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STEREOSELECTIVE S-OXIDATION OF FLOSEQUINAN SULFIDE BY RAT HEPATIC FLAVIN-CONTAINING MONOOXYGENASE 1A1 EXPRESSED IN YEAST

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Abstract—Rat hepatic flavin-containing monooxygenase (FMO) 1A1 expressed in yeast catalysed the S-oxidation of flosequinan sulfide (7-fluoro-1-methyl-3-methylthio-4-quinolone) to *R*(+)-flosequinan (sulfoxide form, *R*(+)-7-fluoro-1-methyl-3-methylsulphinyl-4-quinolone) but not to *S*(−)-flosequinan, and did not catalyse the oxidation of *R*(+)- and *S*(−)-flosequinan to flosequinan sulfone. The K_m and V_{max} for the stereoselective S-oxidation were 33 μM and 6.2 nmol per min per mg of microsomal protein, respectively. The S-oxidation was inhibited by 1-(1-naphthyl)-2-thiourea and thiobenzamide. *n*-Octylamine activated the S-oxidation with little change in stereoselectivity. The ability of the recombinant yeast to produce *R*(+)-flosequinan from flosequinan sulfide could be maintained for at least 2 days and exemplifies the value of a recombinant yeast expressing FMO as a stereoselective bioreactor.

Key words: flavin-containing monooxygenase; expression; yeast; flosequinan; stereoselective S-oxidation; bioreactor

The microsomal FMO§ (EC 1.14.13.8) is present in many tissues of mammals and catalyses the NADPH-dependent oxygenation of nitrogen-, sulfur- and phosphorous-containing drugs [1]. FMOs have been purified and characterized from liver microsomes of pigs [2], rats [3], mice [2], rabbits [4] and guinea pigs [5], from lung microsomes of mice [6] and rabbits [7], and from kidney microsomes of mice [8] and rats [9]. Recently, cDNAs encoding FMOs were isolated and sequenced from pigs, rats, rabbits, guinea pigs and humans [10, 11]. FMOs were classified as five gene subfamilies [10, 11]. The rabbit FMOs 1A1 and 1B1 were transiently expressed in COS cells [12]. While COS cells have the advantage to study post-translational modifications of mammalian proteins, their use may be limited because of a relatively low level of expression. The low cost, ease of manipulation and potential for large-scale production of recombinant proteins are advantages of expression in yeast and *Escherichia coli*. The ability to generate large amounts of a specific FMO isozyme allows the properties of FMO isozymes to be determined. Thus, pig FMO 1A1 [13], rabbit FMO 1B1 [14] and rabbit FMO 1C1 [15] were stably expressed in *Saccharomyces cerevisiae* and *E. coli*. In our laboratory, we isolated the cDNA clone encoding rat FMO 1A1 and expressed it in yeast [16].

Flosequinan [(±)-7-fluoro-1-methyl-3-methylsulfinyl-4-quinolone] is a peripheral vasodilator with therapeutic effects on both arterial and venous vascular beds [17, 18]. This compound has a chiral sulfur yielding two stereoisomers. The estimated metabolic pathway of flosequinan in rats investigated by Kashiyama *et al.*‖ is shown in Fig. 1. A major metabolite, flosequinan sulfone (7-fluoro-1-methyl-3-methylsulphonyl-4-quinolone), is produced as an end product by S-oxidation from *R*(+)- and *S*(−)-flosequinan. A minor metabolite, flosequinan sulfide (7-fluoro-1-methyl-3-methylthio-4-quinolone), is formed by S-reduction from *R*(+)- and *S*(−)-flosequinan.

In this study, rat hepatic FMO 1A1 expressed in yeast was used to investigate the S-oxidation of the sulfur-containing drug. We found that FMO expressed in yeast catalysed the S-oxidation stereoselectively and demonstrates the usefulness of the recombinant yeast expressing FMO as a stereoselective bioreactor.

MATERIALS AND METHODS

Materials. The recombinant yeast transformed with an expression plasmid containing a cDNA encoding rat hepatic FMO 1A1 was used [16]. NADPH, $NADP^+$, NADH, NAD^+, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were obtained from Oriental Yeast (Tokyo, Japan). Thiobenzamide was from Aldrich Chemicals (Milwaukee, WI, U.S.A.), *n*-octylamine from Wako

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§ Abbreviations: FMO, flavin-containing monooxygenase; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride.

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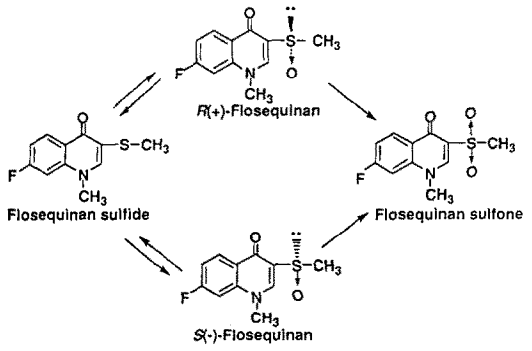

Fig. 1. Proposed metabolic pathways of $R(+)$- and $S(-)$-flosequinan in rats.

Pure Chemicals (Osaka, Japan), 1-(1-naphthyl)-2-thiourea from Tokyo Kasei Kougyo (Tokyo, Japan) and SKF-525A hydrochloride from Funakoshi (Tokyo, Japan). Flosequinan and flosequinan sulfone were supplied by the Boots Company Plc (Nottingham, U.K.). The enantiomers of flosequinan and flosequinan sulfide were prepared in the Research Laboratory of Otsuka Pharmaceutical (Tokushima, Japan).

Preparation of yeast microsomes. Microsomes from a recombinant yeast carrying cDNA coding for rat liver FMO 1A1 were prepared by the method of Itoh *et al.* [16]. Recombinant yeast was grown in Burkholder medium [19] containing 10 mM phosphate without L-leucine at 30°. When cell density monitored by the absorbance at 600 nm reached 0.3–0.4, the yeast cells were harvested. The cells were washed twice with distilled water and collected. For expression of the FMO, the cells were resuspended in the Burkholder medium without phosphate and L-leucine and cultured at 30° for 12 hr aerobically. Control yeast which was not transfected with the expression plasmid was also treated as described above, except that L-leucine was added to the culture. The yeast cells were harvested by centrifugation and washed with 10 mM Tris–HCl buffer (pH 7.4) containing 100 mM NaCl and 1 mM EDTA, and then with solution A [10 mM Tris–HCl buffer (pH 7.4) containing 0.65 M sorbitol, 0.1 mM DTT, 0.1 mM EDTA and 0.1 mg/mL PMSF]. The cells were collected and resuspended in solution B [10 mM Tris–HCl buffer (pH 7.4) containing 2 M sorbitol, 0.1 mM DTT, 0.1 mM EDTA and 0.1 mg/mL PMSF], and incubated with 0.2 mg/mL zymolyase-100T (Seikagaku Kogyo, Tokyo, Japan) at 30° for 2 hr with gentle shaking. The yeast cells were washed twice with solution B, resuspended in solution B and disrupted by sonication on ice. After centrifugation at 10,000 *g* for 20 min, the resultant supernatant was centrifuged at 125,000 *g* for 90 min to obtain a microsomal fraction. Microsomes thus prepared were stored at −80° until use.

Oxidation of flosequinan sulfide by a recombinant yeast carrying cDNA coding for FMO. A recombinant yeast carrying cDNA encoding rat liver FMO 1A1 was cultured for the expression of the FMO in Burkholder medium without phosphoric acid and L-leucine at 30° for 12 hr with aeration. After flosequinan sulfide was added to the culture medium at a concentration of 10 or 1000 μM, the medium was cultured aerobically at 30°. A 0.5 mL portion of the medium was taken at 1, 2, 4, 6, 8, 12, 24 and 48 hr, and centrifuged immediately at 3500 *g* for 10 min. The supernatant (200 μL) was extracted with chloroform (3 mL). Extracts were analysed by HPLC.

Analytical procedures. The S-oxidized metabolites of flosequinan sulfide and $R(+)$- and $S(-)$-flosequinan formed were determined by HPLC. A typical reaction mixture consisted of 50 mM Tris–HCl buffer (pH 8.4), 1.0 mM EDTA, and NADPH-regenerating system (5.0 mM glucose-6-phosphate, 0.5 mM $NADP^+$, 1 U/mL glucose-6-phosphate dehydrogenase and 5 mM $MgCl_2$) and microsomes (1 mg/mL) from the recombinant yeast. The reaction mixture was pre-incubated at 37° for 3 min. Reaction was initiated by the addition of 1 mM flosequinan sulfide or $R(+)$- and $S(-)$-flosequinan. After incubation for 10 min, chloroform (3 mL) was added to stop the reaction and to extract the products. A 2 mL portion of the organic layer was transferred to another tube and evaporated under a gentle stream of nitrogen at 40°. The residue was dissolved in methanol (200 μL), and was subjected to HPLC equipped with a HLC-803D system (Tosoh, Tokyo, Japan), a UV-8000 UV-Visible absorbance detector (Tosoh), a C-R6A integrator (Shimadzu, Kyoto, Japan) and an analytical column Chiralcel OD (4.6 mm i.d. × 250 mm; Daicel Chemical Industries, Tokyo, Japan). The elutions of $R(+)$- and $S(-)$-flosequinan and flosequinan sulfone were monitored at 320 nm. The mobile phase consisting of ethanol-methanol (50:50 v/v) was delivered at a flow rate of 0.7 mL/min.

Thiobenzamide S-oxidation was measured as described by Cashman and Hanzlik [20]. The reactions were measured at 37° using a spectrophotometer (Model UV-160, Shimadzu). A reaction mixture consisted of 50 mM Tris–HCl buffer (pH 8.4), 1.0 mM EDTA, an NADPH-regenerating system as described above and microsomes (0.5 mg/mL). The reaction was initiated by addition of thiobenzamide dissolved in acetonitrile at a final concentration of 1 mM. Differences in optical density at 370 nm between sample and reference cuvettes were monitored after addition of thiobenzamide to a sample cuvette and acetonitrile (10 μL) to a reference cuvette. Absorbance changes were recorded for 5 min, and the rate of reaction was determined using a molar extinction coefficient of 2.93/mM·cm at 370 nm. The protein contents were determined by the method of Lowry *et al.* [21] using bovine serum albumin as a standard.

RESULTS

From the analyses of the metabolites of flosequinan, four routes of S-oxidation, as shown in Fig. 1,* can be considered. The possibility that FMO was

* Kashiyama E, Todaka T, Odomi M, Tanokura Y, Johnson DR, Yokoi T, Kamataki T and Shimizu T, Stereoselective pharmacokinetics and interconversions of flosequinan enantiomers containing chiral sulphoxide in rat. *Xenobiotica*, in press.

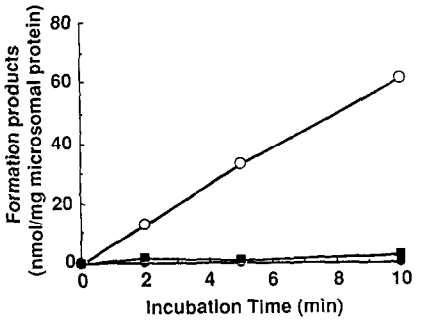

Fig. 2. Stereoselective S-oxidation of flosequinan sulfide and $R(+)$- and $S(-)$-flosequinan by microsomes from yeast transformed with rat FMO cDNA. The formations of $R(+)$-flosequinan from flosequinan sulfide (○), $S(-)$-flosequinan from flosequinan sulfide (●), flosequinan sulfone from $R(+)$-flosequinan (□), and flosequinan sulfone from $S(-)$-flosequinan (■) were measured after incubation of the substrate with microsomes from recombinant yeast.

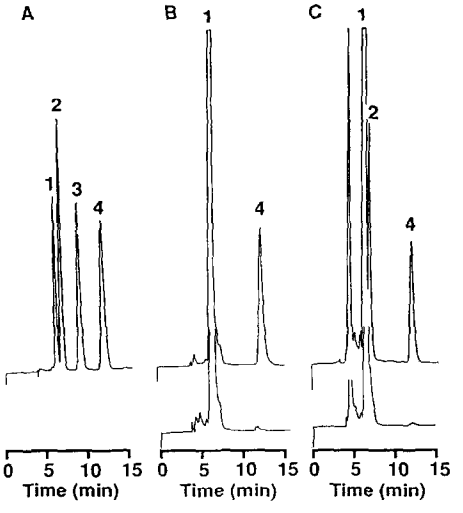

Fig. 3. High-performance liquid chromatograms of metabolites formed from flosequinan sulfide by microsomes from rat liver and yeast transformed with rat FMO cDNA. The elution pattern of authentic standards is shown in (A). Metabolites formed by rat FMO expressed in yeast microsomes and by rat liver microsomes were analysed by HPLC and the results are shown in (B) and (C), respectively. Chromatograms from a reaction mixture incubated in the presence (upper) and absence (lower) of an NADPH-regenerating system are shown. Peak identifications are as follows: 1, flosequinan sulfide; 2, $S(-)$-flosequinan; 3, flosequinan sulfone; 4, $R(+)$-flosequinan.

involved in these reactions was examined using rat FMO 1A1 expressed in yeast microsomes. The FMO expressed in yeast catalysed the S-oxidation stereoselectively; the formation of $R(+)$- but not $S(-)$-sulfoxide from the sulfide was seen (Figs 2 and 3). Since liver microsomes from rats catalysed the S-oxidation to form the $S(-)$-sulfoxide (Fig. 3), the formation of the $S(-)$-sulfoxide in liver microsomes

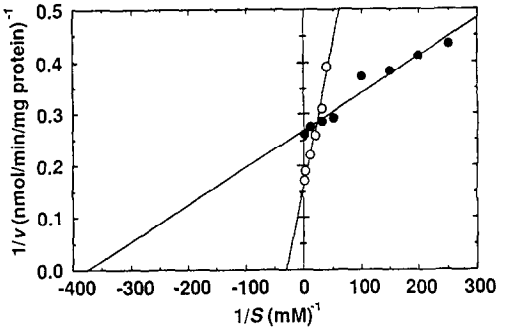

Fig. 4. Double reciprocal plots of the S-oxidation of flosequinan sulfide and thiobenzamide by rat FMO expressed in yeast microsomes. Results are shown as the formation of $R(+)$-flosequinan from flosequinan sulfide (○) and S-oxidation of thiobenzamide (●).

was assumed to be catalysed by an enzyme(s) other than FMO 1A1. In addition, the FMO expressed in yeast did not catalyse the formation of the sulfone from either $R(+)$- and $S(-)$-sulfoxides (Fig. 3).

The double reciprocal plots of the rate of S-oxidation versus the concentrations of flosequinan sulfide and thiobenzamide are shown in Fig. 4. Thiobenzamide was employed as a typical substrate for FMOs [20]. The K_m and V_{max} values for the thiobenzamide S-oxidation, calculated from Fig. 4 were 2.7 μM and 3.7 nmol/min/mg protein, respectively. The K_m and V_{max} values for the $R(+)$-sulfoxide formed from flosequinan sulfide were 33 μM and 6.2 nmol/min/mg protein, respectively.

These results suggest FMO is an enzyme that oxidized flosequinan sulfide stereoselectively and that the FMO expressed in yeast possessed the same properties as that present in liver microsomes. FMO in liver microsomes is known to be unstable towards heat. The unstable nature of this enzyme is probably one of the causes which made it difficult to purify FMO from liver microsomes. Kitchell *et al.* [22] reported that this enzyme could be stabilized by treatment with NADPH. Thus, the stabilization by NADPH of the FMO expressed in yeast was examined using flosequinan sulfide as a substrate. Figure 5 shows the effects of NADPH on the thermal stability of rat FMO expressed in yeast. In the absence of NADPH, the S-oxidation activity to form $R(+)$-sulfoxide gradually decreased with time even at 30°, and the activity was rapidly lost at a temperature higher than 35°. Almost all activity was lost by treatment at 40° for 2 min or at 50° for 1 min (Fig. 5A). In contrast, S-oxidation activity was stable at 40° for 10 min when NADPH was present (Fig. 5B). The stereoselective properties of FMO were not changed by heat treatment and FMO did not catalyse the oxidation of flosequinan sulfide to form $S(-)$-flosequinan even after heat treatment.

To confirm that the FMO expressed in yeast exhibits the same properties as those of FMO from liver microsomes, we examined the effects of cofactors, inhibitors and enhancers to check if the stereoselectivity of the FMO was changed by these modifiers. The FMO expressed in yeast required

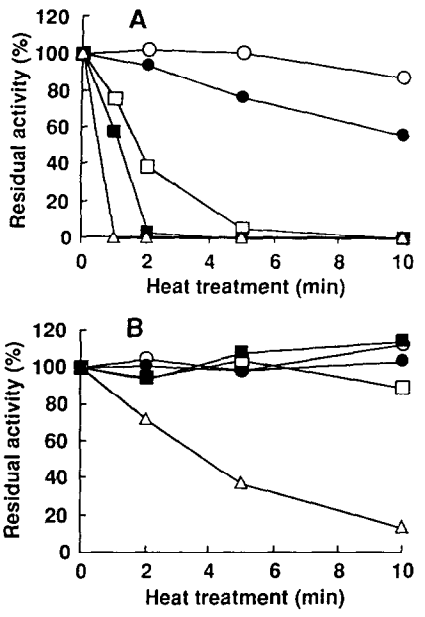

Fig. 5. Effect of NADPH on the thermal stability of rat FMO expressed in yeast microsomes as measured by the S-oxidation activity. The expressed FMO was tested in the absence (A) or presence (B) of 1 mM NADPH at 20° (○), 30° (●), 35° (□), 40° (■) and 50° (△) for the desired periods as indicated in the figure.

NADPH as a cofactor for the S-oxidation of flosequinan sulfide (Table 1). NADPH could not be replaced by NADH in agreement with the results obtained using liver microsomes and purified FMO [9]. FMO in liver microsomes is known to be enhanced and inhibited by *n*-octylamine [6] and 1-(1-naphthyl)-2-thiourea, respectively, [23], and FMO is not affected by carbon monoxide and SKF-525A, known inhibitors of cytochrome P450-dependent monooxygenases [23]. As shown in Table 1, the S-oxidation activities of the FMO expressed in yeast were inhibited by 1-(1-naphthyl)-2-thiourea and activated by *n*-octylamine. These results agree with the catalytic properties of purified FMO. On the other hand, the stereoselective S-oxidation by expressed FMO was enhanced about 2-fold by 5 mM *n*-octylamine. Only *R*(+)-sulfoxide formation from flosequinan sulfide was seen, and no *S*(−)-sulfoxide formation was observed by the addition of *n*-octylamine up to 5 mM concentration (Table 2). The formation of a small amount of the *S*(−)-sulfoxide was noted when *n*-octylamine was added at a concentration of 25 mM. These results indicate that the stereoselectivity was little altered when an excess of *n*-octylamine was added so as to increase the FMO activity.

We found that the FMO expressed in yeast catalysed the S-oxidation of flosequinan stereospecifically. Therefore, we further investigated the possibility that the recombinant yeast could be used as a stereoselective bioreactor for chiral sulfur compounds using flosequinan sulfide. Flosequinan sulfide was added to a culture medium at a final concentration of 10 or 1000 μM, and incubated for different periods. The products in the culture medium were measured periodically (Fig. 6). When the sulfide was added at a concentration of 10 μM, only *R*(+)-sulfoxide was produced rapidly. After 12 hr incubation, flosequinan sulfide was completely converted to the *R*(+)-flosequinan without formation of *S*(−)-flosequinan and flosequinan sulfone. The *R*(+)-flosequinan produced was stable even after 24 hr incubation. When the sulfide was added at a concentration of 1000 μM, the rate of *R*(+)-flosequinan formation was linear for 48 hr and produced at a rate of 17 nmol/hr/mL of culture

Table 1. Effects of additive on the S-oxidations of flosequinan sulfide to give *R*(+)-flosequinan and the S-oxidation of thiobenzamide by rat FMO expressed in yeast microsomes

Additions	Concn (mM)	Substrate: Flosequinan sulfide (nmol/min/mg protein)	Substrate: Thiobenzamide (nmol/min/mg protein)
Standard condition*		6.1 ± 0.2 (100)§	3.8 ± 0.5 (100)
NADPH†	0.5	5.2 ± 0.6 (87)	—‖
NADH†	0.5	0.87 ± 0.39 (14)	—
$NADP^+$†	0.5	0.25 ± 0.43 (4)	—
NAD^+†	0.5	0.33 ± 0.12 (6)	—
Carbon monoxide‡		6.0 ± 0.2 (100)	3.9 ± 0.6 (103)
SKF-525A	0.5	5.7 ± 0.2 (93)	—
1-(1-Naphthyl)-2-thiourea	0.5	0.18 ± 0.08 (3)	0.45 ± 0.49 (12)
Thiobenzamide	1.0	0.08 ± 0.08 (1)	—
n-Octylamine	5.0	12.5 ± 0.7 (206)	9.2 ± 0.1 (242)

Values are means ± SD from three independent determinations.

* Consisted of 50 mM Tris–HCl buffer (pH 8.4), 1.0 mM EDTA, an NADPH-regenerating system and microsomes from yeast.

† Added to the standard mixture in place of an NADPH-regenerating system.

‡ Bubbled for 2 min before reaction.

§ Numbers in parentheses represent the percentage of the value obtained by the standard condition.

‖ Not determined.

Table 2. Enhancement of the S-oxidation of flosequinan sulfide by *n*-octylamine in microsomes from the recombinant yeast

n-Octylamine concn (mM)	Products (nmol/min/mg protein)		Selectivity (%)
	R(+)-Flosequinan	*S*(−)-Flosequinan	
0	7.42 ± 0.96	<0.01	>99.9
1	14.2 ± 0.9	<0.01	>99.9
5	17.3 ± 0.4	<0.01	>99.9
25	13.6 ± 1.5	0.17 ± 0.36	98.7

Values are means ± SD from three independent determinations.

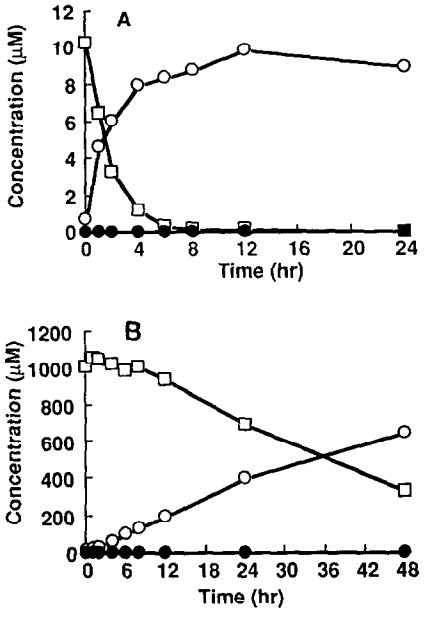

Fig. 6. Stereoselective formation of *R*(+)-flosequinan from flosequinan sulfide in culture medium containing recombinant yeast. Ten micromolar (A) or 1000 μM (B) flosequinan sulfide was added to the culture medium after 12 hr preincubation without phosphoric acid. A 0.5 mL portion of the medium was taken periodically, and the concentrations of *R*(+)-flosequinan (○), *S*(−)-flosequinan (●) and flosequinan sulfide (□) in the culture medium measured.

medium. When *n*-octylamine, an enhancer of FMO activity, was added with flosequinan sulfide to the culture medium, no enhancement was observed (data not shown). Only *S*(−)-flosequinan was detected in the culture medium when *S*(−)-flosequinan was added to the culture (data not shown), indicating that *S*(−)-flosequinan was not metabolized by this recombinant yeast.

DISCUSSION

Molecular forms of FMO, which showed different biochemical and immunological properties, have been purified from tissues of animals [2–9] and classified on the basis of the primary structures [10, 11]. The presence of five distinct FMO isoforms was confirmed in rabbits by the cloning of cDNAs [10]. The primary structures of cDNAs encoding FMOs from other mammalian species including humans are more than 80% identical to the rabbit forms [10, 11]. The rabbit liver FMO 1A1 and rabbit lung FMO 1B1 were expressed in COS-1 cells, yeast and *E. coli*. These FMOs have the same properties as FMOs purified from rabbit livers and lungs [12, 14]. Although rat liver FMO 1A1 expressed in yeast was catalytically active in oxidizing typical substrates of FMO, the properties of this enzyme were not examined in detail [16]. In this study, S-oxidation reactions in the metabolic pathways of the sulfur-containing drug, flosequinan, by the rat FMO 1A1 expressed in yeast were investigated. In addition, the use of this recombinant yeast as a stereospecific bioreactor was also investigated. Microsomes from the recombinant yeast carrying cDNA encoding rat FMO 1A1 catalysed the S-oxidation of flosequinan sulfide stereoselectively. This S-oxidation activity was not detectable in microsomes from a non-transformed yeast, indicating that the S-oxidation was catalysed by the expressed FMO and did not stem from endogenous enzymes present in yeast.

FMO showed stereoselectivity in the oxidation of flosequinan sulfide. Similar stereospecific oxidation by purified FMO has been reported using phorate as a substrate [24, 25]. Since the stereoselective S-oxidation was also demonstrated using FMOs purified from livers and lungs [26–28], it is likely that the stereoselectivity is a common property of FMO isoforms. It was reported that *n*-octylamine changed the stereoselectivity of FMO purified from pig livers [29]. However, *n*-octylamine acted as an activator causing little change in stereoselectivity in the enzyme from yeast (Table 2). Although the reason for this difference is unclear, it might be caused by the difference in the chiral substrates employed at the source of FMOs, i.e. use of FMO from pigs as opposed to enzyme from rats. In contrast to the results of this study, it was reported that the stereoselectivity of the S-oxidation of two different substrates by the pig liver FMO expressed in *E. coli* showed differences from that of the native FMO purified from pig livers [13]. The stereoselectivity of FMO may be useful for analysis of the 3-dimensional structure of the active site of FMO.

While *n*-octylamine enhanced by 2-fold the S-oxidation activity when added to the reaction mixture containing yeast microsomes, *n*-octylamine did not enhance the activity when added to the culture medium. It has been reported that *n*-octylamine modulates FMO activity by binding to the active site [29] but the *n*-octylamine may not penetrate the yeast membranes.

Many clinical drugs are used as a racemate despite the fact that there are many examples of enantiomers showing different pharmacological or side effects [30–32]. In addition to pharmacological studies, the pharmacodynamics and safety of each enantiomer have been investigated for the products obtained by different chiral separations and asymmetric synthesis. Although there are still some difficulties found in asymmetric synthesis of an enantiomer, the bioreactor system using enzymes and microorganisms has a great advantage in some cases [33, 34]. Thus, we investigated the application of yeast expressing FMO to produce a single isomer of the sulfoxide. When flosequinan sulfide was added to the culture medium of the recombinant yeast, only *R*(+)-flosequinan was produced. Moreover, the stereoselective reaction was maintained for long periods, thus, making this recombinant yeast a useful stereoselective bioreactor.

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