國立交通大學
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碩士論文

醇亞硫酸基轉移酶及3′磷酸腺甘酸5'磷酸硫酸合成酶之螢光檢測法

Fluorometric Assay for Alcohol Sulfotransferase & PAPS synthetase through Regeneration of PAPS

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中華民國九十四年七月

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摘要

亞硫酸化普遍地存在於生物系統中，而且被亞硫酸化的生物分子會明顯改變其生理化學性質。過去的研究已經證實 3′磷酸腺甘酸 5'磷酸硫酸合成酶(3'-phosphoadenosine 5'-phosphosulfate synthetase, PAPSS)和亞硫酸基轉移酵素(sulfotransferase, ST)兩類酵素涉及亞硫酸化反應。前者可活化無機硫酸根成為 PAPS，後者則催化產生的 PAPS 之亞硫酸基至接收分子。本論文主要是發展螢光檢測法來探討此兩類酵素在亞硫酸化的重要性，如探討四級結構對於亞硫酸基轉移酵素的影響，並分成三個章節敘述之。首先，我們建立了不同類的亞硫酸基轉移酵素的表達與純化方法。而且首次報導關於醇亞硫酸基轉移酵素(alcohol sulfotransferase, AST)之螢光檢測法。此方法有別於放射性同位素的終點式檢測，屬於連續性的螢光檢測法。檢測系統的主旨是利用重組的酚亞硫酸基轉移酵素(phenol sulfotransferase, PST)催化受質 4-methylumbelliferyl sulfate (MUS)做為亞硫酸基提供者並轉移至 3'-phosphoadenosine 5'-phosphate (PAP)再生 3'-phosphoadenosine 5'-phosphosulfate (PAPS) ，此核苷酸的亞硫酸基再由 AST 催化。在設計的環境中，重組的 PST 不會催化 AST 的受質，而且反應後產生 4-methylumbelliferone (MU)之螢光變化不但可以定量 AST 的活性，也有足夠的靈敏度來測出 ng 或 pmole 的酵素活性。在此研究中，許多 *h*DHEA-ST 的生化特性可被測得，文中並將此法與放射性同位素法就靈敏度等特質相比較，以及於第二章中研究四級結構對於亞硫酸基轉移酵素的影響。第三章，我們成功地利用分生技術得到重組的 3′磷酸腺甘酸 5'磷酸硫酸合成酶。並偶合使用亞硫酸基轉移酵素的生理反應條件，開發螢光檢測法以測量出 3′磷酸腺甘酸 5'磷酸硫酸合成酶的活性。此兩套 AST 及 PAPSS 之螢光檢測法不僅對於常規且詳細的酵素動力學有幫助外，也有潛力利用微量盤成為高效能分析法。

Fluorometric Assay for Alcohol Sulfotransferase & PAPS synthetase through Regeneration of PAPS

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ABSTRACT

The sulfoconjugation of biomolecules occurred widely in biological system which resulted in a dramatic change in the physicochemical property of the sulfonated compounds. Previously, 3'-phosphoadensine 5'-phosphosulfate synthetase (PAPSS) and sulfotransferases (STs) were found to involve in the course of sulfonation. The former activates inorganic sulfate to form PAPS, and the latter catalyzes the sulfuryl group of PAPS transferring to an acceptor molecule. The thesis is divided into three chapters, and introduces sensitive fluorometric assays developed for investigation into their fundamental issues concerning the role of sulfonation, such as the effects of quaternary structure on sulfotransferase function. First chapter described the convenient expression and purification procedure of recombinant STs by affinity column. Furthermore, a first continuous fluorometric assay was developed for alcohol sulfotransferase (AST). It utilized PAPS regenerated from 3'-phosphoadenosine 5'-phosphate (PAP) by a recombinant phenol sulfotransferase (PST) using 4-methylumbelliferyl sulfate (MUS) as the sulfuryl group donor. The change of fluorescence intensity of 4-methylumbelliferone (MU) corresponded directly to the amount of active AST and was sensitive enough to measure ng or picomole amount of the enzyme activity. Some properties of *h*DHEA-ST were determined by this method and were found comparable to published data. In second chapter, this coupled-enzyme assay was applied to study some effects of quaternary structure on AST. Some catalytic properties and thermal stability were compared between dimeric (wild-type) and monmeric (mutant) cytosolic STs. Final chapter described molecular cloning, expression, and purification of recombinant PAPSS. By coupling with the physiological reaction of PST, the activities of recombinant PAPSS were also determined fluorometrically. The two fluorometric methods developed not only are useful for the routine and detailed kinetic study of these important class of enzymes but also have the potential for the development of a high-throughput procedure using microplate reader.

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Abbreviation and Symbol

Abbreviation and Symbol	Full name
PST	Phenol sulfotransferase
AST	Alcohol sulfotransferase
*h*DHEA-ST	Human dehydroepiandrosterone sulfotransferase
*h*PAPSS	Human PAPS synthetase
ε	Absorption (extinction) coefficient
bis-tris propane	1,3-bis[tris(hydroxymethyl)methylamino]propane
Bromphenol blue	3', 3'', 5', 5''-tetrabromophenolsulfonephthalein
PAP	Adenosine 3',5'-bisphosphate
	3'-phosphoadenosine 5'-phosphate
PAPS	Adenosine 3'-phosphate 5'-phosphosulfate
	3'-phosphoadenosine 5'-phosphosulfate
MUS	4-methylumbelliferyl sulfate
MU	4-methylumbelliferone
DTT	Dithiothreitol, Cleland's Reagent
EDTA	(Ethylenedinitrilo)tetraacetic acid
PMSF	Phenylmethylsulfonyl fluoride
MES	2-[*N*-morpholino]ethanesulfonic acid
SDS	Sodium Dodecyl Sulfate
TEMED	N, N, N', N'-tetramethylethylene diamine
DHEA	Dehydroepiandrosterone
U	Units；μmole/min
RFU	Relative fluorescence unit
A_{280}	Absorption at 280 nm
K_m	Michaelis constant
K_{is}	Inhibitory constant of substrate inhibition
V_{max}	Maximum velocity

Chapter 1 Fluorometric Assay for Alcohol Sulfotransferase

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A sensitive fluorometric assay was developed for alcohol sulfotransferase (AST). This was the first continuous fluorometric assay reported for AST. It utilized 3'-phosphoadenosine 5'-phosphosulfate (PAPS) regenerated from 3'-phosphoadenosine 5'-phosphate (PAP) by a recombinant phenol sulfotransferase (PST) using 4-methylumbelliferyl sulfate (MUS) as the sulfuryl group donor. The recombinant PST did not use the alcohol substrate under the designed condition, and the sensitivity for AST activity was found comparable to that of radioactive assay as reported in the literature. The change of fluorescence intensity of 4-methylumbelliferone (MU) corresponded directly to the amount of active AST and was sensitive enough to measure ng or picomole amount of the enzyme activity. This fluorometric assay was used to determine the activities of AST as purified form and in crude extracts of pig liver, rat liver, and *Escherichia coli*. Some properties of *h*DHEA-ST were determined by this method and were found comparable to published data. Under similar assay conditions, the contaminated activities of arylsulfatase in crude extracts were also determined. This method not only is useful for the routine and detailed kinetic study of this important class of enzymes but also has the potential for the development of a high-throughput procedure using microplate reader.

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Introduction

Sulfotransferases (STs) are a large family of enzymes that catalyze the transfer of sulfuryl group from PAPS to numerous endogenous and exogenous compounds (Jakoby and Ziegler, 1990). Cytosolic STs catalyze the sulfonation of steroid hormones, catecholamines/neurotransmitters, drugs and environmental chemicals, and are involved in hormone homeostasis and metabolic detoxication/activation of xenobiotics (Coughtrie *et al.*, 1998; Duffel and Guengerich, 1997; Falany *et al.*, 1993; Mulder and Jakoby, 1990; Weinshilboum *et al.*, 1994). In contrast, Golgi membrane-bound STs catalyze the sulfonation of macromolecules including glycosaminoglycans and proteins, and play important roles in the modulation of receptor binding, intercellular communication, and signaling processes (Bowman and Bertozzi, 1999; Weinshilboum *et al.*, 1997).

As a member of cytosolic STs, AST catalyzes the sulfonation of various steroids and their derivatives as well as many xenobiotic alcohols (Falany, 1997; Weinshilboum *et al.*, 1997). Substrates of human AST include hydroxysteroids such as dehydroepiandrosterone (DHEA), testosterone, β-estradiol, and many other endogenous steroids (Chen *et al.*, 1996; Kakuta *et al.*, 1998). Steroid sulfonation has been recognized as an important function for maintaining steroid hormone level *in vivo* (Chang *et al.*, 2004). A prominent example is that the bulk of DHEA produced by adrenal glands is sulfonated and secreted into circulation, which served as a precursor for the androgenic and estrogenic steroids in extra-adrenal tissues (Kroboth *et al.*, 1999; Labrie *et al.*, 1995).

Suitable assays for STs are essential for investigation into their physiological functions. To date, the most common assays for AST activities are usually conducted by monitoring the transfer of radioisotopic sulfate from [^{35}S]PAPS to specific

products (Ramaswamy and Jakoby, 1987), which involve stopping the reactions by heat treatment after a fixed time interval, centrifugation to remove precipitates formed, and changing the solvent system prior to thin-layer or paper chromatography. These procedures are tedious for routine and detailed kinetic studies of AST enzymes. Other reported AST assays are also end-point analyses requiring the determination of PAP using high-performance liquid chromatography (Sheng and Duffel, 2001). Routine spectrophotometric assays, however, have been available only for PST (Ramaswamy and Jakoby, 1987). Despite the considerable progress made in recent years on AST enzymes, several fundamental issues concerning the role of neurosteroids in neuron morphogenesis, regulation, and physiological involvement still remain to be fully elucidated. The present study was prompted by an attempt to develop a convenient assay in order to address theses important issues.

Here we report the development of a fluorometric assay for AST (**Figure 1**). In this assay, the regeneration of PAPS from PAP with MUS as the sulfuryl group donor was catalyzed by PAP-free PST in a reverse physiological reaction (Lin and Yang, 1998). This reaction was coupled to AST, where purified human dehydroepiandrosterone sulfotransferase (*h*DHEA-ST) was used. The product, MU, served as fluorometric indicator to monitor *h*DHEA-ST activity.

Experimental Procedures

Materials. MUS, MU, DHEA, PAP, PAPS, tris[hydroxymethyl]aminomethane (Tris), 2-[N-morpholino]ethanesulfonic acid (MES), phenylmethylsulfonyl fluoride (PMSF), [ethylenedinitrilo] tetracetic acid (EDTA), glutathione (reduced form) and dithiothreitol (DTT) were purchased from Sigma (St. Louis, MO). Potassium phosphate (dibasic), glycine, and sodium dodecyl sulfate (SDS) were obtained from J. T. Baker (Phillipsburg, NJ 08865 U.S.A.). Glutathione S-transferase Sepharose fast flow was obtained from Amersham Pharmacia Biotech Asia Pacific (Hong Kong). All other chemicals were of the highest purity commercially available.

Preparation of PAP-free PST. The β-form of recombinant PST (Yang *et al.*, 1996) was used as the PAP-free enzyme. Recombinant mutant PST, K65ER68G, was cloned into an expression vector, pET3c, and transformed into *Escherichia coli* BL21 (DE3) (Chen *et al.*, 1992). The enzyme isolation procedure was the same as described earlier (Yang *et al.*, 1996), which resulted in a homogeneous protein as determined by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970).

Preparation of hDHEA-ST. Recombinant *h*DHEA-ST was cloned into an expression vector, pGEX-2TK and transformed into *Escherichia coli* BL21 (DE3). The expression and purification of *h*DHEA-ST was described previously (Chang *et al.*, 2001) and a homogeneous protein was obtained as determined by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970).

Determination of protein concentration. Protein concentration of the homogeneous form of K65ER68G and *h*DHEA-ST was estimated on the basis of

absorbency at 280 nm (1.7 ml/mg cm^{-1} and 2.4 ml/mg cm^{-1}, respectively) (Gill and von Hippel, 1989) with an UV/Vis spectrophotometer (Hitachi UV/Vis-3300, Japan).

PST assay. The activity of PAP-free PST, K65ER68G, was determined based on the change of fluorescence due to the production of MU from MUS as measured using a spectrofluorometer (Hitachi F-4500, Japan). The excitation and emission wavelengths were 360 nm and 450 nm, respectively. The reaction mixture included 5 mM 2-mercaptoethanol, 4 mM MUS, 20 μM PAP, 100 mM potassium phosphate buffer (pH 7.0), and 0.5-2 μg K65ER68G. This assay was also referred to as a reverse physiological reaction catalyzed by K65ER68G. One unit was defined as 1 μmol of PAP converted to PAPS per minute with 4-methylumbelliferyl sulfate under the reaction conditions described above.

Coupled-enzyme assay for AST. Reaction mixture (1ml) contained 100 mM potassium phosphate buffer (pH 7.0), 5 mM 2-mercaptoethanol, 20 μM PAPS, 4 mM MUS, 5 μM DHEA, and 3.2 mU (5.4 μg) K65ER68G. AST (*h*DHEA-ST or crude extract) was added following a preincubation period so as to start the reaction at 37°C. Because commercial PAPS contained significant amounts of PAP which is an inhibitor of sulfotransferase (Duffel and Jakoby, 1981; Yang, *et al.*, 1996) the assay mixture was preincubated for 15 min prior to the addition of DHEA to ensure that all PAP had been converted to PAPS by K65ER68G before complete enzymatic reaction was started. The production of MU was monitored by fluorescence as previously described. The change of fluorescence was linear for over 20 min of initial reaction time.

Preparation of biological samples for enzymatic assay. The liver extracts were

prepared from 20 g each of frozen rat or pig liver which was mixed with 20 ml buffer A (10 mM Tris-HCl at pH 7.4 plus 125 mM sucrose, 10% glycerol, 1 mM DTT, l.5 mM PMSF, and 1 mM EDTA) and homogenized with liquid nitrogen. Bacterial cell extract was prepared from about 1 g *Escherichia coli* pelleted from 250 ml cell culture, which was mixed with 20 ml buffer A and sonicated three times, then collect supernatants.

Results and Discussion

K65ER68G as catalyst for the regeneration of PAPS. In this proposed coupled-enzyme assay, PST was used to regenerate PAPS from PAP and MUS as illustrated in **Figure 1**. Under the similar conditions, arylsulfatase activity not only could be determined in the presence of only MUS but PST activity could be also determined in the absence of AST or its substrate. Two characteristics of wild-type PST, however, might prevent the effective production of PAPS. It had been shown that wild-type PST contained tightly bound PAP (Yang *et al.,* 1996), a sulfotransferase inhibitor which exists in all sulfotransferase-catalyzed reactions. Moreover, phenols were also inhibitors of PST (Duffel and Jakoby, 1981). Under reducing conditions, the activity of PST could be significantly affected by the presence of PAP and/or phenol in the proposed PAPS regenerating system. A ternary complex of PST, PAP, and phenol might form to slow down the regeneration of PAPS from PAP (Marshall *et al.,* 2000; Vakiani *et al.,* 1998; Whittemore *et al.,* 1985). Fortunately, previous studies had demonstrated that a PST mutant, K65ER68G, was free of the complications mentioned above (Yang *et al.,* 1996). Preliminary studies showed that K65ER68G could efficiently catalyze the production of PAPS, and PAP and MU did not inhibit the reaction under the conditions adopted in this report. As shown in **Table 1**, K_m for MUS determined was not significantly affected by the pH of the solution. In contrast, V_{max} of the same reaction was significantly decreased at pH 9.0. These data were useful for the design of coupled-enzyme assay.

Selection of excitation and emission wavelengths for the coupled-enzyme assay. To bring about a virtually irreversible auxiliary reaction with regard to the initial product (Rudolph *et al.,* 1979) in the proposed coupled-enzyme assay, high

concentration of MUS (4 mM) was used to saturate the PAPS regenerating system based on data compiled in **Table 1**. A suitable excitation wavelength was determined to prevent the "inner filter effect" (Lackowicz, 1983) caused by the absorption of MUS. Briefly, due to different absorption spectra of MUS and MU the excitation (absorption) wavelength of MU must be selected carefully to avoid absorption from MUS. The excitation wavelength, therefore, was selected according to the absorption spectra of MU and MUS as shown in **Figure 2**. The suitable excitation wavelength was chosen by comparing the absorption spectra of the two compounds. Therefore, AST activity was in effect determined by the increase of fluorescence of MU at 450 nm upon excitation at 360 nm. The relative emission coefficient of the fluorescence at 450 nm was determined under different conditions as shown in **Table 2**. The sensitivity of this method could thus reach nM range since the pmole amount of MU could be reliably determined. The intensity of the fluorescence was significantly dependent on the pH value ranging from 6 to 10, primarily due to the deprotonation of MU at alkaline pH (pK_a of MU = 7.8 (Sun *et al.*, 1998)). The temperature effect was less significant as shown in **Table 2**.

Coupled-enzyme assay. It is expected that the reaction rate of AST, which was in the range of sub-μmole/min/mg (Chang *et al.*, 2001), could be easily monitored using this coupled-enzyme system. It is worth pointing out that the major requirements for this assay were the adequate amount of MUS, an excess of K65ER68G activity, and a saturating PAPS concentration.

Figure 3 shows the time course of the changes in fluorescence for the coupled *h*DHEA-ST/K65ER68G reaction and corresponding controls, where specific components of the reaction mixture were omitted. Only for the complete reaction did we observe an increase in fluorescence, reflecting the enzymatic activity of

*h*DHEA-ST. Some background fluorescence due to the auxiliary reaction was observed. This was probably because of the presence as an impurity of PAP in commercially available PAPS (Duffel and Jakoby, 1981; Yang *et al.*, 1996;). The fluorescence background was, however, low in the absence of PAPS, K65ER68G, or MUS. Under these conditions, the auxiliary reaction did not take place. No effects were observed on the measured rates by raising the concentration of PAPS (data not shown), which indicated that PAPS concentration was saturating enough for the coupling system to reach its maximum activity. Taking together the results shown in **Figure 3**, it was concluded that the continuous changes in fluorescence were specifically attributable to *h*DHEA-ST activity.

To determine the linear range of AST assay at a given K65ER68G concentration (3.2 mU), the rate of MUS reduction was measured using a concentration range (0.07-1.35 μg) of *h*DHEA-ST as shown in **Figure 4**. Although this linear range and sensitivity of *h*DHEA-ST assay could be further extended, it was suitable for our needs under the present situation.

To test our coupled-enzyme assay and compare the results with the data appeared in the literature, the *h*DHEA-ST was characterized by this fluorometric assay as shown in **Figures 5** to **7**. These results fitted perfectly with previously reported data using radioactive assay procedures (Chang *et al.*, 2001). **Figure 5** shows the pH-dependency of the DHEA-sulfonating activity. The optimum pH spanned from 7 through 9. The enzyme activity at pH 6.0 was approximately 50% of that in the optimum pH range. Virtually no activity was detected at pH 10.0. The effects of temperature on the DHEA-sulfonating activity was examined over 25-50℃ as shown in **Figure 6**. Maximum sulfonating activity was observed at approximately 40℃. The enzyme activity at 25℃ and 50℃ were approximately 50％ of maximal activity at 45℃. At 55℃ or higher, *h*DHEA-ST became inactive. **Figure 7** shows the effects of

DHEA concentration on the DHEA-sulfonating activity. Significant substrate inhibition was observed as previously reported (Chang *et al*, 2004; Duffel and Jakoby, 1981). Substrate inhibition leading to the formation of a nonproductive enzyme-PAP-substrate complex (Marchall *et al*, 2000; Vakiani *et al*, 1998; Whittemore *et al*, 1985) in fact is a rather common feature among member of the sulfotransferase family (Falany *et al*, 1989; Marcus *et al*, 1980; Otterness *et al*, 1992). These results compare favorably to published K_m values determined by a noncoupled, radioisotopic thin-layer chromatography assay (Chang *et al*, 2001; 2004). DHEA were reported to yield K_m and K_{is} values of 2.1 and 3.8 μM, respectively (Chang *et al*, 2001). This coupled assay determined these values to be 4.7 and 4.3 μM, respectively. We suggest that the discrepancy between these values is due to the inherent inaccuracy of the radioisotopic assay. This coupled-enzyme assay allows the continuous measurement of initial reaction velocity more accurately than end-point assays. Besides, it was found that the sensitivity of this fluorometric assay was comparable to that of the radioactive assay for AST reported in the literature (Chang *et al*, 2001; 2004). The activity of the amount of enzyme used (100 ng) previously could be easily determined by the present method as shown in **Figure 4**.

Determination of AST activity in biological samples. We further demonstrated the feasibility of present assay for measuring AST activity in biological samples. In addition, several other enzyme activities associated with sulfonation/desulfonation could also be determined under similar conditions. Sulfatase, alcohol and phenol sulfotransferase activities might all exist in biological samples, and we could take advantage of the coupled-enzyme system to determine all these enzyme activities. As shown in **Table 3**, the complete system (**I**) could detect not only AST but also arylsulfatase activities due to the presence of MUS. The sulfotransferase activity

could not be observed in the absence of PAPS, and therefore reaction condition II gave a background activity exhibited mainly by arylsulfatase. The sulfatase activity was further confirmed and quantified under the reaction conditions that contained only MUS and buffer (or in the absence of DHEA and PAPS, data not shown). PST activity could also be determined using a similar procedure (data was not shown since it did not interfere with the AST activity). In the absence of MUS, the fluorescence observed was close to the experimental error (data not shown) and represented the background derived from the interference of biological samples. Thus, the AST activity could be calculated simply by subtracting arylsulfatase activity from that measured in the complete coupled-enzyme reaction. Based on this method, the AST activity in rat liver was found to be significantly higher than that in pig liver or *E. coli* (**Table 3**). Moreover, the AST activity extracted from transformed *E. coli* cells (with pGEX-2TK) was approximately 100 times than that in untransformed cells.

Conclusion

We developed in this study a continuous fluorometric AST assay whose sensitivity was comparable to that of the end-point radioactive assay reported in the literature. This method was demonstrated to be useful for the determination of AST activities associated with homogeneous AST or those present in crude extracts from biological samples. This new assay procedure could be adapted for high-throughput assay using a microplate reader.

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Table 1

Production of PAPS from PAP and MUS catalyzed by K65ER68G[a]

pH	K_m (μM)	V_{max} (nmol min^{-1} mg^{-1})
6.0	161 ± 14	204 ± 7
7.0	183 ± 15	399 ± 9
8.0	196 ± 21	142 ± 5
9.0	167 ± 51	2.9 ± 0.2

[a] The reaction mixture included 5 mM β-mercaptoethanol, 20 μM PAP, and MUS (50 μM - 3.2 mM) plus 0.55 μg enzyme in 100 mM buffer (MES at pH 6.0, potassium phosphate at pH 7.0, and Tris at pH 8.0 and pH 9.0). The K_m and V_{max} were obtained using nonlinear regression by SigmaPlot 2001, V7.0 and Enzyme Kinetics Module, V1.1 (SPSS Inc., Chicago, IL).

Table 2

The Relative Emission Coefficient of MU at 450 nm[a]

	Relative Emission coefficient ($cm^{-1}nM^{-1}$)						
Temperature (℃) / pH	25	30	35	37	40	45	50
6.0	—	—	—	0.12	—	—	—
7.0	0.4	0.43	0.45	0.48	0.50	0.52	0.55
7.5	—	—	—	0.64	—	—	—
8.0	—	—	—	1.14	—	—	—
9.0	—	—	—	2.14	—	—	—
10.0	—	—	—	4.10	—	—	—

[a] The fluorescence of MU (100, 200, and 400 nM) in 100 mM buffer (MES at pH 6.0, potassium phosphate at pHs 7.0 and 7.5, Tris-base at pHs 8.0 and 9.0 and glycine at pH 10.0) was determined with a spectrofluorometer (Hitachi F-4500, Japan). The excitation and emission wavelengths were at 360 and 450 nm, respectively.

Table 3

Activities of AST and arylsulfatase in biological samples [a]

		Rat liver		**Pig liver**		***E. coli***		***E. coli* (with pGEX-2TK)**	
Reaction conditions	Enzyme activity involved	Specific activity (pmole/min/A_{280})	Total activity (nmole/min/g)	Specific activity (pmole/min/A_{280})	Total activity (nmole/min/g)	Specific activity (pmole/min/A_{280})	Total activity (nmole/min/g)	Specific activity (pmole/min/A_{280})	Total activity (nmole/min/g)
Ⅰ. Complete	AST+ sulfatase	245 ± 4	39.6 ± 0.7	132 ± 4	7.2 ± 0.2	6.1 ± 1.0	2.9 ± 0.7	280 ± 10	236 ± 8.1
Ⅱ. – PAPS [b]	sulfatase	34.5 ± 1.5	5.6 ± 0.2	21.2 ± 5.1	0.8 ± 0.3	0.4 ± 0.1	0.6 ± 0.1	5.0 ± 1.3	0.8 ± 0.2
Ⅰ – Ⅱ	AST	210 ± 6	34.0 ± 0.9	113 ± 12	6.2 ± 0.4	5.6 ± 1.0	2.3 ± 0.7	274 ± 11	231 ± 9

[a] Detailed procedures were described under *Coupled-enzyme assay for AST* in *Materials and Method* except that purified *h*DHEA-ST was replaced by the extract of biological sample. Specific activity referred to MU produced following the addition of extract whose protein concentration was determined by absorption at A_{280}. Total activity referred to MU produced with one gram of wet cell or liver.

[b] AST activity was eliminated in the absence of PAPS as shown in **Figure 3**.

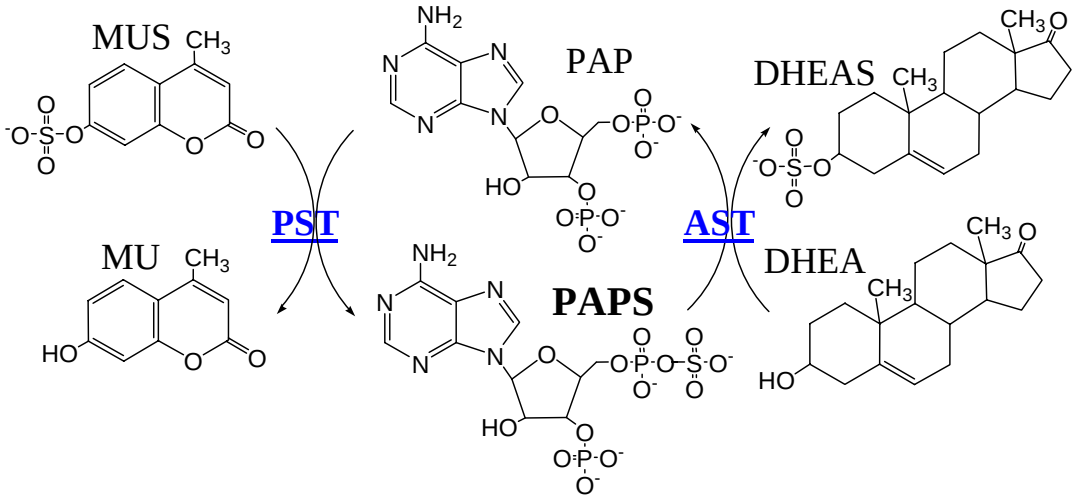

Figure 1. Scheme for the determination of AST activity. This assay was based on the regeneration of PAPS from PAP catalyzed by a recombinant PST (K65ER68G) using MUS as the sulfuryl group donor. In coupled-enzyme assay, PST represented an auxiliary enzyme, and the product, MU, was used as a fluorescent indicator of enzyme turnover. Moreover, the biosynthesis of PAPS from ATP and SO_4^{2-} was catalyzed by *h*PAPS synthetase, which was a bifunctional enzyme with activity of ATP sulfurylase and APS kinase.

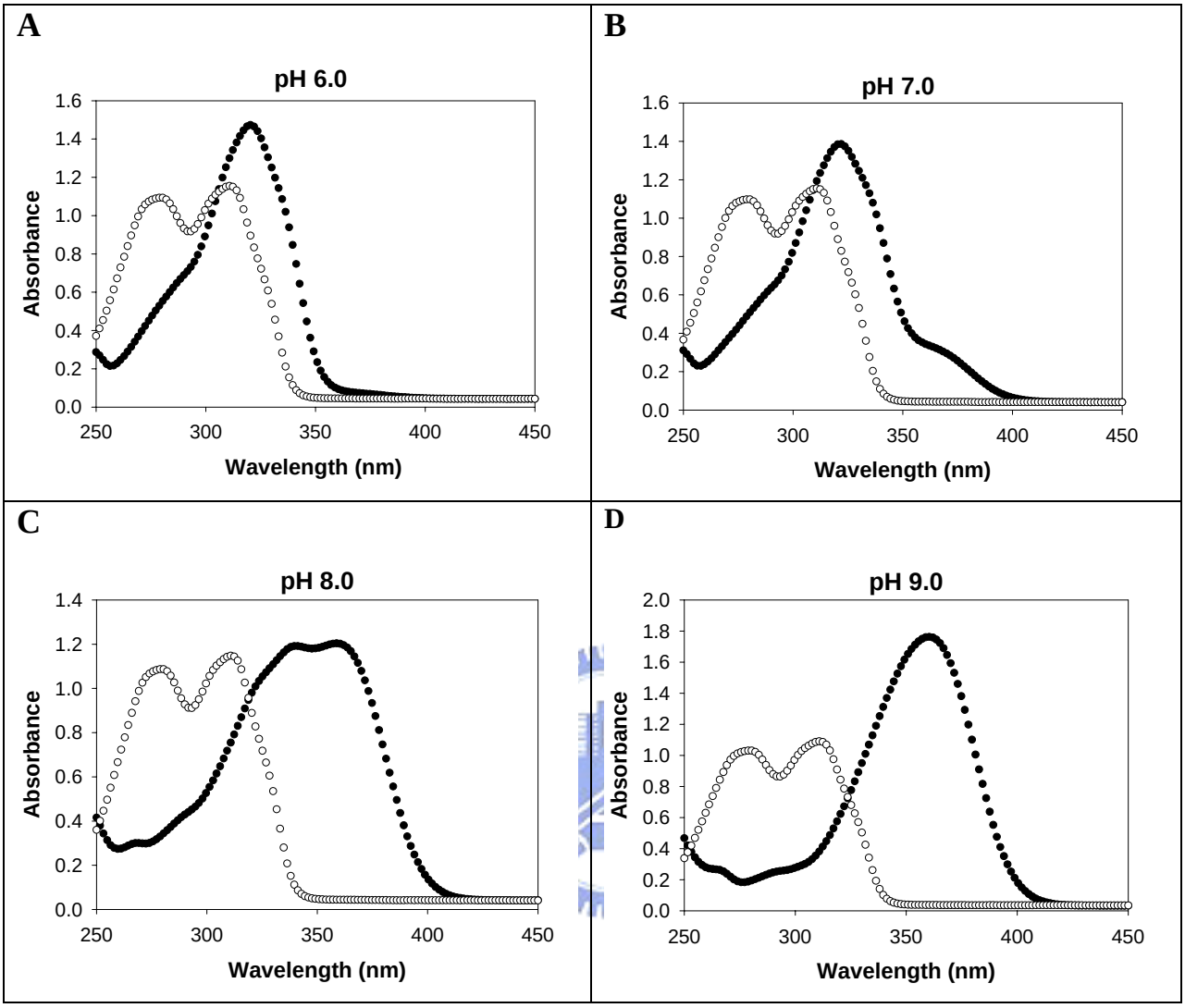

Figure 2. Absorption spectra of MUS and MU. The spectra were obtained in 1ml aqueous solutions contained 100 μM MUS (◯) or MU (●) and 100 mM buffer (MES at pH 6.0, potassium phosphate at pH 7.0, and Tris-base at pH 8.0 and pH 9.0.)

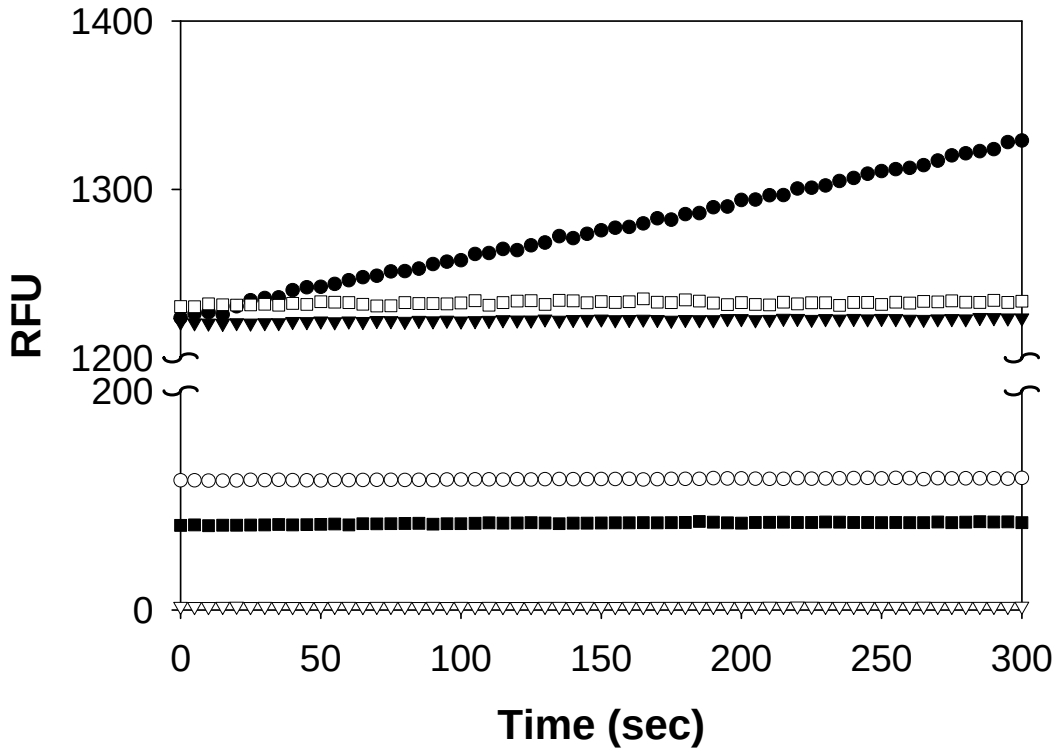

Figure 3. Progress curves of the coupled-enzyme assay for AST. Complete reaction (●) and control reactions without PAPS (○), DHEA (▼), MUS (▽), K65ER68G (■), or *h*DHEA-ST(□) of coupled-enzyme assay for AST were conducted at pH 7.0, 37°C. Detailed procedures were described under *Coupled-enzyme assay for AST* in *Materials and Methods.*

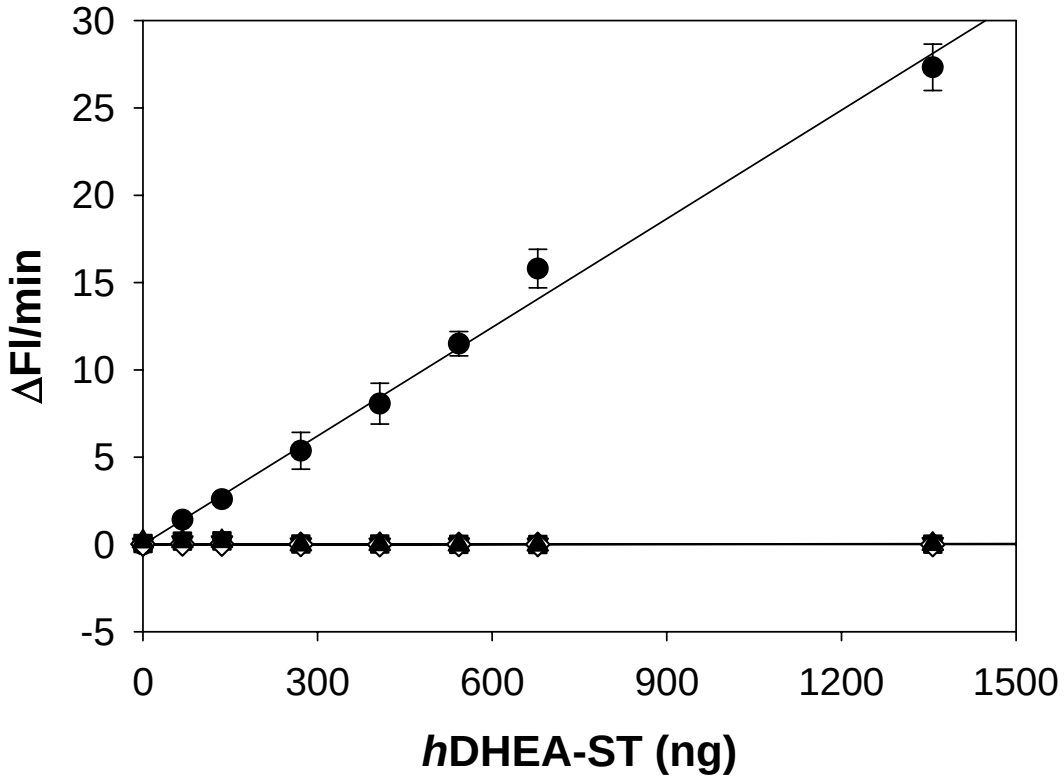

Figure 4. Effective range of fluorometric assay for *h*DHEA-ST. Complete reaction (●) and control reactions without PAPS (○), DHEA (▼), MUS (▽), or K65ER68G (■) of coupled-enzyme assay were run in a total volume of 1000 μl at 37°C. Detailed procedures were described under *Coupled-enzyme assay for AST* in *Materials and Methods.* Each point was determined from triplicate assay data and standard error was obtained by SigmaPlot 2001, V7.0 (SPSS Inc., Chicago, IL).

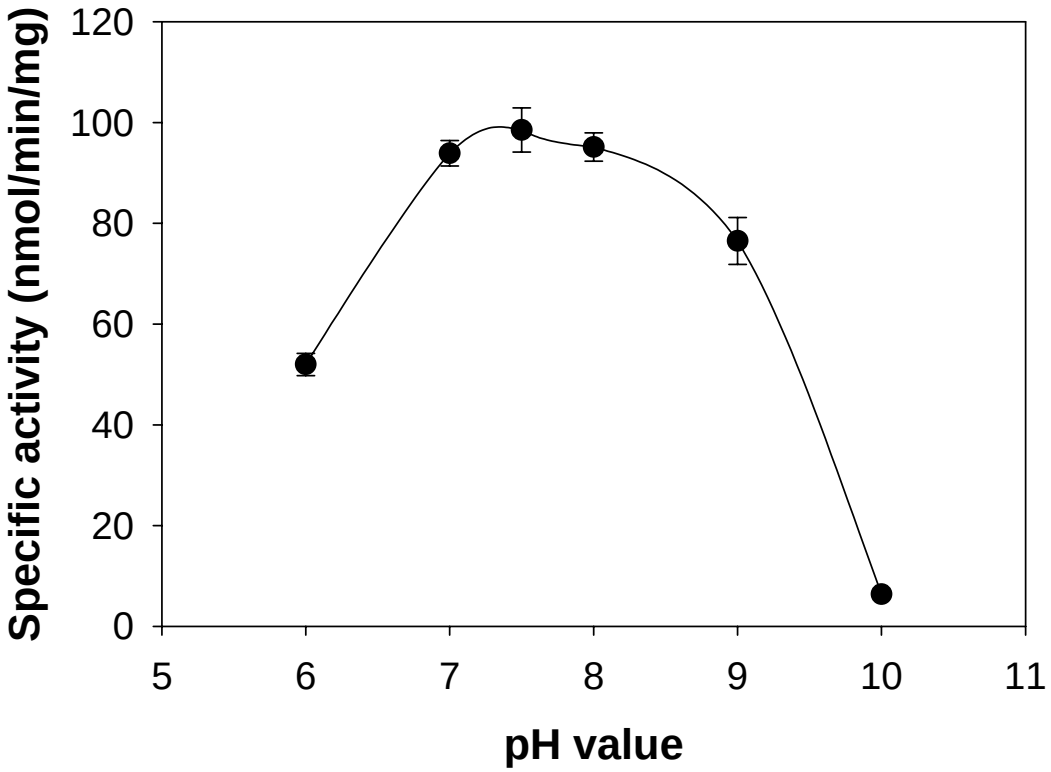

Figure 5. pH profile of recombinant *h*DHEA-ST. The enzymatic assays were carried out with about 0.5 μg recombinant *h*DHEA-ST under the condition of *Coupled-enzyme assay for AST* as described in *Materials and Methods* using different buffer systems (100 mM MES at pH 6.0, potassium phosphate at pH 7.0, Tris-base at pH 8.0 or 9.0, and glycine at pH 10.0). Each point was determined from triplicate assay data and standard error was obtained by SigmaPlot 2001, V7.0 (SPSS Inc., Chicago, IL).

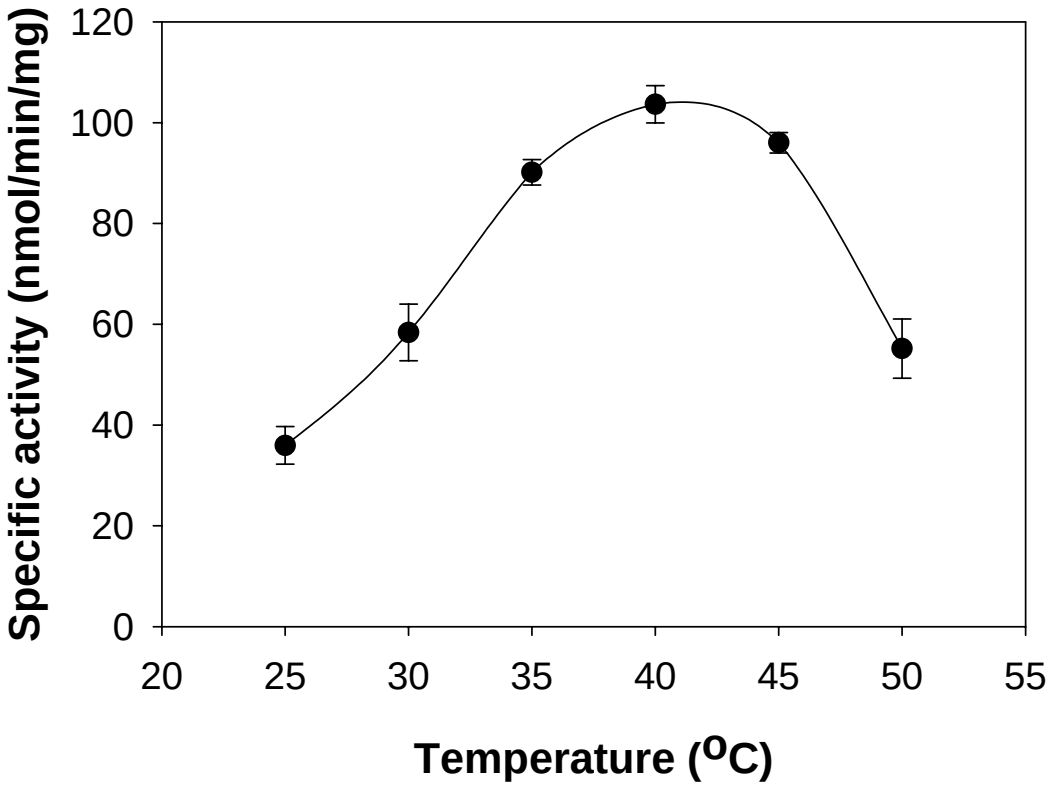

Figure 6. Temperature effect on the activity of recombinant *h*DHEA-ST. The enzymatic assays were carried out with about 0.5 μg recombinant *h*DHEA-ST under the condition of coupled-enzyme assay for AST at pH 7.0 in different temperature. Each point was determined from triplicate assay data and standard error was obtained by SigmaPlot 2001, V7.0 (SPSS Inc., Chicago, IL).

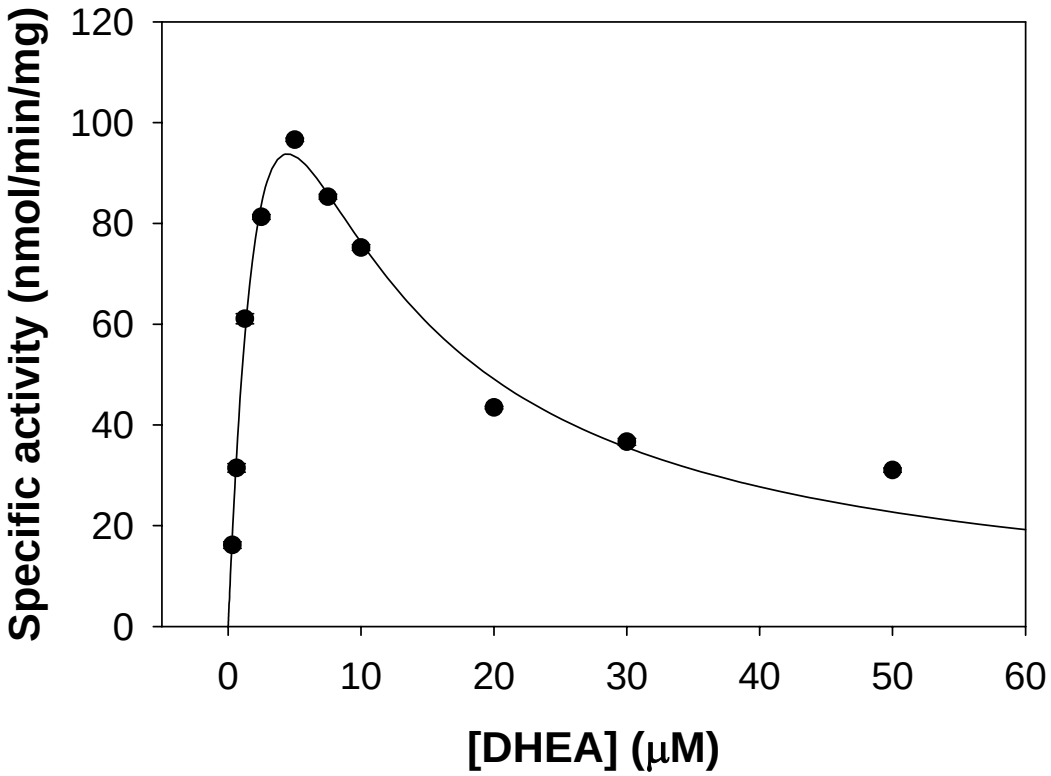

Figure 7. Substrate inhibition of DHEA on recombinant *h*DHEA-ST. The enzymatic assays were carried out under the condition of coupled-enzyme assay for AST at pH 7.0 with about 0.5 μg recombinant *h*DHEA-ST and different DHEA concentrations (0.3 ~ 50.0 μM). The kinetic parameters were obtained using the substrate inhibition equation $v = V[S]/\{K_m + [S](1 + [S]/K_{is})\}$by SigmaPlot 2001, V7.0 and Enzyme Kinetics Module, V1.1 (SPSS Inc., Chicago, IL)

Chapter 2 Effects of Quaternary structure on Cytosolic Sulfotransferase Function

Cytosolic sulfotransferases (STs) catalyze the sulfonation of small molecules including xenobiotics, bioamines, hormones, and steroids. The enzymes, including mammalian phenol sulfotransferase (PST) and alcohol sulfotransferase (AST), are generally homodimers in solution and play a role in detoxication, hormone homeostasis and signal transduction, such as neurotransmission. Previous structural characterization of mutations within the dimer interface of cytosolic ST have demonstrated that KTVE motif are the common protein-protein interaction motif that mediates their homo- as well as heterodimerization. Through site-directed mutagenesis, and gel filtration, we also confirm that a single mutation of Val266 to Glu was sufficient to convert rat PST (*r*PST) to a monomer by breaking hydrophobic KTVE motif. The similar result was found in AST by mutation of Val260 to Glu. First, kinetic constants of substrates (*p*NPS and DHEA) and cofactors (PAP and PAPS) were determined to identify the effect of dimerization on cytosolic ST catalysis. Second, due to stimulatory and inhibitory effects of metal ions on cytosolic STs, metal effect on dimeric and monomeric STs was also compared. No significant difference on catalysis between dimer and monomer was observed either in PST and AST. Therefore, thermal inactivation was investigated into the thermostability in dimeric and monomeric STs. Although the importance of dimerization still cannot be elucidated by these studies, redox effect and stiochiometry of binary (PAP-ST) or ternary complex (PAP-substrate-ST) need to be characterized for understanding this special protein-protein interaction in cytosolic STs.

Introduction

Sulfotransferases (STs) are a large family of enzymes that catalyze the transfer of sulfuryl group from the common 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to numerous endogenous and exogenous compounds (Jakoby and Ziegler, 1990). Cytosolic STs catalyze the transfer of the sulfuryl group to numerous small molecules including steroids, bioamines, therapeutic drugs, and environmental chemicals. Therefore, sulfonation is not only considered to be deactivation and secretion processes of biological signal molecules and xenochemicals, but also be involved in maintaining physiological homeostasis such as steroid hormones (Falany, 1997).

Cytosolic STs consist of around 300 amino acid residues, and are found as dimers in solution. It has been reported that STs are capable of forming not only homodimers but also heterodimers. For example, the sulfonation activity of N-hydroxy-2-acetylaminofluorene is associated with three different dimers of STs from rat liver cytosol. Interestingly, they are aryl ST-IV dimer, ST1C1 dimer, and AST-IV/ST1C1 heterodimer determined through purification and mass analysis (Kiehlbauch *et al.*, 1995). However, protein-protein interaction of STs has not been well studied and the functional significance of the dimerization process is not known at the present time. The crystal structures of numerous cytosolic STs have recently been solved (Bidwell *et al.*, 1999; Dajani *et al.*, 1999; Kakuta *et al.*, 1997; Kakuta *et al.*, 1998; Pedersen *et al.*, 2000), and they reveal a conserved PAPS-binding site and similar reaction mechanism. In some of the solved structures the enzymes crystallized as dimers, but these were unlikely to be the catalytic species, as the substrate-binding site is blocked

by the dimer interface. Previously, structural comparisons and mutagenesis studies were undertaken with mouse SULT1E1 (a monomer) and human SULT1E1 (a dimer) in an attempt to identify a common structural motif. It was found that the mutations V269E and V260E converted the homodimers SULT1E1 and SULT2A1, respectively, into monomers. It was further shown that the mutations P269T and E270V in mouse SULT1E1 precipitated the formation of a homodimeric structure. It was concluded that the common motif KxxxTVxxxE (KTVE motif) is responsible for the dimerization of STs (Petrotchenko *et al.*, 2001).

To date, despite protein-protein interaction was found since 2001 (Petrotchenko *et al.*, 2001), the effect of quaternary structure on cytosolic sulfotransferase catalysis was still not elucidated. In this report, we show a single mutation of valine to glutamic acid was sufficient to convert dimeric ST to a monomer by breaking hydrophobic KTVE motif. To identify effects of the dimerization on sulfotransferase function, we generated two monomeric STs from rat phenol sulfotransferase and human DHEA sulfotransferase to investigate metal effect, substrate inhibition, redox effect, and stiochiometry of binary or ternary complex.

Experimental Procedures

Materials. *p*NPS, *p*NP, DHEA, MUS, β-naphthol, PAP, PAPS, tris[hydroxymethyl]aminomethane (Tris), phenylmethylsulfonyl fluoride (PMSF), [ethylenedinitrilo] tetracetic acid (EDTA), PAP-agarose and dithiothreitol (DTT) were purchased from Sigma (St. Louis, MO). Potassium phosphate (dibasic), glycine, and sodium dodecyl sulfate (SDS), metal ions were obtained from J. T. Baker (Phillipsburg, NJ 08865 U.S.A.). DEAE Sepharose fast flow, Prep Sephacryl S-200 HR, PCR and gel band purification kit, and low molecular weight gel filtration calibration kit were obtained from Amersham Pharmacia Biotech Asia Pacific (Hong Kong). Oligonucleotides were obtained from Mission Biotech (Taipei, ROC). *Taq* polymerase, and reagents for PCR were obtained from New England Biolabs (Taiwan). Plasmid DNA extraction Kit was purchased from VIOGENE (Illkirch Cedex, France) and Agarose was purchased from Ultra Violent (Taipei, ROC). All other chemicals were of the highest purity commercially available.

Site-Directed Mutagenesis. Double-stranded primers (forward primer, 5’-ttcactgaa gcccagaatgag, and reverse primer, 5’-ggcttcagtgaaggtattttt) encoding a single amino acid mutation in the PST gene was used to get mutated PST by means of Quickchange protocol. Wild-type PST cDNA packaged in pET3c prokaryotic expression vector was used as the template in conjunction with specific mutagenic primers. Thirty cycles of PCR were carried out with denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 6 min. The reaction contained 100 ng of DNA template, primers,

400 μM concentrations of each of the four deoxynucleotide triphosphates (dNTPs), 1× *Pfu*Turbo buffer, and 2.5 units of *Pfu*Turbo DNA polymerase. The PCR product was treated with DpnI for 1 h at 37 °C followed by transformation of BL21 (DE3) *Escherichia coli* cells. C. The mutated PST sequence was verified by nucleotide sequencing (Sanger *et al.*, 1977). The same protocol was also utilized to generate monmeric DHEA-ST by mutation residue 260 from valine to glutamic acid using double-stranded primer (forward primer, 5'-ttcactgaagcccagaatgag, and reverse primer, 5'- ggcttcagtgaaggtattttt).

Preparation of wild-type and mutant enzymes. Recombinant wild-type and mutant (V266E) PST were cloned into an expression vector, pET3c (Chen *et al.*, 1992), while recombinant wild-type and mutant (V260E) DHEA-ST were cloned into an expression vector, pGEX-2TK (Chang *et al.*, 2001). Four plasmids were transformed into *Escherichia coli* BL21 (DE3) for overexpression. The enzyme isolation procedures were the same as described earlier (Chang *et al.*, 2001; Yang *et al.*, 1996), which resulted in a homogeneous protein as determined by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970).

Determination of protein concentration. Protein concentration of the homogeneous form of wild-type and mutant form of PST and DHEA-ST were estimated on the basis of absorbency at 280 nm (1.7 ml/mg cm^{-1} and 2.4 ml/mg cm^{-1}, respectively) (Gill and von Hippel, 1989) with a UV/Vis spectrophotometer (Hitachi UV/Vis-3300, Japan).

Enzyme assay for PST. PST activity was determined based on the change of absorbency at 400 nm due to the production of *p*NP (ε = 10,500 $cm^{-1}M^{-1}$ at pH 7.0) from *p*NPS as measured using a UV/Vis spectrophotometer (Hitachi UV/Vis-3300, Japan). The reaction mixture included 5 mM 2-mercaptoethanol, 1 mM *p*NPS, 2 μM PAP, 100 mM Bis-Tris propane (at pH 7.0), and 2 μg PST. The α-form activity was determined in the absent PAP, and the β-form activity was the difference between total and α-form activity. The spectrophotometric physiological assay followed the decrease in absorbance of freshly prepared *p*NP (100 μM) when PAPS (300 μM) was the sulfuryl group donor. Rate was linear with time when average absorbance changed at 400 nm of less than 0.025 per minute was followed for 3 minute.

Coupled-enzyme assay for AST. Reaction mixture (1ml) contained 100 mM potassium phosphate buffer (pH 7.0), 5 mM 2-mercaptoethanol, 20 μM PAPS, 4 mM MUS, 5 μM DHEA, 3.2 mU (5.4 μg) K65ER68G. AST (*h*DHEA-ST or crude extract) was added following a preincubation period so as to start the reaction at 37°C (Chen *et al.*, 2005). Because commercial PAPS contained significant amounts of PAP which is an inhibitor of sulfotransferase (Duffel and Jakoby, 1981; Yang, *et al.*, 1996) the assay mixture was preincubated for 15 min prior to the addition of DHEA to ensure that all PAP had been converted to PAPS by K65ER68G before complete enzymatic reaction was started. The production of MU was monitored by fluorescence as previously described. The change of fluorescence was linear for over 20 min of initial reaction time.

Size-exclusion chromatography (SEC) and calibration curve preparation. Prior to

size exclusion chromatography, proteins were exchanged into 20 mM Tris base (pH 7.5), 50 mM sodium acetate, 250 mM NaCl, 0.1 mM EDTA (SEC buffer), using a Millipore ultrafiltration unit. Aliguots (2 ml of 10 mg/ml protein solution) were injected onto a Pharmacia Sephacryl S-200 HR column and eluted with SEC buffer at 4℃ at flow rate of 0.5 ml/min. Protein elution was monitored by absorbance at 280 nm, and fractions were collected and analyzed by enzyme assay to confirm the presence and retention time of sulfotransferase. Molecular weights were estimated against calibration standards: ribonuclease A (15.6 KDa), chymotrypsinogen A (19.4 KDa), ovalbumin (47.6 KDa), and Albumin (62.9 KDa).

Kinetic constants determinations. Measurement of the kinetic constants of each substrate was performed by varying the concentrations of one substrate, while keeping the other substrate at a fixed and near saturating concentration. The apparent K_m and V_{max} were determined using nonlinear regression by Sigmaplot 2001, V7.0 and Enzyme Kinetics Module, V1.1 (SPSS inc., Chicago, IL).

Determination of the Stimulatory/Inhibitory Effects of Divalent Metal Cations on PST, AST, and their mutants. To determine the stimulation/inhibition patterns of divalent metal cations on purified sulfotransferases, enzymatic assays in the presence or absence of divalent metal cations were performed. The standard assay mixture (phosphate buffer was replaced by HEPES buffer) was similar as described under enzymatic assay but also contained 10 mM of the divalent cation tested, while controls containing all the reagents, but without divalent metal ion or with 10 mM EDTA, were assayed in parallel.

Thermal inactivation studies. Activity measurements were preincubated in the dry-bath at the desired temperature, and were performed by enzymatic assay after a 10 min incubation. The reaction mixture was the same as described in enzyme assay.

Results and Discussion

Mutated enzyme preparation. Previous structural studies have delineated the physiological dimerization interface of cytosolic sulfotransferases to the conserved sequence KXXXTVXXXE that occurs near the C-terminus (Petrotchenko *et al.*, 2001). The KTVE motifs of two given monomers appear to form a zipper-like, anti-parallel interface, leading to the dimerization of two monomers. The main feature of the interface can be described as complementary hydrophobic interactions and backbone hydrogen bonds in the central portion of motif that are reinforced by an ion pair at each end of the motif (Petrotchenko *et al.*, 2001). For the reason, residue 266, valine, of PST was selected and mutated to glutamic acid, and the product of site-directed mutagenesis was seen in **Figure 1a**. The same protocol was also utilized to generate monmeric DHEA-ST by mutation residue 260, valine, to glutamic acid (data not shown). Using bacterial expression, V266E mutant of PST was purified through DEAE sepharose, Sephacryl S-200 HR, and PAP-agarose to get homogeneous forms analyzed by SDS-PAGE in **Figure 1b**. For the expression and purification of V260E DHEA-ST, GSTrap Fast Flow, Sephacryl S-200 HR, and PAP-agarose were used (data not shown).

Calibration curve preparation and molecular weight estimation. SEC (Sephacryl S-200 HR) was used to examine the quaternary structure of V266E and V260E mutants compared to the wild-type PST and DHEA-ST, respectively. Using a set of molecular weight standards, the calibration curve in which Vo (the retention time of eluted blue dextran) was 35.70 ml was prepared by plotting log(molecular weight) versus retention

time in **Figure 2**. Detail parameters under this condition were described in **Table 1**. By means of the calibration curve, the apparent molecular weight of wild-type and mutant PST and DHEA-ST were calculated as described in **Table 2**. The estimated molecular weight of wild-type PST was approximately 2-fold than that of V266E. These data indicated that the interface of dimerization was easily disrupted through site-directed mutagenesis to form monomeric PST because glutamic acid might spoil hydrophobic zipper-like KTVE motif as similar in previous studies (Petrotchenko *et al.*, 2001). Similar result was observed between wild-type and V260E DHEA-STs.

Kinetic analysis. Kinetic constants of *p*NPS and PAP catalyzed by wild-type and V266E PST were determined by transfer reaction as described in **Table 3**. Apparently K_m values for PAP of the monomer and dimmer were 75 and 69 nM, respectively, and the K_m values for *p*NPS were 2.28 and 2.29 μM, respectively. This experiment indicated that the behavior of monomer (V266E) with respect to *p*NPS and PAP was significantly yet different with comparison from wild-type PST. However, this data conflicted with previous studies that approximately 0.5 equivalents of PAP bound per subunit for wild-type (dimeric) PST (Yang *et al.*, 1996). Similar kinetic data were observed in wild-type and V260E DHEA-ST as described in **Table 4**. Substrate inhibition was found both in wild-type and V260E DHEA-ST, but kinetic constants either in K_m and K_{is} were not apparently different.

Effect of divalent metal ion on PST, AST, and their mutants. Previously, Liu's group showed that some divalent metal cations may exert stimulatory or inhibitory effects on

cytosolic STs (Sakakibara *et al.*, 1994; Suiko *et al.*, 1996). For example, the activity of human monoamine PST was strongly stimulated by Mn^{2+}, and this result may be corresponding to neurodegenerative disease (Suiko *et al.*, 1996). In this experiment, we report a systematic investigation of the effects divalent metal cations on the activities of PST, AST, and their mutants as shown in **Table 5**. The degrees of inhibition or stimulation were calculated by comparing the activities determined in the presence of metal cations with the activities determined in the absence of metal cations or with 10 mM EDTA. Both in PST and V266E PST, stimulatory effect was observed by Mg^{2+}, Na^{2+}, Mn^{2+}, Ca^{2+}, and Co^{2+}, while inhibitory effect was observed by Fe^{2+}. However, in DHEA-ST and V260E DHEA-ST, stimulatory effect was only observed by $Mg^{2+}$and Ca^{2+}, while inhibitory effect was observed by Mn^{2+} and Co^{2+}. Although there was different pattern between PST and DHEA-ST, there was not significantly distinctness between wild-type and defects in quaternary structure of PST and DHEA-ST.

Thermal inactivation. The quaternary structure of protein was shown to be responsible for its thermal stability (Julia *et al.*, 2003). Therefore, independence of sulfotransferase catalysis, we assessed the conformational ability of wild-type and dimer interface mutant (V266E) by determining the effects of temperature on their catalytic activity (**Figure 3**). Steep inactivation was observed between 35 to 40 ℃ in both enzymes. The experiments showed that the V266E mutant has similar thermal ability to the wild-type enzyme in this assay. Similar pattern was observed in wild-type and V260E DHEA-ST except steep inactivation occurred between 40 to 50 ℃ (data not shown).

Conclusion

Monomeric ST was easily obtained by disrupting KTVE motif. Using site-directed mutagenesis, rat PST and human DHEA-ST, were mutated to the monomer analyzed by gel filtration (**Table 1-2**). Preliminary results (**Table 3-5**) had shown that catalytic properties of monomeric ST might not be significantly different from those of the homodimeric form. However, the data also conflicted with previous studies that approximately 0.5 equivalents of PAP bound per subunit for wild-type (dimeric) PST (Yang *et al*., 1996). These results indicatesthat previous data with the wild-type enzyme may require reinterpretation. Moreover, independence of sulfotransferase catalysis, thermal stability of wild-type and defect in quaternary structure was similar. Therefore, to understand the importance of quaternary structure on sulfotransferase catalysis, redox effect and stiochiometry of binary (PAP-ST) or ternary complex (PAP-substrate-ST) needed to be characterized.

Table 1

Calibration standard proteins and parameters of gel filtration

Standard [a]	Mw	log(Mw)	Ve [b]	K_{av} [c]
Albumin	67000	4.83	50.59	0.2
Ovabumin	43000	4.63	56.78	0.3
Chymotrypsinogen A	25000	4.40	69.96	0.4
Ribonuclease A	13700	4.14	77.79	0.5

[a] Standard proteins were purchased from Amersham Pharmacia Biotech Asia Pacific (Hong Kong).

[b] Ve represents the retention time of eluted protein under the condition as described under *Preparation of Calibration Curve* in MATERIALS AND METHODS.

[c] K_{av} was calculated by the equation ($K_{av} = (Ve – Vo) / (Vt – Vo)$)

Table 2

Molecular weight determination on Sephacryl S-200 HR

sample	Ve [a]	K_{av} [b]	log(Mw)	Mw [c]
rPST(wt[d])	52.8	0.20	4.75	56514
rPST(V266E)	64.5	0.34	4.47	29246
hDHEA-ST(wt)	49.2	0.16	4.84	69921
hDHEA-ST(V260E)	61.5	0.30	4.55	35700

[a] Ve represents the retention time of eluted protein under the condition as described under *Preparation of Calibration Curve* in MATERIALS AND METHODS.

[b] K_{av} was calculated by the equation (K_{av} = (Ve – Vo) / (Vt – Vo))

[c] The apparent molecular weight (Mw) was calculated by comparison of retention time (Ve) to data obtained from molecular weight standards described under **Table 1**.

[d] wt represents wild-type.

Table 3

Kinetics of *p*NPS and PAP with rat wild-type and V266E PST [a]

	*p*NPS [a]		PAP [b]	
PST	K_m mM	V_{max} nmol/min/mg	K_m μM	V_{max} nmol/min/mg
Wild type	2.29 ± 0.14	1218 ± 44	0.075 ± 0.011	1322 ± 64
V266E	2.28 ± 0.19	1320 ± 62	0.069 ± 0.019	1225 ± 41

[a] The reaction mixture included 5 mM β-mercaptoenthanol, 2 μM PAP, and *p*NPS (0.75 - 5 mM) plus 2 μg enzyme in 100 mM buffer (Bis-Tris propane at pH 7.0). The K_m and V_{max} were obtained using nonlinear regression by SigmaPlot 2001, V7.0 and Enzyme Kinetics Module, V1.1 (SPSS Inc., Chicago, IL).

[b] The reaction mixture included 5 mM β-mercaptoenthanol, 1 mM *p*NPS, and PAP (0.01 - 2 μM) plus 2 μg enzyme in 100 mM buffer (Bis-Tris propane at pH 7.0). The K_m and V_{max} were obtained using nonlinear regression by SigmaPlot 2001, V7.0 and Enzyme Kinetics Module, V1.1 (SPSS Inc., Chicago, IL).

Table 4

Kinetics of DHEA and PAPS with rat wild-type and V260E DHEA-ST [a]

	DHEA [a]			PAPS [b]	
AST	K_m μM	K_{is} μM	V_{max} nmol/min/mg	K_m μM	V_{max} nmol/min/mg
Wild type	4.7 ± 0.3	4.3 ± 0.3	119 ± 10	3.9 ± 0.4	125 ± 12
V266E	5.6 ± 0.5	4.9 ± 0.5	125 ± 15	4.2 ± 0.6	131 ± 20

[a] The reaction mixture included 5 mM β-mercaptoenthanol, 20 μM PAPS, and DHEA (0.3 - 50 μM) plus 0.5 μg enzyme in 100 mM buffer (potassium phosphate at pH 7.0). The K_m, K_{is}, and V_{max} were obtained using nonlinear regression by SigmaPlot 2001, V7.0 and Enzyme Kinetics Module, V1.1 (SPSS Inc., Chicago, IL).

[b] The reaction mixture included 5 mM β-mercaptoenthanol, 5 μM DHEA, and PAPS (0.5 - 20 μM) plus 0.5 μg enzyme in 100 mM buffer (potassium phosphate at pH 7.0). The K_m and V_{max} were obtained using nonlinear regression by SigmaPlot 2001, V7.0 and Enzyme Kinetics Module, V1.1 (SPSS Inc., Chicago, IL).

Table 5

Inhibitory/stimulatory effects of divalent metal cations on the activities of PST, AST, and their mutants [a].

Enzymes / Metal cations	PST	V266E PST	DHEA-ST	V260E DHEA-ST
$LiCl_2$	97%	94%	100%	101%
$MgCl_2$	113%	109%	122%	115%
$MnCl_2$	110%	104%	50%	50%
$CaCl_2$	120%	110%	128%	110%
$ZnCl_2$	N.D. [b]	N.D. [b]	N.D. [b]	N.D. [b]
$FeCl_2$	25%	20%	N.D. [b]	N.D. [b]
$CoCl_2$	115%	110%	17%	10%
$NaCl_2$	100%	99%	100%	105%

[a] Specific activities of the STs are determined in the concentration of the divalent metal cations tested was 10 mM. Data shown represent percentage compared with control (100 %).

[b] N.D. refers to "no activity detected" and, therefore, the complete inhibition of the enzyme.

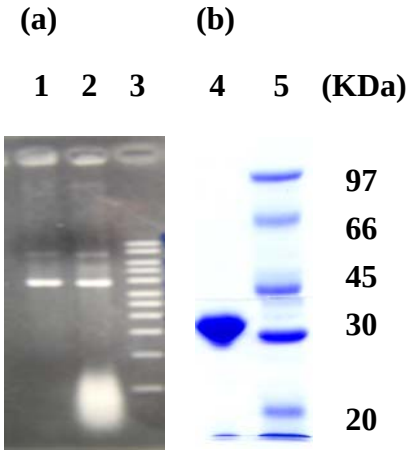

Figure 1. PCR product of mutagenesis and SDS-PAGE. (a) Lane 1 and 2 were the plasmid of wild-type and V266E, respectively. Lane 3 was DNA marker made of 500, 1000, 1500, 2000, 2500, 3000, 4000, 5000, 6000, 8000, 10000 bp. (b) Lane 4 was homogeneous form of V266E *r*PST, and lane 5 was protein marker made of phosphorylase b (97 kDa)，bovine serum albumin (66 kDa)，ovalbumin (45 kDa)，carbonic anhydrase (30 kDa)，soybean trypsin inhibitor (20.1 kDa).

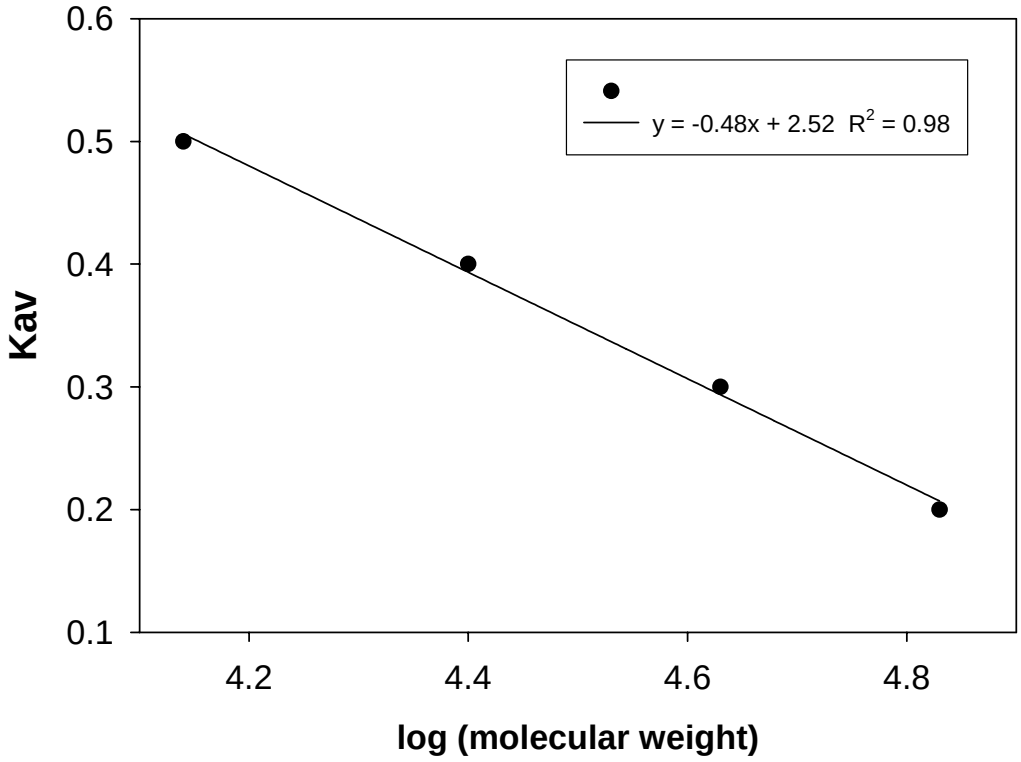

Figure 2. Calibration curve on Sephacryl S-200 HR. A molecular weight calibration curve, which defined the relationship between the elution volumes of a set standards and the logarithm of their respective molecular weights, was determined with Prep Sephacryl S-200 HR as described under MATERIALS AND METHODS. Calibration standards used were ribonuclease A (15.6 KDa), chymotrypsinogen A (19.4 KDa), ovalbumin (47.6 KDa), and Albumin (62.9 KDa).

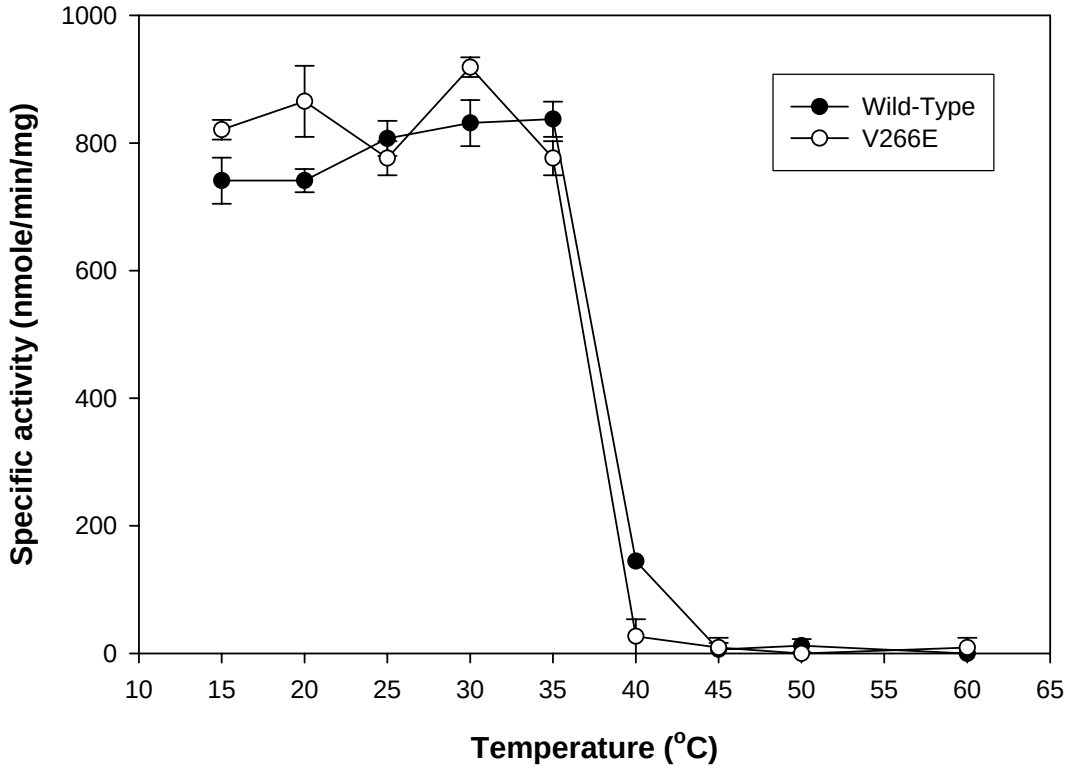

Figure 3. Thermal stability of wild-type and V266E PSTs. The experiment was performed as described under MATERIALS AND METHODS. Each point was determined from triplicate assay data and standard error was obtained by SigmaPlot 2001, V7.0 (SPSS Inc., Chicago, IL).

Chapter 3 Molecular Cloning, Expression, Purification, and Fluorometric assay of human PAPS synthetase

Sulfonation is a major pathway in the biotransformation of many xenobiotic compounds, such as drugs and toxic chemicals. 3'-phosphoadenosine 5'-phosphosulfate (PAPS) plays a key role in offering sulfuryl group to sulfotransferases for mediating these reactions. In higher organism, such as human and mouse, the synthesis of PAPS from inorganic sulfate and ATP is catalyzed by bufunctional enzyme called PAPS synthetase (PAPSS). In this project, we successfully cloned the gene for human PAPSS1 as a step forward understanding tissue-specific expression of this enzyme. In addition, a convenient fluorometric assay for PAPSS had been developed. This assay replaced end-point radioisotopic method and was the first continuous method in this report. PAP-free PST proved to be ideal under the present situation because of its higher tolerance to substrate inhibition by PAP and MU and inhibition by ATP, a substrate for PAPSS-catalyzed step, than wild-type PST. PAP-free PST, therefore, was used to catalyze PAPS, which biosynthesized by human PAPSS, with MU as sulfuryl group acceptor in the literature. The reactant, MU, served as fluorometric indicator to monitor *h*PAPSS activity. This method not only is useful for the routine and detailed kinetic study of this important class of enzymes but also has the potential for the development of a high-throughput procedure using microplate reader.

Introduction

Sulfonation, an important pathway in the metabolism of many drugs, other xenobiotics, and biomolecules, is mediated through the universal sulfuryl group donor, 3'-phosphoadenosine 5'-phosphosulfate (PAPS) (Gregory and Robbins, 1960). PAPS plays a role in offering sulfuryl group for the sulfotransferases that catalyze these reactions (Klaassen and Boles, 1997; Weinshilboum and Otterness, 1994). In prokaryotes, PAPS is synthesized from 2 moles of ATP and 1 mole of SO_4^{2-} by two separate enzymes, ATP sulfurylase and adenosine 5'-phosphate kinase (Farooqui, 1980; Klaassen and Boles, 1997). The former enzyme catalyzes the synthesis of adenosine phosphosulfate (APS) from ATP and SO_4^{2-}, while the latter enzyme phosphorylates APS in the presence of another molecule of ATP to form PAPS. However, in higher organisms, such as human and mice, these reactions are catalyzed by a single bifunctional enzyme, PAPS synthetase (PAPSS) (**Figure 1**, step A). Previously, two isoforms of PAPSS, *PAPSS1 and PAPSS2*, have been identified in both humans and mice (Girard et al., 1998; Kurima et al., 1998; Li et al., 1995; ul Haque et al., 1998; Venkatachalam et al., 1998). Northern blot analysis showed different patterns of tissue expression: PAPSS1 mRNA was highly expressed in human brain, whereas PAPSS2 mRNA was not, but the opposite was true of human liver (Xu et al., 2000).

Despite the considerable progress made in recent years on PAPSS enzymes, rare evidences demonstrated different PAPSS patterns of tissue expression, and no common simple nucleotide polymorphism (SNP) that change the biochemical activity or expression level of these enzymes have been described in human. Therefore, suitable assays for PAPSS are essential for investigation into their physiological functions. To date, the most common assays for PAPSS activities are usually

conducted by monitoring [^{35}S]PAPS from free carrier [^{35}S]SO_4^{2-} (Hazelton *et al.*, 1985; Vargas, 1988; Wong *et al.*, 1990) which involve stopping the reactions by heat treatment after a fixed time interval, removing precipitates formed by centrifugation, and changing the solvent system prior to thin-layer or paper chromatography. These procedures are tedious for routine and detailed kinetic studies of PAPSS enzymes. Other reported PAPSS assays are also end-point analyses requiring the determination of PAPS using radiochemical coupled-enzyme assay (Xu *et al.*, 2000). In an attempt to begin the process of characterizing PAPSS activity, the present study was to develop a convenient assay in order to address theses important issues.

Here, we report the molecular cloning, expression, and purification of human PAPSS (*h*PAPSS) for the biosynthesis of PAPS (**Figure 1**, step A). This reaction was coupled to PAP-free PST, and a continuous fluorometric assay was developed. In this assay, the depletion of MU to MUS with PAPS as the sulfuryl group donor was catalyzed by PAP-free PST in physiological reaction (**Figure 1**, step B). The reactant, MU, served as fluorometric indicator to monitor *h*PAPSS activity.

Experimental Procedures

Materials. MUS, MU, DHEA, PAP, PAPS, tris[hydroxymethyl]aminomethane (Tris), 2-[N-morpholino]ethanesulfonic acid (MES), phenylmethylsulfonyl fluoride (PMSF), [ethylenedinitrilo] tetracetic acid (EDTA), glutathione (reduced form) and dithiothreitol (DTT) were purchased from Sigma (St. Louis, MO). Potassium phosphate (dibasic), glycine, and sodium dodecyl sulfate (SDS) were obtained from J. T. Baker (Phillipsburg, NJ 08865 U.S.A.). PCR and gel band purification kit, Hitrap chelating column, Hitrap Q column, and glutathione S-transferase Sepharose fast flow was obtained from Amersham Pharmacia Biotech Asia Pacific (Hong Kong). Oligonucleotides were obtained from Mission Biotech (Taipei, ROC). *Taq* polymerase, T4 ligase, and reagents for PCR were obtained from New England Biolabs (Taiwan). Plasmid DNA extraction Kit was purchased from VIOGENE (Illkirch Cedex, France) and Agarose was purchased from Ultra Violent (Taipei, ROC). All other chemicals were of the highest purity commercially available.

Construction of Full-length of hPAPSS for Escherichia coli. The experimental method was modified from Satishchandran and Markham (1998). Bacterial clone containing the huamn ATP sulfurylase/ APS kinase coding sequence cloned into the vector pCMV-SPORT6 was purchased from OPEN Biosystems. The plasmid was isolated and verified for the presence of the *h*PAPSS insert and the CMV promoter in the proper orientation by DNA sequencing using T7 and pCMV-SPORT6 reverse primer. In addition, the plasmid was transformed into DH5α competent cell for storage at -80℃. The full-length *h*PAPSS was amplified by PCR. Briefly, an aliquot of the *h*PAPSS cDNA was used as a template for PCR amplification using primers designed to contain EcoRI and HindIII restriction sites: forward primer,

5'-CCGGAATTCCATGCAGAGAGCAACCAATGT-3' and reverse primer, 5'-GCCAAGCTTCTAAGCTTTCTCCAAGGATTT-3'. All PCR amplifications were performed in a Perkin-Elmer thermal cycler using *Taq* polymerase from New England Biolabs (Taiwan). Standard cycling parameters included a 2 min preincubation at 94 °C followed by 35 cycles of 1 min at 94 °C, 1 min at 60 °C, and 2 min at 72 °C. Following the cycling phase, there was a final extension for 7 min at 72 °C. The PCR product was analyzed on a 1% low melting agarose gel; the expected product (1.9 kilobase pairs) was sliced out of the gel and purified with a spin cartridge (Pharmacia) according to the manufacturer's instructions. The Novagen pET-21b system was chosen for bacterial expression of the cloned human bifunctional enzyme. All DNA fragments to be expressed were inserted into the EcoRI / HindIII doubly- digested plasmid, and transformed into DH5α competent cell. Positive clones were identified by both restriction mapping and DNA sequencing of the entire insert.

Preparation of hPAPSS. The experimental method was modified from Satishchandran and Markham (1998). The positive clones were sequenced in their entirety before transformation into BL21 (DE3) cells by the $CaCl_2$ method. A single colony was used to inoculate an LB/ampicillin liquid culture which was then placed on a shaker at 37 °C. Growth was continued to an A_{600} of 0.4–0.6 and then the culture was centrifuged, and the cells were resuspended in LB/ampicillin and stored overnight at 4 °C. This suspension culture was subsequently used to inoculate a second, larger LB/ampicillin liquid culture which was grown with shaking at 37 °C for approximately 4 h; then cDNA expression was induced with 1 mM isopropyl-thio-β-D-galactoside (IPTG) during additional 12 hr incubation. The cultures were centrifuged at 8000 rpm for 20 min, and the pellet was sonicated in IMAC 5 buffer (5 mM imidazole, 50 mM Tris, pH 7.9) many times. Following

removal of cellular debris by ultracentrifugation at 15,000 rpm for 30 min, the supernatant was diluted 1:2 in IMAC 5 sonication buffer. To purify the expressed protein, the chelating column from Pharmacia was used following the supplier's protocol. Briefly, the resin was washed with double distilled H_2O to remove ethanol from the storage solution, charged with 100 mM $NiSO_4$, and then equilibrated in sonication buffer. And then crude supernatant was loaded onto the 5-ml column in a 1:1 dilution with sonication buffer and incubated at 4 °C for 20 min with intermittent mixing. Following incubation, the flow-through was collected, and the column was washed with 30 mM imidazole to remove the majority of nonspecifically bound bacterial proteins, after which the expressed protein was eluted with 400 mM imidazole. The purified protein was dialyzed against Q column buffer (50 mM Tris, pH 8.0, 1 mM dithiothreitol, 1 mM EDTA, 10% glycerol) overnight for purification with Hitrap Q. Then the Q column was eluted with 0.5 M NaCl gradient (total volume 500 mL with flow rate at 1 mL/min).

Preparation of PAP-free PST. The β-form of recombinant PST (Yang *et al.*, 1996) was used as the PAP-free enzyme. Recombinant mutant PST, K65ER68G, was cloned into an expression vector, pET3c, and transformed into *Escherichia coli* BL21 (DE3) (Chen *et al.*, 1992). The enzyme isolation procedure was the same as described earlier (Yang *et al.*, 1996), which resulted in a homogeneous protein as determined by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970).

Determination of protein concentration. Protein concentrations of K65ER68G and *h*PAPSS in the homogeneous form were estimated on the basis of absorbency at 280 nm (1.7 ml/mg cm^{-1} and 1.3 ml/mg cm^{-1}, respectively) (Gill and von Hippel, 1989) with an UV/Vis spectrophotometer (Hitachi UV/Vis-3300, Japan).

PST assay. The activity of PAP-free PST, K65ER68G, was determined based on the change of fluorescence due to the depletion of MU as measured using a spectrofluorometer (Hitachi F-4500, Japan). The excitation and emission wavelengths were 360 nm and 450 nm, respectively. The reaction mixture included 5 mM 2-mercaptoethanol, 20 μM MU, 20 μM PAPS, 100 mM potassium phosphate buffer (pH 7.0), and 15 μg K65ER68G. This assay was also referred to as a physiological reaction catalyzed by K65ER68G. One unit was defined as 1 μmol of PAPS converted to PAP per minute with 4-methylumbelliferone under the reaction conditions described above.

Coupled-enzyme assay for PAPSS. Reaction mixture (1ml) containing 100 mM potassium phosphate buffer (pH 7.0), 5 mM 2-mercaptoethanol, 1 mM ATP, 4 mM Na_2SO_4, 1 mM $MgCl_2$, 20 μM MU,3 mU (5 μg) K65ER68G, and 1-2 μg *h*PAPSS. ATP or Na_2SO_4 was added last so as to start the reaction at 25°C.

Preparation of APS and PAPS. This method was modified from Satishchandran and Marjham (1989). In brief, the reaction mixture for the preparation of APS and PAPS contained 1 mM $MgCl_2$, 1 mM ATP, 4 mM carrier free Na_2SO_4, 100 mM Tris-HCl (pH 7.0), 5.0 units of inorganic pyrophosphatase, and 2.0 unit of purified ATP surturylase/APS kinase (PAPSS) in a total volume of 50 μl. The reaction after 30 min was stopped by placing the reaction vessel in a boiling water bath for 2 min. The precipitate formed was removed by centrifugation (stage I). HPLC analysis showed that the SO_4^{2-} was completely converted to PAPS. In order to facilitate purification of PAPS, 10 units oh hexokinase and 5 mM D-glucose (final concentration) were added and the solution was incubated at 30℃ for 30 min to convert ATP into ADP. This

reaction was stopped by boiling, and the precipitate was removed by centrifugation (stage II). For the synthesis of APS, 25 units of either 3'-nucleotidase or P1-nuclease were added to stage I material, and the mixture incubated for 30 min in order to convert PAPS to APS. The reaction was terminated by placing the reaction vessel in a boiling water bath, and the precipitate was once again removed by centrifugation (stage III). Pure PAPS and APS could be obtained by HPLC with RP-18 column with pump A (0.1 M KH_2PO_4 and 10 mM TBHS at pH 5.8, 0.9 ml/min) and pump B (acetonitrile, 0.1 ml/min) (Lin and Yang, 1998).

Results and Discussion

Molecular cloning, expression, and purification of human PAPS synthetase. The experiment of molecular cloning was shown in **Figure 2**. Briefly, amplified PCR products (*h*PAPSS cDNA) were ligated into expression vector, pET-21b, using T4 DNA ligase at 3:1 molar ratio of insert to vector. The resulting plasmid was verified for the presence of the *h*PAPSS by DNA sequencing, and then transformed into *E coli* strain. The expressed protein had hexa-His in C-terminal. So we purified *h*PAPSS to homogeneity from overproducing strain of *E coli* BL21 (DE3), which encoded *h*PAPSS, through Ni^{2+}-chelating column and then Hitrap Q column. The isolated enzyme analyzed by SDS-PAGE existed ~ 70 kDa shown in **Figure 3**.

K65ER68G as catalyst for the depletion of PAPS. In this proposed coupled-enzyme assay, PST was used to deplete PAPS to PAP with MU as sulfuryl group acceptor. For maintaining the coupled-enzyme assay, a suitable ST was required for use in the PAPS measurement step (**Figure 1**, step B) All STs need PAPS as cofactor (Klaassen and Boles, 1997), so any ST-catalyzed reaction could potentially be used for this purpose. However, the sensitivity of previous coupled PAPSS assays was limited (Hazelton *et al.*, 1985; Wong *et al.*, 1990; Wong and Wong, 1994; Vargas, 1988), because ATP, one of the cosubstrates for PAPSS, inhibits all STs (Lin and Yang, 1998; Rens-Domiano and Roth, 1987). Fortunately, previous studies had demonstrated that a PST mutant, K65ER68G, could tolerate the complications mentioned above (Chen *et al.*, 2005; Yang *et al.*, 1996). **Figure 4** describes the sulfonating activity of MU catalyzed by wild-type and K65ER68G PST. It indicated that the maximal sulfonating activity of MU by K65ER68G was almost four times

than that by wild-type PST. In addition, K65ER68G could tolerate substrate inhibition to higher extent than wild-type PST as mentioned above. In physiological reaction catalyzed by K65ER68G with 1 mM ATP and 20 μM MU, still 60-70% activity was observed (shown in **Figure 5**) compared nearly no activity with that of wild-type PST (data not shown) (Xu *et al.*, 2001). In addition, preliminary studies also showed that K65ER68G could efficiently catalyze PAPS for the transfer of sulfuryl group, and PAP and MU did not form ternary complex to inhibit the reaction under the conditions adopted in this report (Chen *et al.*, 2005; Yang *et al.*, 1996).

Selection of excitation and emission wavelengths for the coupled-enzyme assay. A suitable excitation wavelength was determined to prevent the "inner filter effect" (Lackowicz, 1983) caused by the absorption of MUS.. Therefore, PAPSS activity was in effect determined by the decrease of fluorescence of MU at 450 nm upon excitation at 360 nm (Chen *et al.*, 2005). The sensitivity of this method could thus reach nM range since the pmole amount of MU could be reliably determined.

Coupled-enzyme assay. It is expected that the rate of AST, which was in the range of nmole/min/mg (Xu *et al.*, 2001), could be easily monitored using this coupled-enzyme system. It is worth pointing out that the major requirements for this assay were the adequate amount of MU and an excess of K65ER68G activity.

Figure 6 shows the time course of the changes in fluorescence for the coupled *h*PAPSS/K65ER68G reaction and corresponding controls, where specific components of the reaction mixture were omitted. Only for the complete reaction did we observe an decrease in fluorescence, reflecting the enzymatic activity of *h*PAPSS. The fluorescence background was, however, almost constant in the absence of K65ER68G, or MU. Under these conditions, the coupled reaction did not take place. No effects

were observed on the measured rates by raising the amount of K65ER68G (data not shown), which indicated that the reaction rate of K65ER68G is fast enough for the coupling system to reach its maximum activity. Taking together the results shown in **Figure 6**, it was concluded that the continuous changes in fluorescence were specifically attributable to *h*PAPSS activity.

To determine the linear range of PAPSS assay at a given K65ER68G concentration (6 mU), the rate of MU reduction was measured using a concentration range (0.5-8 μg) of *h*PAPSS as shown in **Figure 7**. In addition, this linear range and sensitivity of *h*PAPSS assay could be further extended for other needs

Conclusion

In summary, we have developed a nonradioisotopic coupled-enzyme assay for the determination of PAPSS activity. This is the first report for continuous fluorometric assay for PAPSS. The selection of recombinant PAP-free PST (K65ER68G) for use in the PAPS measurement step represented a critical factor not only to tolerate substrates inhibition by PAP and MU but also inhibition by ATP to higher extent. Not only is this method useful for the routine and detailed kinetic study of this important class of enzymes but also has the potential for the development of a high-throughput procedure using microplate reader. In the future work, this new assay could be modified in other condition with more sensitivity and applied to determine biological samples.

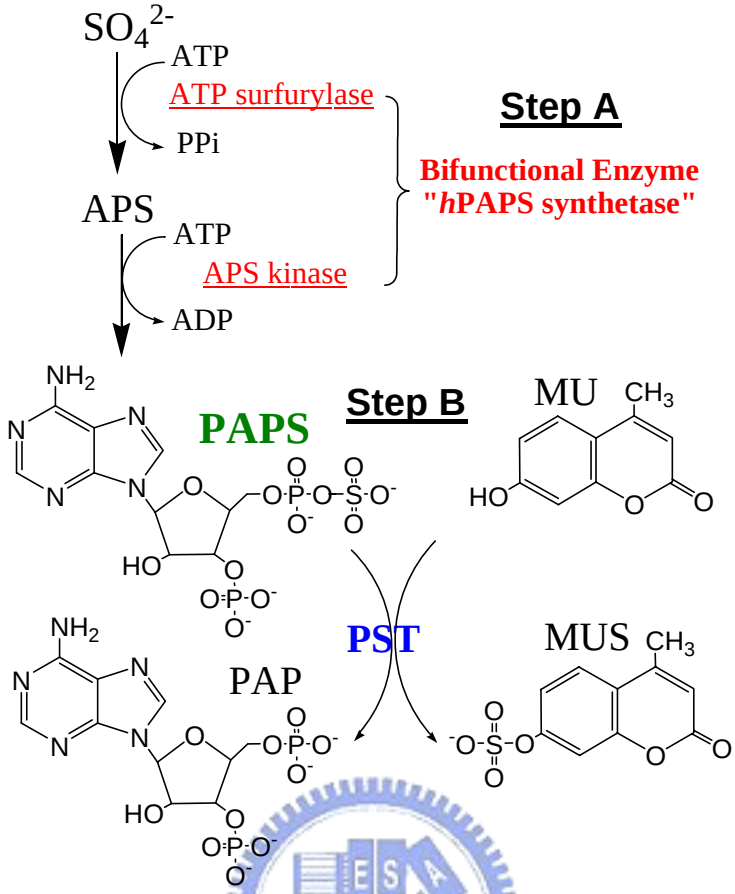

Figure 1. Scheme for the determination of PAPSS activity. The biosynthesis of PAPS from ATP and SO_4^{2-} was catalyzed by *h*PAPS synthetase, which was a bifunctional enzyme with activity of ATP sulfurylase and APS kinase (Step A). This assay was based on the depletion of PAPS to PAP catalyzed by a recombinant PST (K65ER68G) using MU as the sulfuryl group acceptor. In coupled-enzyme assay, PST represented an auxiliary enzyme, and MU was used as a fluorescent indicator of enzyme turnover (Step B).

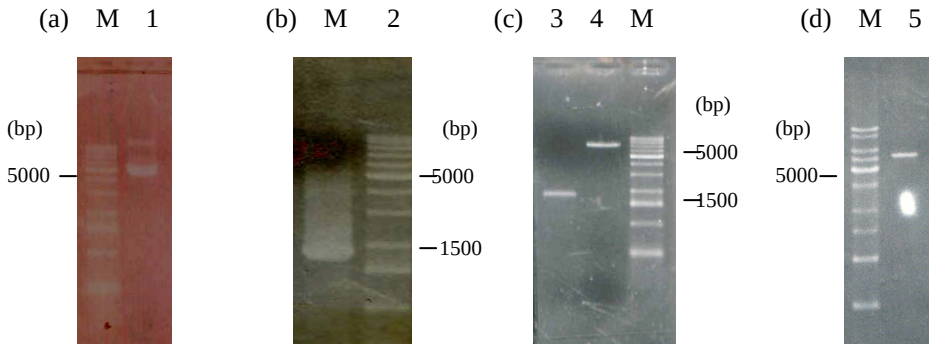

Figure 2. DNA electrophoresis of constructing human PAPS synthetase cDNA .(a) Lane 1 indicated full length human PAPS synthetase (PAPSS) was obtained and cloned into pCMV-SPORT6 vector. (b) PCR amplification of PAPSS cDNA with NdeI and BamHI cutting site using forward and reverse primer was shown in lane 2. (c) PAPSS cDNA and expression vector pET-21b were digested by EcoRI and HindIII restriction enzyme shown in lane 3 and 4. (d) Lane 5 indicated the complete plasmid encoded PAPSS cDNA after ligation by T4 DNA ligase. Lane M represented DNA marker ladder. Detailed procedures were described under *Construction of Full-length of hPAPS Synthetase for Escherichia coli* in *Materials and Methods*

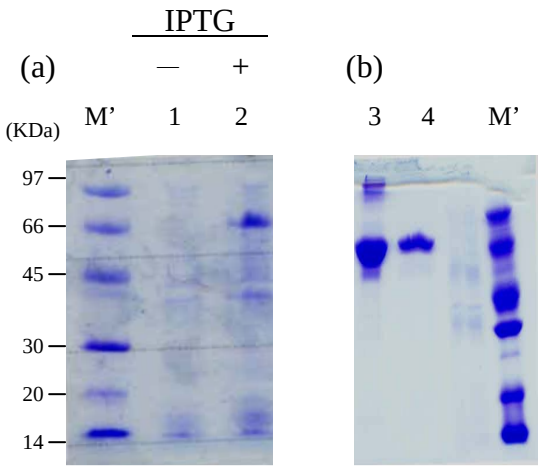

Figure 3. SDS-PAGE for determination of human PAPSS. (a) Ligation of PAPSS cDNA and pET-21b was prepared and transformed into BL21 (DE3) for expression. The extracted materials were analyzed by SDS-PAGE, and only in which had induction with 1 mM IPTG (when O.D. ~ 0.8) was determined the isolated ~70 kDa full length PAPSS (shown in lane 1 and 2). (b) Lane 3 and 4 indicated the expressed proteins were purified by Ni^{2+}-chelating column and then Hitrap Q column, respectively. Lane M’ represented protein marker.

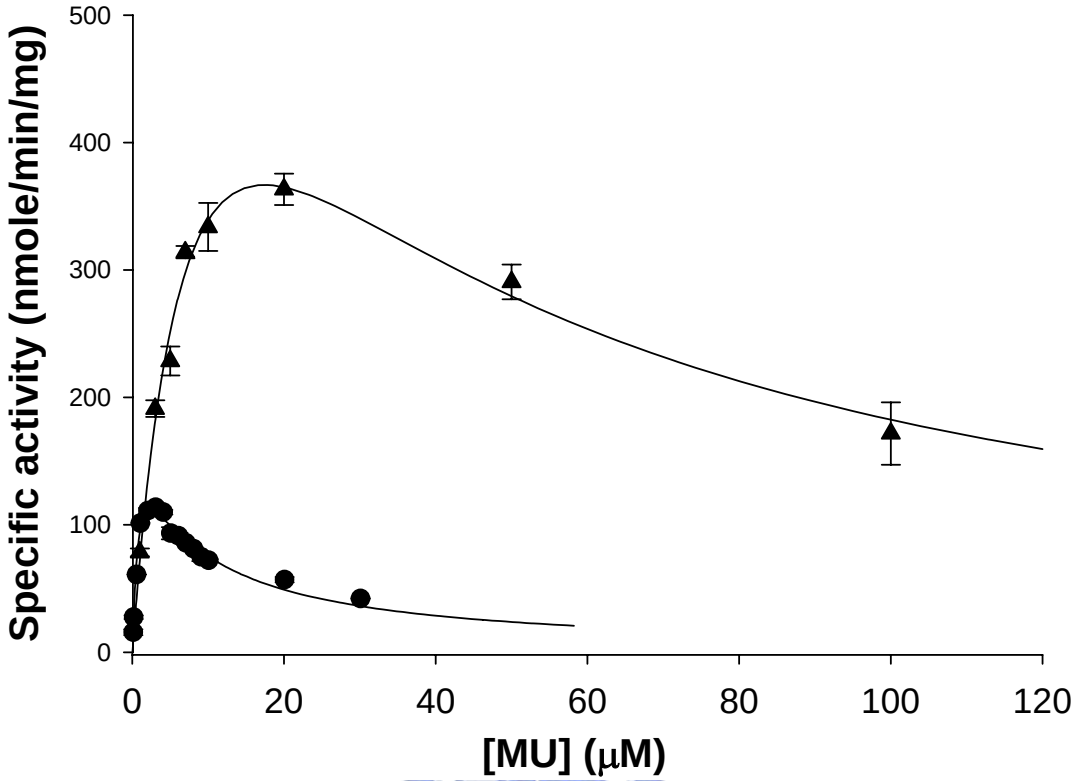

Figure 4. Substrate inhibition of MU on recombinant wild-type and PAP-free (K65ER68G) PSTs. The enzymatic assays were carried out under the condition of *PST assay* at pH 7.0 with about 5 μg recombinant wild-type (●) or PAP-free (▲) PST and different concentrations of MU (0.05 ~ 30.0 μM for wild-type PST and 1 ~ 100.0 μM for PAP-free PST). The kinetic parameters were obtained using the substrate inhibition equation $v = V[S]/\{K_m + [S](1 + [S]/K_{is})\}$by SigmaPlot 2001, V7.0 and Enzyme Kinetics Module, V1.1 (SPSS Inc., Chicago, IL)

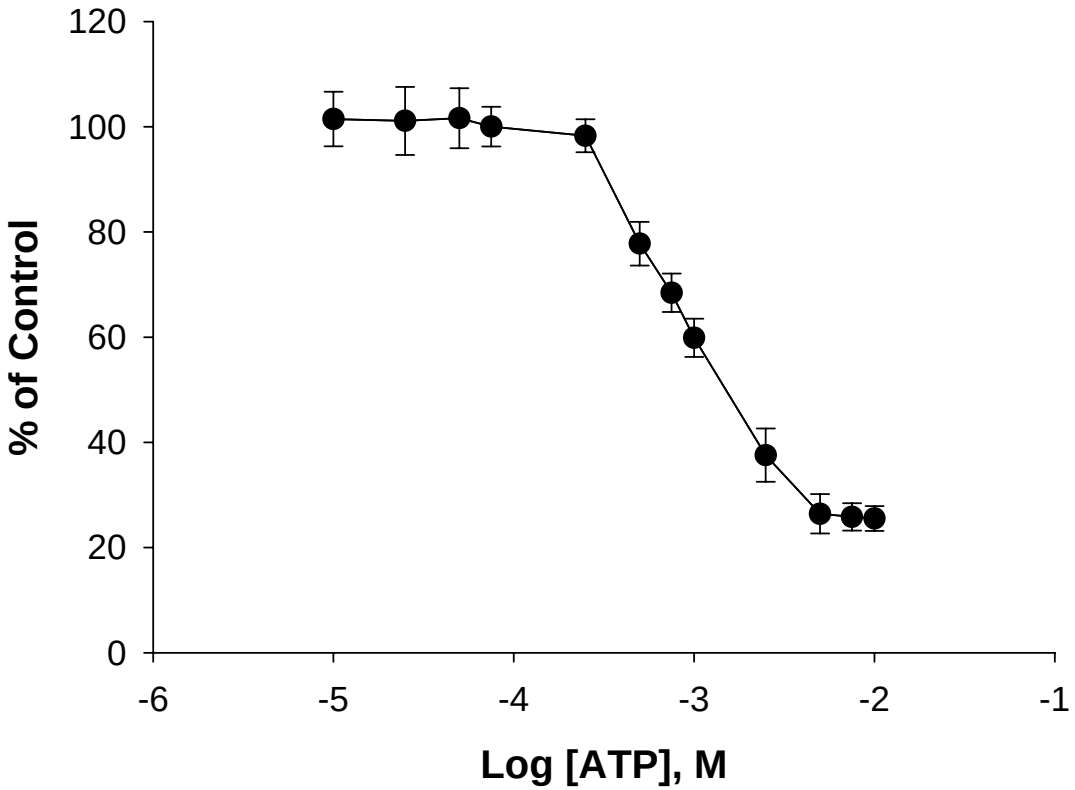

Figure 5. Inhibition of PAP-free PST by ATP. The enzymatic assays were carried out under the standard condition of *PST assay* at pH 7.0 in the absence of ATP and in the presence of a series of concentration of ATP (5 μM ~ 10 mM) using about 5 μg recombinant K65ER68G and 20 μM MU. Each point was determined from triplicate assay data and standard error was obtained by SigmaPlot 2001, V7.0 (SPSS Inc., Chicago, IL).

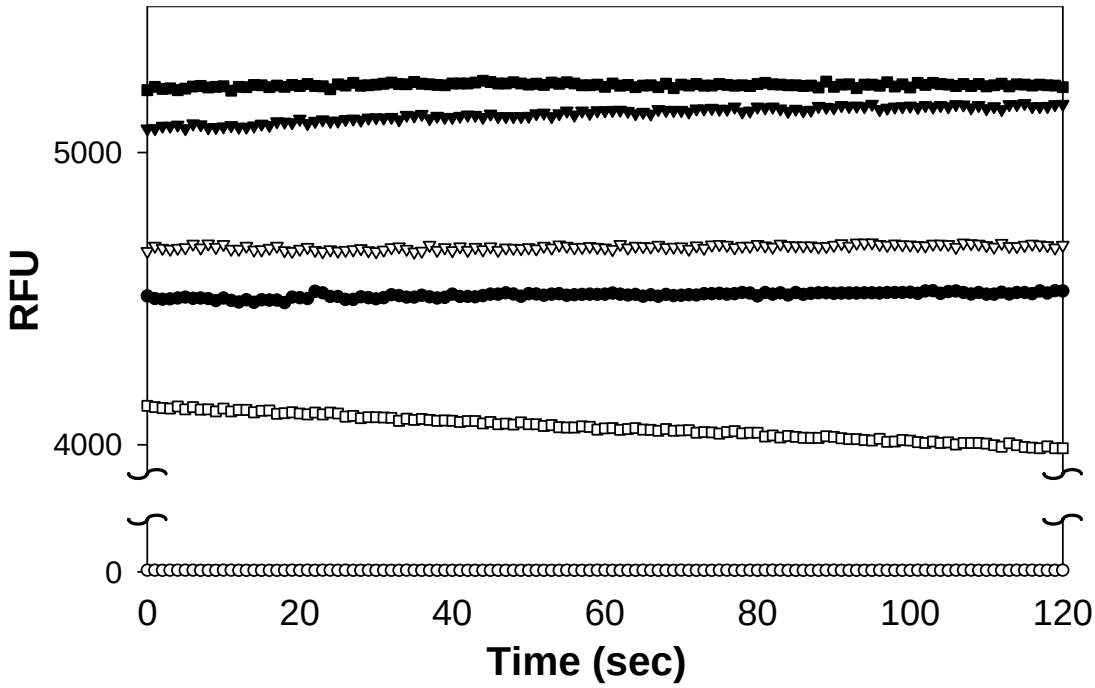

Figure 6. Progress curves of the coupled-enzyme assay for PAPSS. Complete reaction (□) and control reactions without ATP (■), Na_2SO_4 (▼), MU (○), K65ER68G (●), or *human* PAPSS(▽) of coupled-enzyme assay for PAPSS were conducted at pH 7.0, 25°C. Detailed procedures were described under *Coupled-enzyme assay for PAPSS* in *Materials and Methods.*

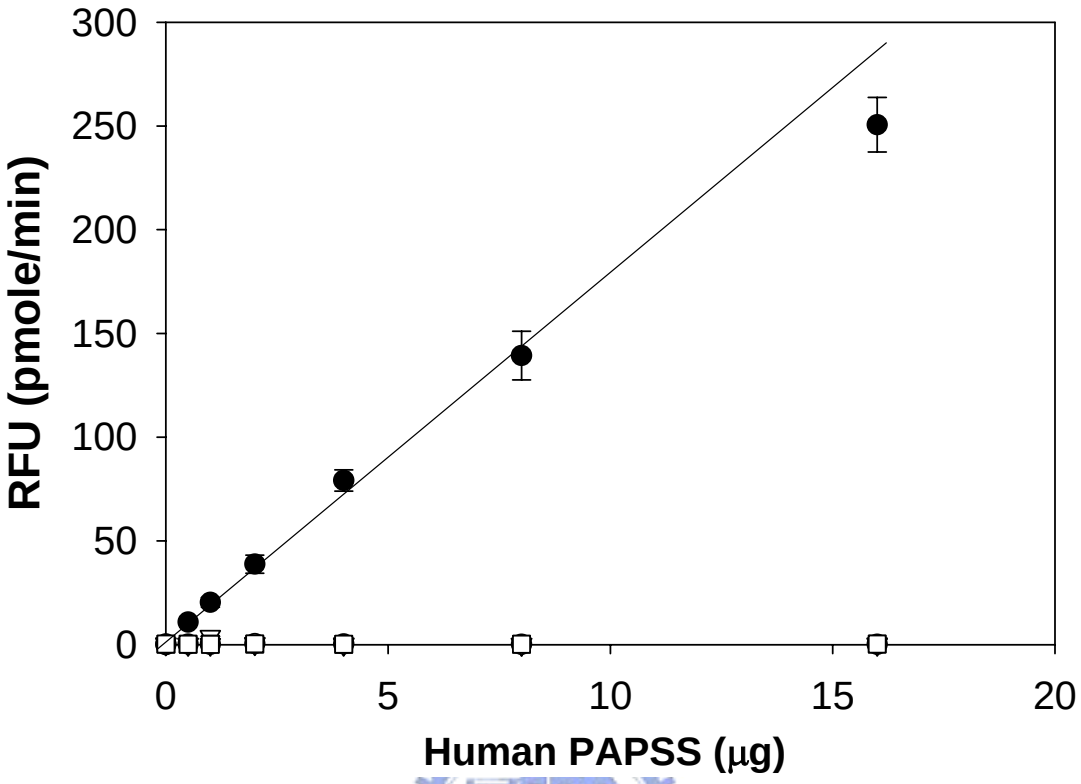

Figure 7. Effective range of fluorometric assay for *h*PAPSS. Complete reaction (●) and control reactions without K65ER68G (○), MU (▼), Na_2SO_4 (▽), PAPSS (■) or ATP (□)of coupled-enzyme assay were run in a total volume of 1000 μl at 25°C. Detailed procedures were described under *Coupled-enzyme assay for PAPSS* in *Materials and Methods.* Each point was determined from triplicate assay data and standard error was obtained by SigmaPlot 2001, V7.0 (SPSS Inc., Chicago, IL).

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Appendix

Materials and Methods

藥品與方法

Materials and Methods

A. Equipments

Equipment	Company
550 Sonic Dismenbrator	Fisher Scientific
FPLC	Pharmacia (Hong Kong)
Column	Amicon or Pharmacia
GSTrap Fast Flow	Pharmacia (Hong Kong)
Hitrap Q column	Pharmacia (Hong Kong)
Hitrap chelating column	Pharmacia (Hong Kong)
Commercial Blendor Incubator	Waring
Orbital Shaker Incubator	DEHG YNG Instruments Co., LTD.
DNA 迷你電泳槽	MUPID-2
Biologic low pressure liquid chromatography system	Bio-Rad Co., LTD.
GelAir Drying System	Bio-Rad Co., LTD.
Mini-Protein II electrophoresis System	Bio-Rad Co., LTD.
Instant camera	Polaroid
U3300 UV/VIS Spectrophotometer	Hitachi
F-4500 Fluorescence Spectrophotometer	Hitachi
Orbital Shaker Incubator	DEHG YNG
Ultrafiltration System	Amicon
桌上型微量離心機 (Eppendrof centrifuge 5410)	Eppendorf
高速離心機 (himac CR 22G)	Hitachi
Laminer Flow	海天科學股份有限公司
PCR Mechaine (Perkin Elmer 2400)	Perkin Elmer
pH meter	Fisher Scientific

B. Bacteria and Plasmids

a. *Escherichia coli* DH5α and BL21 (DE3)

分別用於產生大量質體 (plasmids)和表現蛋白質。

購於食工所菌種中心。

b. 大鼠酚亞硫酸基轉移酵素之野生和突變菌株

由美國 NIH Drs. W. B. Jakoby 和 D. Marshall 贈與。

c. 人類亞硫酸基轉移酵素之野生(1A1, 1A3, and 2A1)和突變菌株

由美國 Dr. Ming Cheh Liu 贈與。

C. Miniprep. of E. coli plasmid DNA

以下抽取大腸桿菌的質體乃使用 VIOGENE DNA/RNA Extraction Kit。

根據 Sambrook *et al.* 的 alkaline lysis 方法抽取質體 DNA。挑單一菌落 *E.coli* DH5α 接種至 5 mL 的 LB 液體培養基 (1% tryptone, 0.5% yeast extract, 0.5% NaCl)，培養基內含 ampicillin (100 μg / mL) 或其他合適的抗生素，於 37˚C 振盪(150 rpm)培養約 12-16 小時。吸取 1.5 mL 菌液至微量離心管中，於 4˚C 以 12,000 rpm 離心 30 秒，將菌體離下。移除上清液後，加入 250 μL 冰的 MX I (含有 50 μg/mL RNase A)，劇烈振盪，使菌體完全懸浮。隨後加入 250 μL 的 MX II ，輕輕的將微量離心管搖動數下，使溶液充份混和均勻後呈現黏稠狀，置於冰上 1-5 分鐘。再加入 350 μL MX III 中和溶解物，反覆搖動，使溶液充份混和均勻後產生白色沉澱，隨後以 12,000 rpm 離心 10 分鐘，小心吸取上清液至

mini-M column 後離心 1 分鐘。再依序用 500 µL WF buffer 和 700 µL WS buffer 清洗 mini-M column，最後再用 50 µL 二次去離子水沖提出 DNA。所得的溶液 (約 45 µL)放入離心真空乾燥機中，乾燥 15 分鐘。乾燥的沉澱物以 20 µL 的二次去離子水重新溶解，然後取 2 µL 以 1 %洋菜膠體作電泳分析。

D. DNA Agarose

取 1 克 agarose (Ultra Violent)加入 100 mL 1X TAE (40 mM Tris-Base, 40 mM glycial acetic acid, and 1 mM EDTA)電泳緩衝液。利用微波爐加熱至融化，置於室溫下約 50- 60ºC，在倒入插有樣品梳片(comb)的膠體型成模(gel-forming mold)中，待約 30-45 分鐘後完全凝固，小心將樣品梳片取出即可使用。將製備好的膠體放入電泳槽中，加入適量 1X TAE 電泳緩衝亦使液面略高於膠體。混合 5 μL DNA 與 1 μL 1X loading dye，再將混合液注入膠體之孔洞(well)，於另一孔洞加入標準分子量 DNA 混合物。蓋上安全蓋，以 100 伏特進行電泳約 30 分鐘。關掉電源，取出膠體，置於溴化乙基碇(EtBr, 0.5 μg / mL)溶液中染色約 1 分鐘，再將膠體取出，置於清水中退染約 5 分鐘。取出膠體，置於紫外光下觀察 DNA 與照相。

E. Gel band Purification

以下洋菜膠內質體的純化乃使用 GFX^{TM} PCR DNA and Gel Band Purification Kit

(from Pharmacia, Hong Kong)

經由 1%洋菜膠電泳分析後，在紫外光下觀察 DNA 並切割下 DNA 與洋菜膠混合物，放入 1.5 mL eppendorf 中，隨後加入等重的 capture buffer，放入 dry bath 中以 57 度溶解混合物約 10 分鐘。溶解後的溶液立即加入 mini-M column 中，靜置 1 分鐘後離心 30-60 秒，隨後用 500 μL Wash Buffer 清洗，最後再用 50 μL 二次去離子水沖提出 DNA。所得的溶液(約 45 μL)放入離心真空乾燥機中，乾燥 15 分鐘。乾燥的沉澱物以 20 μL 的二次去離子水重新溶解，然後取 2 μL 以 1 %洋菜膠體作電泳分析純化結果。

F. Transformation

依據 Sambrook *et al.* 之方法，將 *E.coli* DH5α 接種於 LB 平板培養基（其成分除含有 1 % tryptone, 0.5 % yeast extract, 0.5 % NaCl，但內含 2 %的洋菜膠）上，在 37 ºC 培養 16-20 小時。取單一菌落接種入 2 mL LB 液體培養基內，於 37 ºC 振盪隔夜培養。再取其中 100 μL 至 5 mL LB 培養液中生長至 OD_{600}~0.5，以 5000xg 在 4 ºC 下離心 10 分鐘。收集菌體加入 2.5 mL 之 100 mM $CaCl_2$，置於冰上 1 小時後離心，倒掉上清液後，重複上述步驟一次。菌體加入 250 μL 之 100 mM $CaCl_2$ (含 10 % glycerol)，使完全懸浮後，分裝於微量離心管，保存於-20 ºC 下。此即為勝任細胞(competent cell)。

於 1.5 mL 微量離心管內加入 50 μL 勝任細胞即 5 μL 質體或經限制酵素 Dpn I 反應後之混合液，混合均勻後置於冰上 30 分鐘。冰浴後，將離心管由冰浴取出，立即放入 42 ºC 加熱板中進行熱休克(heat shock)處理兩分鐘，隨後立刻取出離心管再放回冰浴 2 分鐘中。加入 500 μL LB 培養液，於 37 ºC 反應 1 小時

使菌體恢復。取 500 μL 菌液塗佈於含有抗生素(如 100 μg /mL)的 LB 平板培養基上，塗佈均勻後的平板置於室溫，直到菌液完全平板吸收後，再於 37 ℃ 培養約 16~18 小時。待菌落長出後，即可由轉型菌株抽取質體，以鑑定質體中是否帶有選殖的 DNA 片段。另外，藉由加入已知的質體量，可求出勝任細胞的轉殖效率(transformation efficiency)。

G. Protein Expression

a. Recombinant rat phenol sulfotransferase

E.coli BL21(encoded pET3c-11/PST)在 1 mL LB/Amp 培養液中，於 37 ℃、150 rpm 做隔夜培養後，取 2 %體積之菌液放大至 5 mL LB/Amp 培養液。待菌體 OD_{600} 大約 0.8 時，再取 2 %體積之菌液放大至 500 mL LB/Amp 培養液中。於不同時間取出 1 mL 作 OD_{600} 吸光值測量以觀察其生長曲線。另外，於不同時間取出 10 mL 菌體離心後，溶於 1 mL 的純化用緩衝液並置於冰上，以超音波震盪打破，週期為震盪 2 秒然後靜置 1 秒，打 10 次為一循環，共打 6-10 循環。離心後分為細胞殘體及細胞質溶液，個別取出與全細胞比較，在 SDS-PAGE 電泳的分析結果以觀察蛋白質表現。

b. Recombiant human dehydroepiandrosterone sulfotransferase

E.coli BL21(encoded pGEX-2TK/*h*DHEA-ST)在 1 mL LB/Amp 培養液中，於 37 ℃、150 rpm 做隔夜培養後，取 2 %體積之菌液放大至 5 mL LB/Amp 培養液。待菌體 OD_{600} 大約 0.8 時，再取 2 %體積之菌液放大至 500 mL LB/Amp 培養液中。待菌體 OD_{600} 大約 0.6 時加入 1 mM IPTG，於 25℃、150rpm 下開始表現蛋白質。於不同時間取出 1 mL 作 OD_{600} 吸光值測量以觀察其生長曲線。另外，於不同時間取出 10 mL 菌體離心後，溶於 1 mL 的純化用緩衝液並置於冰上，以超音波震盪打破，週期為震盪 2 秒然後靜置 1 秒，打 10 次為一循環，

共打 6-10 循環。離心後分為細胞殘體及細胞質溶液，個別取出與全細胞比較，在 SDS-PAGE 電泳的分析結果以觀察蛋白質表現。

c. Recombiant human PAPS synthetase

E.coli BL21(encoded pET-21b/*hPAPSS*)在 1 mL LB/Amp 培養液中，於 37 ºC、150 rpm 做隔夜培養後，取 2 %體積之菌液放大至 5 mL LB/Amp 培養液。待菌體 OD_{600} 大約 0.8 時，再取 2 %體積之菌液放大至 500 mL LB/Amp 培養液中。待菌體 OD_{600} 大約 0.6 時加入 1 mM IPTG，於 25℃、150rpm 下開始表現蛋白質。於不同時間取出 1 mL 作 OD_{600} 吸光值測量以觀察其生長曲線。另外，於不同時間取出 10 mL 菌體離心後，溶於 1 mL 的純化用緩衝液並置於冰上，以超音波震盪打破，週期為震盪 2 秒然後靜置 1 秒，打 10 次為一循環，共打 6-10 循環。離心後分為細胞殘體及細胞質溶液，個別取出與全細胞比較，在 SDS-PAGE 電泳的分析結果以觀察蛋白質表現。

H. 聚丙醯胺凝膠電泳分析 (SDS-PAGE)

蛋白質的表現量與純化後蛋白質純度以及其單體 (monomer)分子量 (Mr)之估計，都可由 SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis)分析(HOEFER SE250 Mighty Small II)，根據 Laemmli (1970)的方法製備單體濃度(concentration of monomers)為 10 %的分析膠(Resolving gel)與堆積膠(Stacking gel)之電泳膠片，並以 Coomassie Brilliant Blue R-250 染色。蛋白質染色帶可與範圍從 14.4 kDa 到 97 kDa 的標準蛋白(standard marker)作比較，估計其分子量。

H. Protein Purification

a. 菌體培養

挑選單一菌落於 1 mL 之 LB / Amp 液態培養基在 37 ºC、150 rpm 做隔夜培養後，取 2 %體積之菌液放大至 5 mL LB/Amp 培養液。待菌體 OD_{600} 大約 0.8 時，再取 2 %體積之菌液放大至 300 mL LB/Amp 培養液中，在菌體 OD_{600}~5.0 時收下菌體，以 9000xg、4 ºC 下離心 30 分鐘，將菌體保存於-80ºC。但是表現 human PAPSS 的菌體不可培養過久(約 12 小時即可)，會有 inclusion bodies 的產生。

b. 粗酵素液的製備

收集的菌體懸浮於純化用緩衝液，以超音波震盪打破，週期為震盪 2 秒然後靜置 1 秒，打 15 次為一循環，共打 6-10 循環，然後再 4 ºC 下以 17000xg 離心 20 分鐘 ，回收上清液，共重複 4 次。收集以上 4 次的上清液以同樣條件再離心一次，回收之上清液即為粗酵素液。

c. 酵素純化

I. *Recombinant rat phenol sulfotransferase*

本實驗所用的分離管柱是 DEAE 陰離子交換樹脂(Pharmacia, Hong Kong)，我所選擇的是直徑 2.5 cm ，膠體高度約 30 cm 由公式 $V=\pi R^2 H$ 可得知膠體體積約 147 cm^3 。膠體在裝填前先使用超音波震盪機來去除空氣，裝填後用緩衝液 A (10mM Tris-Base at pH 7.4、125mM Sucrose、10% Glycerol、1mM PMSF(2-propanol)、1mM EDTA、1mM Dithiothreitol)平衡膠體。純化的原理主要是利用兩種溶液混合比例產生鹽梯度差，使吸附在膠體上的酵素溶離出。將粗酵素液注入分離管柱中，使用 5 倍體積的緩衝液 A 沖洗管柱，隨後再與緩衝液 B(除緩衝液 A 的內容物外，仍含有 0.3 M NaCl)產生鹽梯度。

b. Recombiant human dehydroepiandrosterone sulfotransferase

本實驗所用的分離管柱是 GSTrap Fast Flow(Pharmacia, Hong Kong)。以下是純化的步驟(modified from affinity handbook of pharmacia):

1. Fill the pump tubing or syringe with Lysis buffer (10 mM Tris-HCl at pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 125 mM sucrose, and 10% glycerol). Connect the column to pump tubing “drop to drop” to avoid introducing air into the column.
2. Remove the twist-off end. Equilibrate the column with 5 column volumes of binding buffer.
3. Apply the sample by pumping it onto the column. For best results, use a flow rate of 1-5 ml/min (5 ml column) during sample application.
4. Wash with 5-10 column volumes of binding buffer or until no material appears in the effluent.
5. Prepare the thrombin mix: GSTrap FF 5 ml column (40mg GST fusion protein bound): Mix 400 µl thrombin solution with 4.6 ml Lysis buffer.
6. Load the thrombin solution onto the column using a syringe and the adaptor supplied. Seal the column with top cap and the domed nut supplied.
7. Incubate the column at room temperature (+22 to +25 ℃) for 2-16 hours.
8. Avoid introducing air into the column. Elute the column and collect the eluate (0.5 ml-1 ml/tube). The eluate will contain the protein of interest and thrombin, while the GST moiety of the fusion protein will bound to GSTrap FF.

c. Recombiant human PAPS synthetase

本實驗所用的分離管柱是 HiTrap Chelating HP (Pharmacia, Hong Kong)。以下是純化的步驟(modified from affinity handbook of pharmacia):

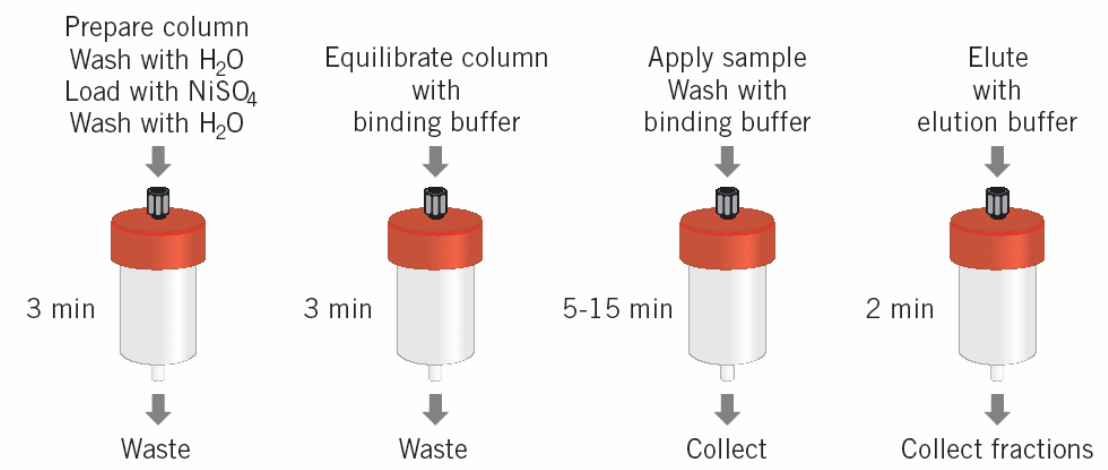

Figure. HiTrap Chelating HP and a schematic overview of poly (His) fusion protein purification.

1. This avoids the risk of nickel salt precipitation in the next step. If air is trapped in the column, wash the column with distilled water until the air disappears.
2. Load 0.5 column volumes of the 0.1 M nickel solution onto the column.
3. Wash with 5 column volumes of distilled water.
4. Equilibrate the column with 10 column volumes of binding buffer.
5. Apply sample at a flow rate 5 ml/min. Collect the flow-throughfraction. A pump is more suitable for application of sample volumes greater than 15 ml.
6. Wash with 10 column volumes of binding buffer. Collect wash fraction.
7. Elute with 5 column volumes of elution buffer. Collect eluted fractions in small fractions such as 1 ml to avoid dilution of the eluate.
8. Wash with 10 column volumes of binding buffer. The column is now ready for a new purification and there is rarely a need to reload with metal if the same $(His)_6$ fusion protein is to be purified.

I. 蛋白質的濃縮

利用 Amicon (Amicon, Inc. U.S.A.)之透析膜濃縮器進行蛋白質樣品之濃縮。使用的透析膜(ultrafiltration membranes)可以過濾掉分子量小於 10 kDa 的蛋白質。將欲濃縮的蛋白質水溶液至入 Amicon 中，並通入氮氣，調節壓力閥約至 4 kg / cm^2，利用氮氣的高壓使蛋白質通過透析膜，而分子量大於 10 kDa 者則留存在 Amicon 中，如此即可達到濃縮的效果。以上蛋白質濃縮操作應於 4 ℃ 下進行。

J. 蛋白質濃度測定

根據 Gill 等人於 1989 年發表的文章所述的方法，利用已知的胺基酸序列中的 Trytophan、Tyrosine 及 Cysteine 的數量，計算出蛋白質在波長 280 nm 時分子消滅常數(molar extinction coefficient，ε)，以此常數換算蛋白質濃度。將紫外光/可見光光譜儀波長設定 280 nm。用二次水將酵素溶液作適當稀釋，以二次水當 blank，測量酵素溶液吸收值，由消滅常數換算酵素濃度。

K. p-nitrophenyl sulfate (pNPS) 之純化

秤取約 100 mg 之 pNPS (Fluka)，將其溶解於 95% 酒精中，震盪搖晃使其均勻混合，置於冰浴中約 1 分鐘，而後於 4℃下離心約 5 分鐘，取其沉澱物，並重複此步驟約 3~10 次。將最後得到之沉澱物於真空乾燥機中乾燥約 30 分鐘，而後秤重計算純化回收率；以 2 mL 去離子水將純化後之 pNPS 溶出，並測量其 A280 與 A400 吸收值以計算內含之 pNPS 濃度。

L. 酵素活性分析

a. UV/VIS

標準活性分析是利用酵素進行轉移反應而生成*p*-nitrophenol，並以紫外光 / 可見光(UV/VIS)光譜儀波長設定 400 nm 吸收的變化，再由消滅常數(extinction coefficient，ε = 10.5 × 10 $M^{-1}cm^{-1}$)求得酵素活性。將紫外光 / 可見光光譜儀波長設定 400 nm，反應時間為兩分鐘。在 1 mL 石英管中依序加入 100 mM / pH 7.0 Bis-tris propane、5 mM 2-Mercaptoethanol、2 μM PAP、1 mM *p*NPS、50 μM β-naphthol (in 50% acetone)及適量酵素，迅速混合均勻，並觀察吸收度的變化，如此可以測得α-form 與β-form 酵素的活性。重複上述步驟，但是試劑中不含 PAP，如此可測得α-form 酵素的活性。

b. Fluorescence

標準活性分析是利用酵素進行轉移反應而生成 methylumbelliferone，並以螢光(Fluorescence)光譜儀波長設定以 360 nm 為激發光和 450 nm 放射光的變化，再由消光常數(extinction coefficient，ε = 0.48 $nM^{-1}cm^{-1}$ at pH 7.0 (37℃))求得酵素活性(1 U=1 unit=1 μmole / min)。將螢光光譜儀波長設定 400 nm，反應時間為兩分鐘。在 1 mL 石英管中依序加入 100 mM / pH 7.0 phosphate buffer、5 mM 2-Mercaptoethanol、20 μM PAP、4 mM MUS、50 μM β-naphthol (in 50% acetone)及適量酵素，迅速混合均勻，並觀察吸收度的變化，如此可以測得α-form 與β-form 酵素的活性。重複上述步驟，但是試劑中不含 PAP，如此可測得α-form 酵素的活性。

M. K_m 與 V_{max} 值的測定

本實驗以不同濃度的受質與酵素進行轉移反應或偶合酵素對反應，將求得的酵素活性與受質濃度作圖，並利用電腦軟體求得 K_m 與 V_{max} 值。

1. 計算蛋白質濃度之公式

Rat phenol sulfotransferase (*r*PST)

ε = (5690×7＋1280×14＋120×5) $M^{-1}cm^{-1}$ = 58350 $M^{-1}cm^{-1}$

M. W.: 33904

ε = 58350 $M^{-1}cm^{-1}$ ÷ 33904 = 1.7 $(mg/mL)^{-1}cm^{-1}$

Human alcohol sulfotransferase (*h*AST)

ε = (5690×12＋1280×9＋120×3) $M^{-1}cm^{-1}$ = 80160 $M^{-1}cm^{-1}$

M. W.: 33779

ε = 80160 $M^{-1}cm^{-1}$ ÷ 33779 = 2.3 $(mg/mL)^{-1}cm^{-1}$

Human PAPS synthetase (*h*PAPSS)

ε = (5690×11＋1280×22＋120×14) $M^{-1}cm^{-1}$ = 92430 $M^{-1}cm^{-1}$

M. W.: 70833

ε = 92430 $M^{-1}cm^{-1}$ ÷ 70833 = 1.3 $(mg/mL)^{-1}cm^{-1}$

2. 酵素活性計算公式

酵素活性(μmole/mL) = [A_{400} 吸收值 ÷ 10.5] × [1000 (μL/mL) ÷ 分析體積 (μL)]

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