Experimental Study on Method of Measuring Cytochrome P450 in Fish Liver subcellular fractions

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Abstract. To study the method of measuring cytochrome P450 (CYP450) in fish liver subcelluar fractions (S9 and microsomes). Fish liver microsome was obtained by differential velocity centrifugation, the concentration of protein of liver microsome was measured by Bradford, and the activity of mice CYP450 was detected by spectrophotometer. The result showed that this method is easy and reliable, and has good reproducibility.

Introduction

Study *in vitro* can effectively reduce the dosage of the living creature, well expel the interference of complex factors *in vivo*. The metabolism selectivity of enzyme to substrate could be direct observed. Thus, the study method *in vitro* has been widely used in a variety of study on drugs and pollutants metabolism. At present, two kinds of liver subcellular fractions: one is after the removal of mitochondrial homogenate supernatant(S9), another is the liver microsome were used at most *in vitro* study. Cytochrome P450 (CYP 450) isozymes were the major phase Ⅰ catalyze isozymes in liver. Ethoxycoumarin O-deethylase (ECOD), Ethoxyresorufin O-deethylase (EROD), pentoxyresorufin O-deethylase (PROD) and aniline hydroxylase (AH) were several common used to characterize of CYP450 enzyme activity. Glutathione S-transferase (GST) is one of important phase Ⅱ metabolic enzymes. The freshwater fish crucian carp (*Carassius auratus*) was chosen as the test animal, and the effect on several major metabolic enzyme activities, including extracting methods of liver microsome, incubation temperature and enzyme inducers was investigated.

Materials and methods

Fish were dosed via intraperitoneal (i.p.) injection of normal saline, phenobarbital(PB) (80 mg/kg b.w. /day) and hexadecadrol (DEX)(100 mg/kg b.w. /day), separately. All injections were standardized to ensure a final injection volume of 0.1 mL and fish were injected daily for 3 consecutive days. After the 3-day exposure, the fish were killed by a sharp blow to the head. Livers were quickly removed, rinsed in ice-cold 0.9% NaCl solution, and weighed. The liver tissues were homogenized in a 0.1 M Tris-HCl buffer. S9 fractions were obtained by centrifugation of the liver homogenates at 9,000 g for 20 min at 4 °C. Microsomal pellets were obtained by centrifugation of S9 fractions at 105, 000 g for 60 min at 4 °C. The microsomal pellet was resuspended in a 0.1 M Tris-HCl buffer. The microsomal suspension and S9 were divided into aliquots respectively and stored in liquid nitrogen until utilized for in vitro assays. The protein content analysis of subcellular fractions was based on previously reported methods[1] .

Ethoxycoumarin O-deethylase (ECOD) activity was measured using a modified method of Greenlee and Poland[2] . The product 7-hydroxycoumarin was determined fluorometrically

(excitation/emission wavelengths of 370/450 nm) with an HIACHI F-7000 spectrophotofluorimeter. Ethoxyresorufin O-deethylase (EROD) and pentoxyresorufin O-deethylase (PROD) activities were measured using the modified method of Lubet et al.[3] . The aniline hydroxylase (AH) activity of the CYP2E1 enzyme was measured with aniline as the substrate according to the procedure described previously[4]. Glutathione S-transferase (GST) activities were determined utilizing the broad GST substrate CDNB[5].

Results and discussion

Phase I and II xenobiotic metabolizing enzymes play fundamental roles in metabolism and elimination of enobiotics. CYP450 (Phase I) and GST(Phase II) are important metabolism enzymes in fish liver microsomes and S9. In fish, CYP1A isozymes have been previously reported to catalyze the metabolism of various xenobiotic compounds (PAH, PCBs, dioxins, etc.) [6], whereas CYP3A and CYP2E subfamily are the isozymes presenting predominantly in fish[7] .EROD, PROD and AH can respectively represent CYP1A, CYP2B and CYP2E activity. Another CYP450 enzyme ethoxycoumarin-O-deethylase (ECOD) has been studied in mammals as a marker of activities for CYP1A1, CYP1A2, CYP2B1, CYP2E1, and CYP2B6 [8].

Table 1 compares five enzyme activities in both the liver S9 fraction and microsomes. The phase Ⅰ enzyme in hepatic microsomes showed the higher catalytic viability (24±5 pmol mg^{-1} min^{-1}) than that of the S9 (4±1 pmol mg^{-1} min^{-1}), as measured by CYP1A-mediated EROD activity. The same pattern was seen in the ECOD activity. Mean ECOD activity of microsomes was 93±3 pmol mg^{-1} min^{-1}, and which of S9 fractions was 31±4 pmol mg^{-1} min^{-1}. Hodson et al.found that EROD activity in rainbow trout liver S9 was 5.1 pmol min^{-1} mg^{-1} which was closed to our results[9].The CYP450 enzyme activity in liver microsomes is generally higher than that in the S9, because some phase Ⅱ enzyme and coenzyme was removed in the microsomes due to further centrifugal.

It is generally believed that ECOD activity and EROD activity can be used to characterize CYP1A activity, our results showed that ECOD activities in both microsomes and S9 were higher than EROD activities which suggested Ethoxycoumarin was a wider catalysis substrate than ethoxyresorufin for CYP450. Lange et al. determined the activities of ECOD, EROD and PROD in the liver of postspawning dab (*Limanda limanda*), and a biphasic kinetic of ECOD was found which indicated that in addition to CYP 1A a second isozyme catalyses the O-deethylation of ethoxycoumarin in the liver of dab[10]. Neither microsomes nor S9 showed CYP2B-mediated PROD activities when 1 μM substrate concentration was tested for pentyloxyresorufin. Parente et al. also found this phenomenon in tilapias liver micorosmes[11]. The PROD activity in rainbow trout liver was only 1.7 pmol min^{-1} mg^{-1}[12]. Therefore, The CYP2B activity in teleost was generally low. The activity of AH in microsomes was 20±5 pmol mg^{-1} min^{-1}, whereas in S9 fractions it was not detected. In comparison to the phase Ⅰ enzyme, phase Ⅱ enzyme GST activity in S9 fractions (183±45 nmol mg^{-1} min^{-1}) exhibited higher levels than that in microsomes (92±23 nmol mg^{-1} min^{-1}). Our results were consistent with other reports of teleost, which showed that the methods in this experiment is reliable.

Table 1. Phase Ⅰ cytochrome P450 activities and Phase Ⅱ GST activity in fish liver microsomes and S9 fraction.

Enzyme activity (pmol mg^{-1} min^{-1})	Microsomes	S9
ECOD	93±3	31±4
EROD	24±5	4±1
PROD	ND.	ND.
AH	20±5	ND.
GST	$(9.2\pm2.3)\times10^4$	$(1.83\pm0.45)\times10^5$

ND. : not detected.

We found that effect to EROD and ECOD activities of temperature (25℃ or 37℃) was not significant (Table 2). Generally considered 37 ℃ as the mammals physiological temperature ,

which is also a best temperature of enzyme activity, therefore in the use of rodents or the human body liver subcellular fractions incubation experiment *in vitro* was generally choose 37 ℃ as the incubation temperature. The fish CYP450 enzyme activity of optimum temperature is relatively low compared with mammals, many fish enzyme activity of optimum temperature is about 25 ℃, and even some cold water fish is about 15 ℃.Therefore, at the time of experiment using fish to fully consider the influence of temperature on enzyme activity, the early studies of CYP450 fish to use higher incubation temperature and detect active reason [13].

Table 2 The EROD and ECOD activities in fish liver microsomes and S9 in different temperature, 25 ℃ and 37 ℃

		ECOD activity (pmol mg^{-1} min^{-1})	EROD activity(pmol mg^{-1} min^{-1})
25 ℃	Microsomes	92.4	21.5
	S9	29.7	5.3
37 ℃	Microsomes	89.6	22.4
	S9	28.4	3.8

CYP450 was a kind of isozymes which have many different specificity substrate, and plays an important role in the compounds transormation. Each CYP450 isozymes has its own specific catalytic substrates, and susceptible to induction and inhibition of certain compounds, which affect the enzyme activity. Phenobarbital (PB) and dexamethasone (DEX) is a classic mammals CYP450 inducers. In this study, the induction effect to CYP450 of PB and DEX in fish liver was detected (Table 3).The results showed that the activity of EROD and the concentrations of protein was increased slightly, while the ECOD and PROD activity have no significant changes. It was indicated that the effect of inducer to CYP450 activity between fish and mammals was differently.

Table 3 Effect of PB and DEX on the fish liver enzyme activity

Enzyme activity (pmol mg^{-1} min^{-1})	PB	Dex	Control
ECOD	88.5	85.6	90.1
EROD	25.3	22.4	20.6
PROD	ND.	ND.	ND.
Protein content（mg mL^{-1}）	3.1	4.2	3.5

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