydroxylation (oxidation) and glucuronidation (a major conjugation reaction), are coupled in the endoplasmic reticulum (ER) membranes has been proposed (von Bahr and Bertilsson 1971). Both the duration and intensity of pharmacological and toxicological drug effects are largely dependent on the functional efficiency of the biotransformation systems and their modulation by numerous endogenous and exogenous physiological and pathological factors *in vivo*. U.S. Food and Drug Administration (FDA) guidance in 2008 showed consideration for safety assessment of the metabolites identified only in human plasma or metabolites present at disproportionately higher levels in humans than in any of the animal test species (Anderson et al. 2010). In addition to drugs, many xenobiotic compounds including environmental pollutants, food additives, and dietary compounds are metabolized by a common biotransformation system. Therefore, detailed knowledge of the specific reactions involved in the metabolism of affected xenobiotic compounds is an essential basis for understanding the nature of xenobiotics, including drugs.

Efforts in development of whole cell-dependent production of metabolites using yeast cells have been undertaken by many research groups, including our laboratories. Sakaki et al. established yeast whole-cell biotransformation systems expressing human P450s (Sakaki 2013). They have examined the metabolism of some drugs, vitamin D, and environmental pollutants, indicating the advantage of usage of budding yeast cells for P450-dependent metabolite production. The major phase II enzymes are UGTs, which contributed to the clearance of about 10 % of the top 200 drugs presented in the USA in 2002 (Crettol et al. 2010). There are 19 UGT isoforms belonging to two major families, *UGT1* and *UGT2* (Mackenzie et al. 2005). These enzymes catalyzed the glucuronidation of xenobiotics as well as endogenous substances with a hydrophobic nature, facilitating their elimination from the body. UGT isoforms from two gene families show overlapping but sometimes very distinctive substrate specificities. UGT is an endoplasmic reticulum-localized membrane protein with the catalytically active part facing the luminal side. Their activity is dependent on the localization in the phospholipid membrane environment (Radomska-Pandya et al. 2005).

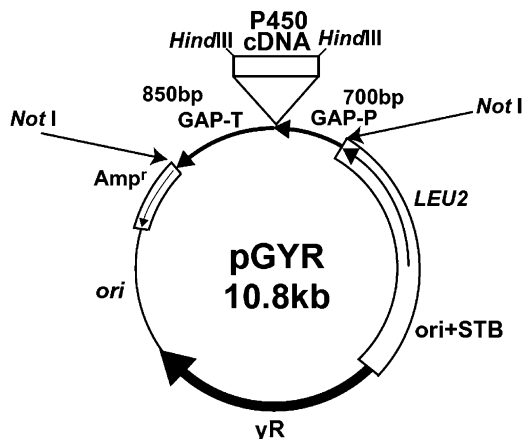
**Table 10.1** Methods of preparation for drug metabolites

Methods	Yield		Disadvantage
	Standard or NMR (~mg)	Toxicity (~g)	
Chemical synthesis	○	○	Often very challenging or sometimes even impossible
Preparation from urine or bile	○	×	Cost- and time-consuming
In vitro enzymatic synthesis	○	Δ	High cost (UDP-glucuronic acid)
Whole-cell biosynthesis using recombinant yeast cells	○	○	

The formation of glucuronides in the body can lead to severe health damage by the formation of pharmacologically active and toxic metabolites such as acyl glucuronides (Spahn-Langguth and Benet 1992). Furthermore, polymorphism of the UGT gene can sometimes cause adverse effects of drug therapy by the accumulation of active or toxic metabolites (Maruo et al. 2005). These findings led to a high demand for purified glucuronides for toxicity studies and as reference standards. Table 10.1 shows the preparation methods of phase II metabolites. The first choice is the chemical synthesis method, but this is often very challenging or sometimes even impossible. Various biological techniques are the second choice: these include metabolite isolation from animal urine or bile, but these techniques are costly and time-consuming processes. Alternative methods are in vitro enzymatic synthesis using liver homogenate, or enzyme preparations from recombinant expression of enzymes in cell culture or microbial systems. However, these in vitro techniques are limited by the expense of cofactors such as UDP-glucuronic acid. To overcome these difficulties of synthesis of metabolites, we have now developed the whole-cell biotransformation system of xenobiotic conjugates (Ikushiro et al. 2010; Sakaki et al. 2011). Furthermore, coexpression of phase I and phase II metabolizing enzymes in yeast cells allows us to synthesize the xenobiotic metabolites of various compounds. This chapter describes our current advances in whole cell-dependent biosynthesis of phase I- and phase II-dependent drug and xenobiotic metabolites using genetically engineered budding yeasts.

## 10.2 Biosynthesis of P450-Dependent Metabolites of Xenobiotics

Among the most frequently used host organisms for the expression of P450s are *Escherichia coli* and the yeasts *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and *Pichia pastoris* (Yun et al. 2006; Sakaki and Inouye 2000; Peters et al. 2009; Andersen and Møller 2002). The production of large quantities of P450 metabolites required for safety testing of developing drugs is often performed using recombinant yeasts. The main advantages of yeast usage are that the yeast cell



**Fig. 10.1** Structure of yeast expression vector pGYR. *yR* yeast NADPH-cytochrome P450 reductase gene (from *Saccharomyces cerevisiae*), *GAP-P* glyceraldehyde-3-phosphate dehydrogenase promoter (from *Zygosaccharomyces rouxii*), *GAP-T* glyceraldehyde-3-phosphate dehydrogenase terminator (from *Z. rouxii*), *LEU2* isopropyl malate dehydrogenase gene (from *S. cerevisiae*), *ori + STB* replication origin in yeast and stabilized region for plasmid DNA (from *S. cerevisiae*), *Amp<sup>r</sup>* ampicillin-resistance gene (from bacterial vector pUC19 DNA), *ori* replication origin in *Escherichia coli* (from bacterial vector pUC19 DNA)

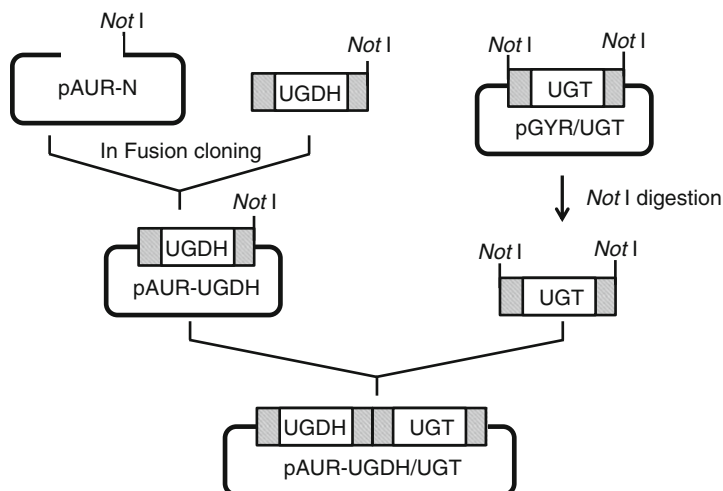
has an endoplasmic reticulum membrane as well as protein expression and processing of maturation that resemble those of higher eukaryotes. Further advantage of the yeast expression system are the availability of the intrinsic P450 redox partner (NADPH-cytochrome P450 reductase, CPR) that functionally interacts with mammalian P450s. *S. cerevisiae* contains only three endogenous P450s that are involved in the metabolism of sterol, indicating that these are unlikely to cause the formation of unwanted side products in a mammalian P450-dependent biosynthesis in yeasts.

For optimization of P450 expression in yeast, the plasmid vector pGYR was developed (Ikezawa et al. 2003) (Fig. 10.1). pGYR contains the promoter and terminator sequences of glyceraldehyde 3-phosphate dehydrogenase from a yeast strain, *Zygosaccharomyces rouxii*, for expression of P450 proteins. pGYR has also a single copy of the yeast CPR gene for enhancement of electron transfer to coexpressed P450. The budding yeast cell strain AH22 as host cells are widely used for heterologous expression of proteins including microsomal P450 isoforms. One of the advantage may be that the AH22 strain is a petite mutant and lacks mitochondrial DNA, resulting in developed endoplasmic reticulum membranes in the yeast cells. Finally, human major P450 isoforms were expressed in *S. cerevisiae* (Imaoka et al. 1996). In some cases, low monooxygenase activity of human P450 3A4 was attributed to the insufficient reduction of the heme iron of P450 by CPR. To enhance the efficiency of electron transfer, a fused enzyme was constructed between human P450 3A4 and yeast CPR gene, with coexpression of human cytochrome *b<sub>5</sub>* as effector (Hayashi et al. 2000).

The alternative application of whole cell-dependent P450 biotransformation allows us to achieve bioremediation for soils and sediments contaminated with industrial chemicals, such as dioxins and PCBs. Previous studies revealed that mammalian P450s are capable of the degradation of mono-, di-, and tri-chlorobenzo-*p*-dioxin (CDD) (Sakaki et al. 2013). Apart from mammalian P450, some P450 isoforms from other organisms are useful for the biotransformation of xenobiotics. Because the white-rot fungus *Phanerochaete chrysosporium* genome contains 148 isoforms of P450 genes, it is possible to assume that some *P. chrysosporium* P450s can metabolize various xenobiotic compounds. Recently, we obtained 120 clones expressing individual P450s of *P. chrysosporium* in budding yeast, and 6 of them could metabolize 2-mono-CDD (Kasai et al. 2010). Watanabe et al. reported that avian P450 isoforms were expressed in yeast and characterized these (Watanabe et al. 2013). According to numerous reports, including our results, *S. cerevisiae* is one of the most established host organisms as a platform for characterization of P450 function and the production of P450 metabolites via whole cell-dependent biosynthesis.

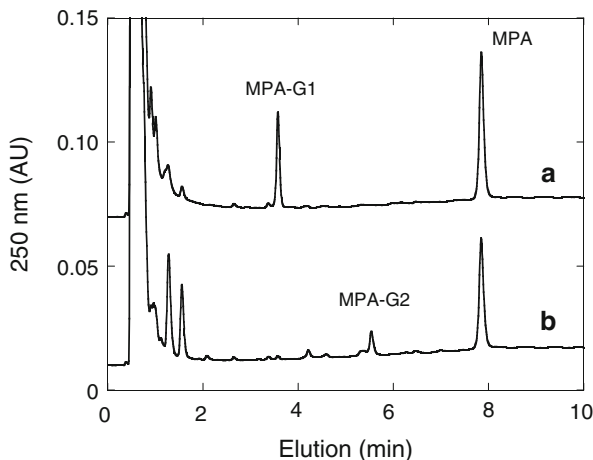
### 10.3 Biosynthesis of UGT-Dependent Metabolites of Xenobiotics

In contrast to the P450-dependent biosynthesis system in yeast, *S. cerevisiae* is incapable of producing the UDP-glucuronic acid as a cofactor of glucuronidation. Oka et al. previously reported that the functional expression of plant UDP-glucose dehydrogenase (UGDH) resulted in the production of UDP-glucuronic acid in *S. cerevisiae* (Oka and Jigami 2006). To effect biosynthesis of glucuronides in a whole-cell system, the coexpression of rat UDP-glucose dehydrogenase with UGT isoforms delivers UDP-glucuronic acid and permits self-sufficient glucuronide production. Coexpression of mammalian UGT isoforms, including human, rat, and mouse, and rat UGDH in *S. cerevisiae* was performed using the genome-integrated yeast expression vector, pAUR. pAUR is commercially available and transformants bearing the gene are selected by aureobasidin A-containing medium (Ikushiro et al. 2004). Figure 10.2 shows the construction of the expression vector. Modified pAUR contains a single copy of the rat UGDH gene with the yeast GAPDH promoter/terminator, and also the *NotI*-digested sequence of the cloning site. Insertion of a *NotI* fragment having the mammalian UGT gene with the yeast GAPDH promoter/terminator results in the coexpression of rat UGDH and various mammalian UGT isoforms. Western blot analysis of selected yeast strains from aureobasidin A-resistant colonies confirmed the protein expression of mammalian UGT and UGDH.  $\beta$ -glucuronidase treatment of more polar metabolites of 7-hydroxycoumarin revealed the production of glucuronides in resting yeast cells. Production of glucuronide was dependent on the concentration of glucose in the reaction medium. Most of the glucuronide of 7-hydroxycoumarin was excreted from



**Fig. 10.2** Strategy of coexpression of UGT with UGDH using genome-integrated expression vector in yeast. *UGT* UDP-glucuronosyltransferase, *UGDH* rat UDP-glucose dehydrogenase

**Fig. 10.3** Biosynthesis of regiospecific glucuronides of mycophenolic acid using resting yeast cells. (A) HPLC profile of MPA metabolite using resting yeast cell having human *UGT1A9* and *UGDH*. (B) HPLC profile of MPA metabolite using resting yeast cell having rat *UGT2B1* and *UGDH*. *MPA* mycophenolic acid, *MPA-G1* phenolic glucuronide of MPA, *MPA-G2* acyl glucuronide of MPA



the yeast cell, indicating the involvement of endogenous transporters in yeast cell membranes. Recently, Bureik's group (at PomBio Tech) successfully established a whole-cell biotransformation system using *Schizosaccharomyces pombe* (Dragan et al. 2010; Zöllner et al. 2010; Buchheit et al. 2011). Comparison of glucuronide production between *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* showed some differences in efficiency in an isoform-dependent manner.

One of the advantages of enzymatic biosynthesis is regiospecific glucuronidation of substrates with multiple conjugation sites. Mycophenolic acid used in immunosuppressant drugs has phenolic and carboxylic hydroxyl groups. Figure 10.3 shows the glucuronide production of mycophenolic acid using resting

**Table 10.2** Biosynthesis of acyl glucuronides using recombinant yeast cells

Substrates	Production (mg/l)
Mefenamic acid	60
Flufenamic acid	132
Diclofenac	260
Zomepirac	47

yeast cells with the UGT-UGDH gene. Human UGT1A9 in yeast cells mainly catalyzed the phenolic glucuronide formation. In contrast, rat UGT2B1 can specifically catalyze acyl glucuronide formation. Selecting the suitable mammalian UGT isoforms in yeast, we can obtain the desired glucuronides of various compounds with multiple conjugation sites.

$\beta$ -1-*O*-Acyl glucuronides have been shown to be reactive metabolites that hydrolyze, acyl migrate, and bind covalently to proteins causing potential toxicity (Spahn-Langguth and Benet 1992). This toxicity may be caused by formation of immunoreactive glucuronide protein adducts (Worrall and Dickinson 1995), but toxicity mechanisms involving modification of active sites of enzymes (Terrier et al. 1999) and interaction with structural proteins (Bailey et al. 1998) have also been suggested. Thus, So acyl glucuronide is one of the most important metabolites for safety testing of drug development. Table 10.2 shows the biosynthesis of acyl glucuronides of various drugs with carboxylic groups using resting yeast cells with the rat UGT2B1-UGDH gene. Based on our results, the genetically modified budding yeast, *Saccharomyces cerevisiae*, with the UGDH gene is one of the useful host organisms for characterization of UGT function and the production of UGT metabolites via whole cell-dependent biosynthesis.

## 10.4 Biosynthesis of Sulfotransferase (SULT)-Dependent Metabolites of Xenobiotics

In addition to glucuronidation, sulfonation is an important reaction in the phase II process for numerous xenobiotics, drugs, and endogenous compounds (Gamage et al. 2005). For example, biotransformation of phenolic drugs and xenobiotics yields products that are water soluble, less biologically active, and in most cases stable enough to be readily excreted from the body. These reactions are catalyzed by sulfotransferases (SULTs), which also are important phase II drug-metabolizing enzymes and have a supergene family. Of these human SULTs, 1A1, 1A3, 1B1, 1E1, and 2A1 are major cytosolic isoforms in liver, intestine, or lung (Riches et al. 2009). For biosynthesis of sulfo-conjugates in yeast, we have attempted to express these SULTs in *S. cerevisiae*, which has intrinsic enzymes to convert ATP to 3'-phosphoadenosine 5'-phosphosulfate (PAPS) as sulfonate donor. Expression of human SULT isoforms in *S. cerevisiae* was performed using yeast expression vector pGYR. Western blot analysis confirmed the protein expression of human

**Table 10.3** Biosynthesis of sulfo-conjugates using recombinant yeast cells

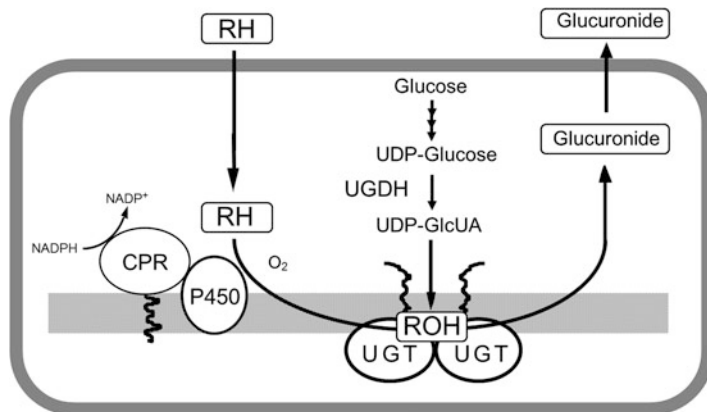
Isoforms	Substrates	Production (mg/l)
SULT1A1	7-Hydroxycoumarine	165
SULT1A3	1-Hydroxypyrene	210
SULT1E1	Minoxidil	13
SULT2A1	Testosterone	52

SULTs. Sulfatase treatment of the more polar metabolite of 7-hydroxycoumarine revealed the production of sulfo-conjugates in resting yeast cells. Table 10.3 shows the conversion rate of sulfo-conjugated metabolites of various compounds. SULT1A1 catalyzed efficiently the sulfo-conjugation of small phenolic compounds such as 7-hydroxycoumarine. Minoxidil is an antihypertensive agent and hair growth promoter that is metabolized by sulfation to the active compound, minoxidil sulfate. In the SULT isoforms, SULT1E1 significantly catalyzed the conversion of the minoxidil sulfate. Selecting the suitable human SULT isoforms in yeast, we can obtain desirable sulfo-conjugates of various compounds including drugs, environmental pollutants, and dietary compounds.

## 10.5 Biosynthesis of Phase I–Phase II-Dependent Metabolites

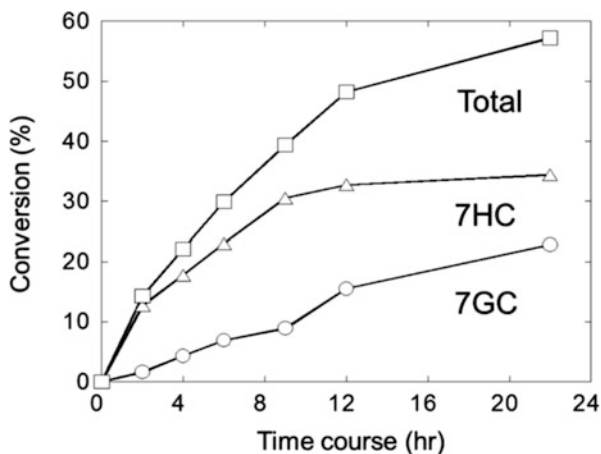
The heterologous expression of xenobiotic-metabolizing enzymes using various host systems has been reported to characterize substrate specificity and to determine the contribution of these enzymes to drug metabolism in vivo (Guengerich et al. 1997). Most xenobiotics are thought to be biotransformed via xenobiotic phase I and II enzyme-dependent multi-step pathways, resulting in the complexity of overall drug metabolism in the body (Tirona and Pang 1996). In contrast to expression systems for individual enzymes, few studies on the coexpression of phase I and II enzymes have been performed. In the current study, a coexpression system was developed that reconstituted xenobiotic phase I and II biotransformation. P450 and the conjugating enzyme, UGT, were introduced into the yeast strain AH22 through plasmid-based, pGYR, and genome integration-based, pAUR, genetic engineering, respectively (Fig. 10.4). To achieve the coupled hydroxylation–glucuronidation reactions in the coexpression system, 7-ethoxycoumarine was used as model substrate. Oxidative deethylation of 7-ethoxycoumarine by human P450 1A1 generates 7-hydroxycoumarine, which is then susceptible to conjugation of the 7-hydroxy group by UGT1A6. Figure 10.5 shows the significant formation of 7-glucuronated coumarine in the whole-cell system. Formation of 7-hydroxycoumarine, which was catalyzed by P450, was linear versus time and then reached a plateau phase. In contrast, 7-glucuronated coumarine formation was detected after 7-hydroxycoumarine accumulation following a lag phase and then linear with time. This formation pattern of the metabolites represents a typical sequential conversion by the two enzymes. The time-course of 7-ethoxycoumarine





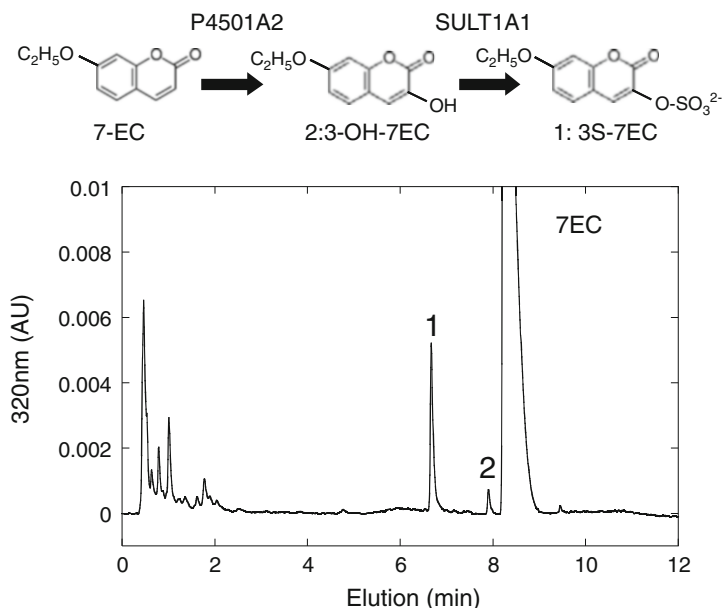
**Fig. 10.4** Yeast coexpression system of P450 with conjugation enzymes for biosynthesis of xenobiotic metabolites phase I and phase II coupled reaction. *CPR* NADPH-P450 reductase, *UGDH* UDP-glucose dehydrogenase, *UDP-GlcUA* UDP-glucuronic acid, *RH* hydrophobic substrates, *ROH* hydroxylated intermediates

**Fig. 10.5** Biosynthesis of 7-hydroxycoumarine and the glucuronide using coexpression system of human P4501A1 and UGT1A6 in yeast. This figure shows the time-course of 7-ethoxycoumarine metabolism in yeast coexpression system of human P450 1A1 and UGT1A6. *7HC* 7-hydroxycoumarine, *7GC* 7-glucuronidated coumarine



metabolism was observed in the yeast coexpression system of P450 1A1 and UGT1A6.

Another phase I- and -II-linked reaction is hydroxylation coupled with sulfo-conjugation. To mimic the reaction in yeast cells, human *SULT1A1* was coexpressed with human P4501A2 using a pAUR-pGYR expression system. Figure 10.6 shows the coproduction of the hydroxylated intermediate, 3-hydroxy-7-ethoxycoumarine, and its sulfo-conjugate. This coexpression of the P450 electron-transfer and glucuronidation or sulfo-conjugate production system allows us to obtain phase I metabolites such as 7-hydroxycoumarine, and phase II metabolites, the conjugated hydroxycoumarine from the parent compound. It is an advantage that phase II



**Fig. 10.6** Biosynthesis of 3-hydroxy-7-ethoxycoumarin and the sulfo-conjugate using coexpression system of human P4501A2 and SULT1A1 in yeast. This figure showed HPLC profile of 7-ethoxycoumarin metabolism in yeast coexpression system of human P450 1A2 and SULT1A1. *7EC* 7-ethoxycoumarin, *3-OH-7EC* 3-hydroxy-7-ethoxycoumarin, *3S-7EC* 3-sulfated-7-ethoxycoumarin

metabolites can be obtained directly when the phase I metabolite is unknown or is barely synthesized by chemical reaction. In addition to metabolite production for safety testing, analysis of a metabolic profile using a combination of P450 and UGT isoforms can lead to a simple and high-throughput screening protocol.

## 10.6 Conclusion

This chapter described one of the practical applications of xenobiotic-metabolizing enzymes including P450, UGT, and SULT for biosynthesis of xenobiotic metabolites using the budding yeast cell, *Saccharomyces cerevisiae*. A coexpression system of P450, CPR, UGT, and UGDH was successfully constructed in *S. cerevisiae* cells to produce glucuronides of various xenobiotics. A coexpression system of P450 and SULT was successfully constructed to produce sulfo-conjugated metabolites of various xenobiotics. The recombinant *S. cerevisiae* cells expressing human drug-metabolizing enzymes are useful to predict drug metabolism in the human body. The recombinant *S. cerevisiae* cells appear to be useful to follow the FDA guidance in 2008 on the safety testing of drug metabolites.

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