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蛋白質亞硫酸基轉移酶及 3' 磷酸腺甘酸 5' 磷酸硫酸合成酶之螢光
檢測法

Fluorometric Assay for Protein Sulfotransferase & PAPS Synthetase

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Abstract

Sulfation is a major pathway in the biotransformation of many drugs and other xenobiotic compounds. The sulfotransferase (ST) enzymes that catalyze these reactions use 3'-phosphoadenosine 5'-phosphosulfate (PAPS) as a sulfate donor co-substrate. The synthesis of PAPS from inorganic sulfate and ATP is catalyzed by PAPS synthetase (PAPSS). Protein sulfation has been known for 50 years. Tyrosine O-sulfation is a common post-translational modification for all multicellular organisms. It was recently discovered that there are similar phenomena in serine and threonine. However, the enzyme which catalyzes this transformation and its mechanism are still unknown. In the first part of this thesis, a continuously fluorescent assay for PAPS synthetase was developed for the first time. It was performed by coupling with the physiological reaction of phenol sulfotransferase (PST). In the second part of the thesis, we developed a very effective assay for the sulfotransferase that catalyzes the sulfation of serine and threonine. It utilized PAPS regenerated from 3-phosphoadenosine 5-phosphate (PAP) by a recombinant PST using 4-methylumbelliferyl sulfate (MUS) as the sulfuryl group donor. The change in the fluorescence intensity of 4-methylumbelliferone (MU) corresponded directly to the amount of active protein sulfotransferase.

摘要

在許多藥物以及異體化合物的生物轉化中，亞硫酸化是一個相當重要的反應路徑，亞硫酸轉移酵素(sulfotransferase, ST)利用3' 硫酸腺甘酸 5' 磷酸硫酸(3'-phosphoadenosine 5'-phosphosulfate, PAPS)當作亞硫酸基的來源，進行以上所述反應的催化，而PAPS則是由無機硫以及三磷酸腺甘(ATP)經由3' 硫酸腺甘酸 5' 磷酸硫酸合成酶(3'-phosphoadenosine 5'-phosphosulfate synthetase, PAPSS)催化而成。蛋白質亞硫酸化的現象已經被證實了五十多年，酪胺酸亞硫酸化為一種普遍存在於所有的多細胞生物中的後修飾作用。最近幾年，這種蛋白質亞硫酸化的現象也被發現會發生在絲胺酸和蘇胺酸上，然而，催化這個反應的酵素尚未被發現。本論文主要分為兩部份，第一部份主要探討的是 PAPSS螢光檢測法，此活性檢測法的操作結合了酚亞硫酸轉移酵素(phenol sulfotransferase, PST)的生理活性，為一靈敏度極高的活性檢測法。第二部份則是探討蛋白質亞硫酸轉移酵素的活性檢測方法，檢測系統的主旨是利用重組的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PAPS，此核苷酸的亞硫酸基再由蛋白質亞硫酸轉移酵素催化，反應後所產生的4-methylumbelliferone (MU)之螢光變化可用來定量蛋白質亞硫酸轉移酵素的活性。

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Abbreviation and Symbol

Abbreviation and Symbol	Full name
PST	Phenol sulfotransferase
AST	Alcohol sulfotransferase
<i>h</i> PAPSS	Human PAPS synthetase
PAP	Adenosine 3',5'-bisphosphate 3'-phosphoadenosine 5'-phosphate
PAPS	Adenosine 3'-phosphate 5'-phosphosulfate 3'-phosphoadenosine 5'-phosphosulfate
ATP	Adenosine 5'-triphosphate
MU	4-methylumbelliferone
MUS	4-methylumbelliferyl sulfate
Tris	tris[hydroxymethyl]aminomethane
EDTA	(Ethylenedinitrilo)tetraacetic acid
DTT	Dithiothreitol
PMSF	Phenylmethylsulfonyl fluoride
MES	2-[<i>N</i> -morpholino]ethanesulfonic acid
SDS	Sodium dodecyl sulfate
TEMED	N, N, N', N'-tetramethylethylene diamine
ϵ	Extinction coefficient
U	Unit : $\mu\text{mole}/\text{min}$
RFU	Relative fluorescence unit
K_m	Michaelis constant
V_{max}	Maximum velocity

Introduction

Sulfation biological system has been recognized for over a century. In 1876, Baumann first described sulfate conjugation as a pathway in biotransformation (Williams, 1959). These reactions are catalyzed by a large family of enzymes, sulfotransferase (STs). Through many years of the development, scientists have determined definitely the mechanism of STs, their substrate specificities, regulation, and evolutionary relationships. In 1954, Bettelheim first discovered the sulfation in a peptide (fibrinopeptide B) derived from fibrinogen (Bettelheim *et al.*, 1954). Since then, protein sulfation in tyrosine residue has emerged as a widespread post-translational modification in multicellular eukaryotes (Huttner, 1982). In the mid-1980s, a series of tyrosine-sulfated proteins was discovered, which includes diverse molecules such as α -choriogonadotrophin and heparin cofactor II (Kehoe and Bertozzi, 2000; Huttner, 1998; Niehrs *et al.*, 1994). In the past few years, tyrosine sulfation has been identified as a key modulator of protein-protein interactions that mediate inflammatory leukocyte adhesion (Wilson and Rider, 1992).

The sulfation reaction of protein is catalyzed by tyrosylprotein sulfotransferase, a membrane-bound enzyme of the trans-Golgi-network (Kehoe and Bertozzi, 2000; Niehrs *et al.*, 1994). Two membrane-bound forms of tyrosylprotein sulfotransferase

were as revealed by phase partitioning in Triton X-114 in Golgi-enriched fractions from bovine adrenal medulla (Xia *et al.*, 2000). In 1998, there was a major advance in the field, The gene of the two tyrosylprotein sulfotransferases, TPST-1 and TPST-2, from human tissue and mouse liver, were cloned(Ouyang *et al.*, 1998; Ouyang *et al.*, 1998; Beisswanger *et al.*, 1998). The human TPSTs share 67% sequence identity. TPST-A, a related protein which was found in *Caenorhabditis elegans*, is 54% identical to human TPST-1 (Ouyang *et al.*, 1998). There was a second gene found in *C. elegans*, it has 39% identity and 62% similarity to TPST-A, but its function is also unknown. Northern analysis indicates that both human TPSTs are expressed in many tissues. However, whether human TPST-1 and TPST-2 are co-expressed in the same cells has not been determined. There might be more than one TPST exist which have not yet been discovered. The additional TPST might explain the diversity of sequences that are tyrosine sulfated ; each of them may act on different amino acid residues of target protein and have a different substrate.

It has been recently discovered that there are similar phenomena occurring in serine and threonine. In 2004, Medzihradszky described this situation for the first time (Medzihradszky *et al.*, 2004). The post-translational modification of serine and threonine sulfations are discovered in four proteins from very different species. This situation suggests that this modification is widespread and may occur ubiquitously in all eukaryotes. The four proteins include a neuronal intermediate filament and a myosin light chain from the invertebrate mollusk *Lymnaea stagnalis*, a cathepsin-C-like enzyme of the unicellular malaria parasite *Plasmodium falciparum*,

and the cytoplasmic domain of the human orphan receptor tyrosine kinase Ror-2. Moreover, the four modified proteins reported by the scientists are targeted to distinct cell compartments, *Lymnaea* intermediate filament locates in cytoplasm, the human orphan receptor tyrosine kinase Ror-2 locates in plasma membrane. The location of *Plasmodium* cathepsin-like enzyme is still unknown. This suggests that sulfation of serine and threonine may be involved in multiple functions including protein assembly and signal transduction and occurs both in the endoplasmic reticulum continuum and the cytoplasm.

To date, there are lots of STs known, and they could be divided into two main groups. One of them is a membrane-bound class which of them is found in the Golgi membrane, this group of STs involved in the modification of protein amino acid residues (tyrosine), proteoglycans and polysaccharides, the other is a soluble cytoplasmic class that modifies small molecules, such as estrogen (Strott, 2002). Among all of the STs, PAPS is the universal sulfate group donor (Robbins and Lippmann, 1956; Moore, 2003; Kehoe and Bertozzi, 2000). Therefore, although we do not know which enzyme(s) might catalyze the sulfation of serine and threonine in proteins yet, one or more of the already known STs are the most likely candidates. However, we cannot figure out whether a different as yet unidentified enzyme is involved, we cannot conceivably figure out a different donor compound, either. Similar considerations apply to any sulfatases, assuming that these modifications are physiologically reversible.

Since this situation has been discovered only recently, the mechanism of this enzyme is not clear yet. In order for the convenience of further study, we decided to develop a continuous assay for protein sulfotransferase. Suitable assays for protein sulfotransferase are essential for investigation into its physiological functions. The most common assays for protein sulfotransferase activities to date are usually conducted by monitoring the transfer of radioisotopic sulfate from [³⁵S]PAPS to specific products (Niers *et al.*, 1990), 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 protein sulfotransferase. Routine spectrophotometric assays have been available only for PST (Ramaswamy and Jakoby, 1987) and AST (Chen *et al.*, 2005). Despite the considerable progress made in recent years on protein sulfotransferase, several fundamental issues concerning the role of regulation in signal transductions 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 these important issues.

Here we proposed a fluorometric assay for protein sulfotransferase (**Figure 10**). 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 protein sulfotransferase. Since we don't have purified protein sulfotransferase, we use rat liver crude extract as the source of protein sulfotransferase, and the product, MU, served as fluorometric indicator to monitor protein sulfotransferase activity.

Sulfation, as described above, is mediated through the universal sulfonyl group donor, 3'-phosphoadenosine 5'-phosphosulfate (PAPS) (Grogory and Robbins, 1960). PAPS plays an important role in offering sulfonyl group for the sulfotransferases that catalyze these reactions (Klaassen and Boles, 1997; Weinshilboum *et al.*, 1994). PAPS is synthesized from two moles of ATP and one mole of SO_4^{2-} . In prokaryotes, this reaction is catalyzed by two separate enzymes, ATP sulfonylase and adenosine 5'-phosphosulfate kinase (Klaassen and Boles, 1997; Farooqui, 1980). ATP sulfonylase catalyzes the synthesis of adenosine phosphosulfate (APS) from ATP and SO_4^{2-} . APS kinase, as its name suggests, phosphorylates APS in the presence of another molecule of ATP to form PAPS. In higher organisms, such as human and mice, these reactions are catalyzed by a single by bifunctional enzyme, PAPS synthetase (PAPSS) (**Figure 1**). There are two different isoforms of PAPSS, *PAPSS1* and *PAPSS2*. They have been identified in both human and mice previously (Girard *et al.*, 1998; Kurima *et al.*, 1998; Li *et al.*, 1995; ul Haque *et al.*, 1998; Venkatachalam *et al.*, 1998).

Despite the considerable progress made in recent years on PAPSS enzymes, rare

evidence 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 [³⁵S]PAPS from free carrier [³⁵S]SO₄²⁻ (Hazelton *et al.*, 1985; Vargas, 1988; Wong and Khoo, 1990) which involve stopping the reactions by heat treatment after 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 PAPS synthetase. Other reported PAPSS assays are also end-point analyses requiring the determination of PAPS using radiochemical coupled-enzyme assay (Xu *et al.*, 2001). In an attempt to begin the process of characterizing PAPSS activity, the objective of the present study was to develop a convenient assay in order to address these important issues.

We report the purification of human PAPSS (hPAPSS) for the biosynthesis of PAPS (**figure 1**). This reaction was coupled to PAP-free PST, and a continuous flourometric assay was developed. In this assay, the deletion of MU to MUS with PAPS as the sulfuryl group donor was catalyzed by PAP-free PST in physiological reaction (**figure 1**). The reactant, MU, served as a flourometric indicator to monitor hPAPSS activity.

Experimental Procedures

Materials.

PAPS, PAP, MUS, MU, 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 (U.S.A.). The P-selectin glycoprotein ligand-1 PSGL-1 peptide (Niers *et al*, 1990) (QATEYEYLDYDFLPEC) and the Ror-2 peptide (QAKLKEISLSAVRFME) were synthesized and purified by Genesis Biotech Inc. (Taiwan). All other chemicals were obtained commercially at the highest purity possible.

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 CaCl₂ 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 A₆₀₀ 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 hours; then cDNA expression was induced with 1 mM isopropyl-thio- β -D-galactoside (IPTG) during additional 12 hours incubation. The cultures were centrifuged at 8000 rpm for 20 minutes, and the pellet was sonicated in IMAC 5 buffer(5mM imidazole, 50mM Tris, pH 7.9) many times. Following removal of cellular debris by ultracentrifugation at 15,000 rpm for 30 minutes, 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 H₂O to remove ethanol from the storage solution, charged with 100mM NiSO₄, and then equilibrated in sonication buffer. 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 minutes with intermittent mixing. Following incubation, the flow-through was collected, and the column was washed with 30mM 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 dithiotreitol, 1mM EDTA, 10% glycerol) overnight for purification with Hitrap Q. Then the Q column was eluted with 0.5 M NaCl gradient (total volumn 500 mL with flow rate at 1mL/min).

Preparation of biological samples for enzyme assay.

The liver extracts were prepared from 20 g of frozen rat liver, which was mixed with a 20 ml buffer (10 mM Tris-HCl at pH 7.4 plus 125 mM sucrose, 10% glycerol, 1 mM DTT, 1.5 mM PMSF, and 1 mM EDTA, 0.1% Triton X-100) and was homogenized by grinding with liquid nitrogen. All further steps were performed at 4°C. Liver were minced, suspended in 30ml of buffer per g of liver and homogenized by homogenizer three times and the homogenate centrifuged (10min, 800 × g). The following steps were described previously (Ouyang *et al.*, 1998).

Preparation of PAP-free phenol sulfotransferase.

The β -form of recombinant phenol sulfotransferase (Yang *et al.*, 1996) was used as the PAP-free enzyme. Recombinant mutant PST, K65ER68G, was cloned into 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).

PST enzyme assay.

The activity of PAP-free PST, K65ER68G, was determined by the change of fluorescence due to the production of MU from MUS by 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 μ M PAP, 100 mM potassium phosphate buffer, and 0.5-2 μ g K65ER68G at

pH 7.0, 25°C. This assay also referred to the 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 condition described above.

Coupled-enzyme assay for hPAPSS.

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 hPAPSS. 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 units of purified ATP surfurylase/APS kinase (PAPSS) in a total volume of 50 μL . The reaction was stopped after thirty minutes by placing the reaction vessel in a boiling water bath for two minutes. The precipitate formed was removed by centrifugation. HPLC analysis showed that the SO_4^{2-} was completely converted to PAPS. In order to facilitate purification of PAPS, 10 units of hexokinase and 5 mM D-glucose (final concentration) were added.

The solution was incubated at 30°C for 30 min to convert ATP into ADP. This reaction was stopped by boiling water, 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 bath of boiling water, 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).

Determination of protein concentration.

The protein concentration of the homogeneous form of K65ER68G was estimated on the basis of absorbency at 280 nm ($1.7 \text{ ml/mg cm}^{-1}$) (Gills and Hippel, 1989) with a UV/Vis spectrophotometer (Hitachi UV/Vis-3300, Japan). Protein concentration of crude extract was estimated by Bradford protein assay kit.

Coupled-enzyme assay for protein sulfotransferase.

Each reaction mixture (1ml) contained 100 mM potassium phosphate buffer at pH 7.0, 5 mM 2-mercaptoethanol, 20 μM PAPS, 4 mM MUS, substrate (Ror2 peptide), 3.2 mU (5.4 μg) K65ER68G. Crude extract was added following the pre-incubation period to start the reaction at 37°C. Because commercial PAPS contained significant amounts of PAP, which is an inhibitor of sulfotransferase (Yang *et al.*, 1996; Duffel and Jakoby, 1998), the assay solution was pre-incubated for 15 min prior to the addition of substrate to ensure that all PAP was transferred to PAPS by K65ER68G

before the start of the complete reaction. The production of MU was monitored by fluorescence as previously described. The change of fluorescence was linear for over 20 min of the initial reaction time.

Sample preparation of mass spectrum.

Before MALSI-TOF MS analysis, we processed the peptides by Ziptip to get a higher purity. The Ziptip must be pre-wetted before we use. This is performed by depressing the pipettor plunger to a dead stop using the maximum volume setting of 10 μ L. Aspirate wetting solution into tip, dispense to waste, repeat this step for several times. After that, equilibrate the tip for binding by washing with the equilibration solution 3 times. Following by these steps after equilibration, binds peptides and proteins to the ZipTip by fully depressing the pipettor plunger to a dead stop. Aspirate and dispense the sample 3 to 7 cycles for simple mixtures and up to 10 cycles for maximum binding of complex mixtures. Wash the tip and dispense to waste using at least five cycles of wash solution. A 5% methanol in 0.1% TFA/water wash improves desalting efficiency. Dispense 1 to 4 μ L of elution solution into a clean vial using a standard pipette tip. Carefully, aspirate and dispense elution through the ZipTip at least three times without introducing air. Sample recovery can be improved (at the expense of concentration) by increasing elution volume to 10 μ L or by performing multiple elutions.

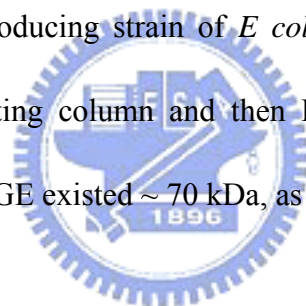
MALDI-TOF MS Analysis.

Peptide mass mapping was performed on a Bruker (Bruker-Daltonics, Bremen, Germany) REFLEX III time-of-flight mass spectrometer equipped with a SCOUT source and delayed extraction. Detection was performed in the positive ion reflector mode with each mass determination being the average of 100 spectra. Samples for mass measurement were prepared using the solution-phase nitrocellulose method (Landry *et al.*, 2000) with α -cyano-4-hydroxycinnamic acid as the matrix. Alternatively, samples were deposited on a Bruker 600 micron AnchorChip according to the manufacturer's instructions. Angiotensin II (1,045.54 amu), adrenocorticotrophic hormone fragment 18–39 (2,465.73 amu), and somatostatin (3,146.47) were used as external calibration standards. The peptide mass spectra obtained on MALDI-TOF analysis were analyzed using PeptideIdent (<http://tw.expasy.org>) and Profound (<http://129.85.19.192/profound/>).

Result and Discussion

Purification of human PAPS synthetase.

The *hPAPSS* cDNA were ligated into expression vector pET-21b using T4 DNA ligase at 3:1 molar ratio of insertion to vector. The resulting plasmid was verified for the presence of the *hPAPSS* by DNA sequencing, then transformed into *E coli* strain. The expressed protein had hexa-His in C-terminal, so we purified *hPAPSS* to homogeneity from an overproducing strain of *E coli* BL21 (DE3), which encoded *hPAPSS*, through Ni²⁺-chelating column and then Hitrap Q column. The isolated enzyme analyzed by SDS-PAGE existed ~ 70 kDa, as shown in **Figure 2**.



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 a sulfuryl group acceptor. For maintaining the coupled-enzyme assay, a suitable ST was required for use in the PAPS measurement step (Chen *et al*, 2005). Since all STs need PAPS as a cofactor (Klaassen and Boles, 1997), it is said that any ST-catalyzed reaction could potentially be used for this purpose. However, the sensitivity of the previous coupled enzyme assays for PAPSS was limited (Hazelton *et al.*, 1985; Wong *et al.*, 1990; Wong and Wong, 1994; Vargas, 1988), because ATP, one of the co-substrates 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 diminish the inhibition of ATP mentioned above (Chen *et al.*, 2005; Xu *et al.*, 2001; Yang *et al.*, 1996). Previous reports indicated that the maximal sulfonating activity of MU by K65ER68G was almost four times that of wild-type PST (Chen *et al.*, 2005). In addition, K65ER68G could tolerate substrate inhibition to higher extent than wild-type PST as mentioned above. In a physiological reaction catalyzed by K65ER68G with 1 mM ATP and 20 μ M MU, 70-80% activity was still observed (shown in **Table 2**) compared to nearly no activity with that of wild-type PST (data not shown) (Xu *et al.*, 2001). This situation could be observed in the reverse physiological reaction (**Table 3**). 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 relative emission coefficient of the fluorescence at 450 nm was determined under different conditions as shown in **Table 1**. 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 (pKa of MU = 7.8 (Sun *et al.*, 1998)). The temperature effect was less significant as shown in **Table 1**.

Coupled-enzyme assay for human PAPS synthetase.

It is expected that the rate of *hPAPSS*, 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 3** shows the time course of the changes in fluorescence for the coupled *hPAPSS*/K65ER68G reaction and corresponding controls, where specific components of the reaction mixture were omitted. Only for the complete reaction did we observe a decrease in fluorescence, reflecting the enzymatic activity of *hPAPSS*. 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. Taken together with the results shown in **Figure 3**, it was concluded that the continuous changes in fluorescence were specifically attributable to *hPAPSS* activity.

To determine the linear range of *hPAPSS* assay at a given K65ER68G concentration (5mU), the rate of MU reduction was measured using a concentration

range (0.5-8 μ g) of *hPAPSS* as shown in **Figure 4**. In addition, this linear range and sensitivity of *hPAPSS* assay could be further extended for other needs.

To test our coupled-enzyme assay and compare the results with the data appeared in the literature, the *hPAPSS* was characterized by this fluorometric assay as shown in **Figures 5 to Figure 9**. These results fit perfectly with previously reported data using radioactive assay procedures (Fuda *et al*, 2002). **Figure 5** shows the pH-dependency of the *hPAPSS* activity. The optimum pH spans from 7 through 9. The enzyme activity at pH 6.0 was approximately 50% of that in the optimum pH range. At pH 10.0, there was no activity be detected. The effects of temperature on the *hPAPSS* activity was examined over 20-55°C as shown in **Figure 6**. Maximum activity was observed at approximately 40°C. The enzyme activity at 20°C and 50°C were approximately 50% of the maximum activity at 40°C. At 55°C or higher, *hPAPSS* became inactive. **Figure 7 to Figure 9** shows the concentration of ATP, sodium sulfate and APS on the *hPAPSS* activity. ATP was determined to yield K_m and V_{max} values of 2.5mM and 24 nmole/min/mg. Sodium sulfate was determined to yield these values of 22mM and 30.3 nmole/min/mg. The K_m and V_{max} values of APS was determined to be 63.3 μ M and 30.9 nmole/min/mg.

Coupled-enzyme assay for serine and threonine protein sulfotransferase .

Figure 11 shows the time course of the changes in fluorescence for the coupled serine and threonine protein sulfotransferase/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 serine and threonine protein sulfotransferase. Some background fluorescence due to the auxiliary reaction was observed. This was probably because of the presence of an impurity of PAP in commercially available PAPS (Duffel and Jakoby, 1981; Yang *et al.*, 1996). The fluorescence in the 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 the PAPS concentration was saturating enough for the coupling system to reach its maximum activity. Taken together with the results shown in **Figure 11**, it was concluded that the continuous changes in fluorescence were specifically attributable to serine and threonine protein sulfotransferase activity.

In the experiment of standard curve (**Figure 12**), we found that the crude extract also has a linear range in the catalyzation of protein sulfation. Besides this, we found that there was a familiar result with the enzyme kinetic (**Figure 13**). This suggested that the crude extract surely has the activity of serine and threonine protein sulfotransferase.

Result of mass spectrum

Figure 14 shows the intensity signal of the peptide QAKLKEISLSAVRFME (Ror-2 peptide) treated with rat liver crude extract. The molecular weight of peptide Ror-2 peptide was 1850.21 Dalton. The intensity of the MS signal represents the

amount of the peptide. After performing the coupled enzyme assay, we have found the MS intensity of Ror-2 peptide to be decreased. It indicates that the amount of the Ror-2 peptide decreased. After sulfation, the Ror-2 peptide would add two sulfuryl groups whose molecular weight was 79.9568 Dalton. Eventually, the molecular weight of peptide Ror-2 peptide becomes 2010.12 Dalton. Since the molecular weight of Ror-2 peptide changed, the signal intensity of Ror-2 peptide decreased. The situation described above could change the intensity signal of the MS data. Fortunately, we found there were apparently decrease at 1850.21 Dalton. This represents that the peptide Ror-2 peptide surely would be used under this coupled-enzyme assay condition.



Conclusion

We have developed a non-radioisotopic coupled-enzyme assay for PAPS synthetase and serine and threonine protein sulfotransferase. This is the first report for continuous fluourometric assay for PAPS synthetase and serine and threonine protein sulfotransferase. In the enzyme-coupled assay for PAPS synthetase, 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 enzyme-coupled assay for serine/threonine protein sulfotransferase, the assay must be modified in the future.

Table 1
The Relative Emission Coefficient of MU at 450 nm^a

		Relative Emission coefficient (cm ⁻¹ nM ⁻¹)						
pH	Temperature (°C)	25	30	35	37	40	45	50
	6.0		—	—	—	0.14	—	—
6.5		—	—	—	0.31	—	—	—
7.0		0.48	0.50	0.52	0.55	0.58	0.60	0.63
7.5		—	—	—	0.73	—	—	—
8.0		—	—	—	1.31	—	—	—
8.5		—	—	—	1.85	—	—	—
9.0		—	—	—	2.35	—	—	—
9.5		—	—	—	3.30	—	—	—
10.0		—	—	—	4.45	—	—	—

^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 2
Inhibition of ATP in Physiological reaction

[ATP](mM)	Activity(pmole/min)	Inhibition rate
0	514.0278	
1	401.0417	22%



Table 3
Inhibition of ATP in reverse physiological reaction

[ATP](mM)	Activity(pmole/min)	Inhibition rate
0	206.6667	
1	147.0347	29%



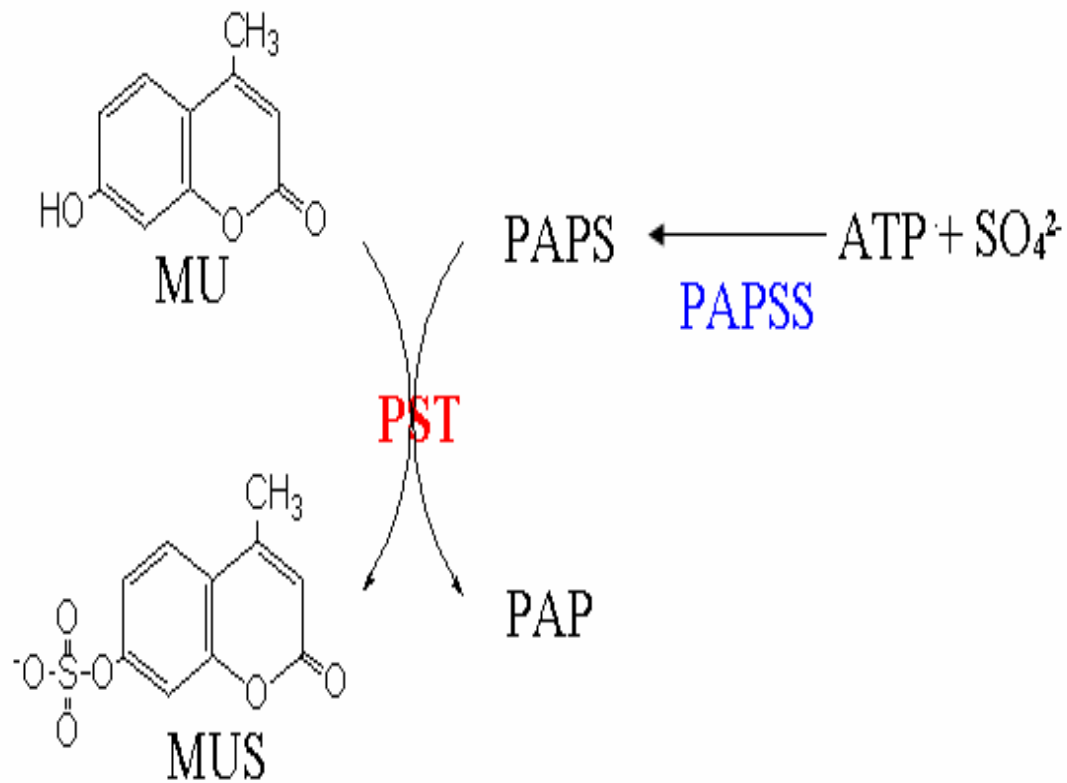


Figure 1. Scheme for the determination of PAPSS activity. Human PAPS synthetase, which was a bifunctional enzyme with ATP sulfurylase and APS kinase, catalyzed the biosynthesis of PAPS from ATP and sodium sulfate. This enzyme coupled assay was based on the depletion of PAPS to PAP catalyzed by a recombinant PST (K65ER68G) which uses MU as the sulfonyl group acceptor. In coupled-enzyme assay, PST represented an auxiliary enzyme, and MU was used as a fluorescent indicator of enzyme turnover.

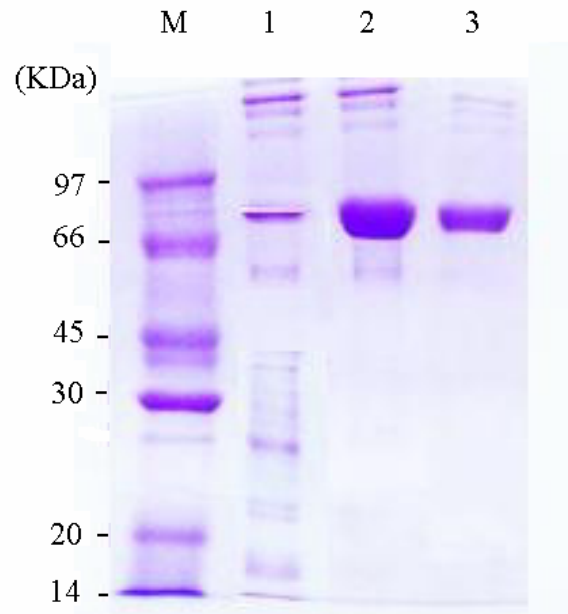


Figure 2. SDS-PAGE for determination of human PAPS synthetase. Lane 1 represented the extract induced by IPTG. Lane 2 and 3 indicated the expressed proteins were purified by Ni²⁺-chelating column and then Hitrap Q column, respectively. Lane M represented protein marker.

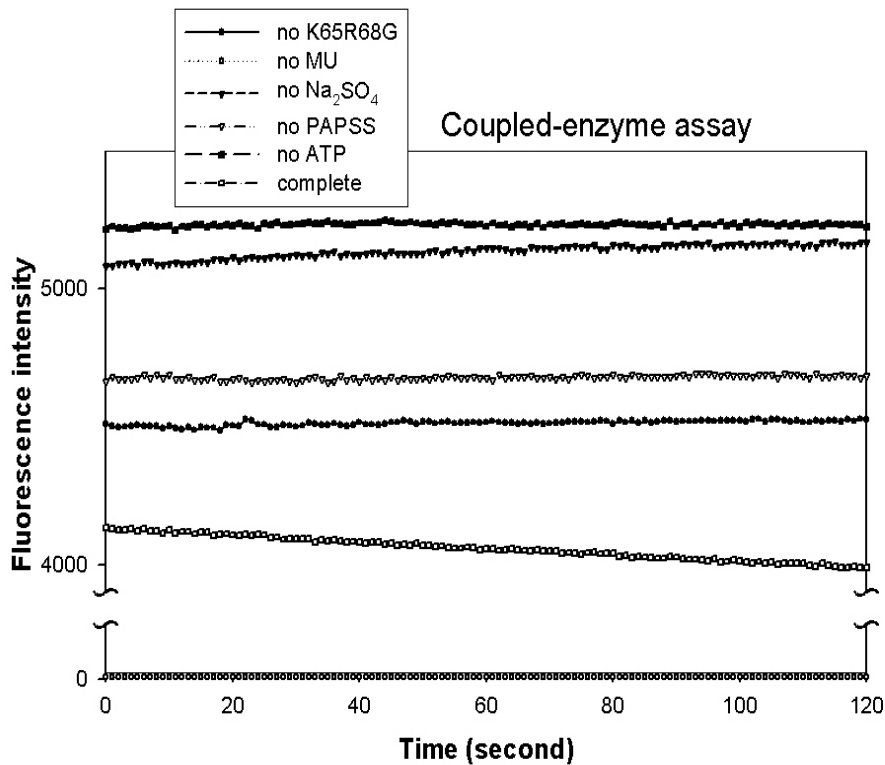


Figure 3. Progress curves of the coupled-enzyme assay for *h*PAPSS. Complete reaction (□) and control reactions without ATP (■), Na₂SO₄ (▼), MU (○), K65ER68G (●), or *human* PAPSS(▽) of coupled-enzyme assay for PAPSS were conducted at pH 7.0, 25°C. Detailed procedures were described in *Materials and Methods*

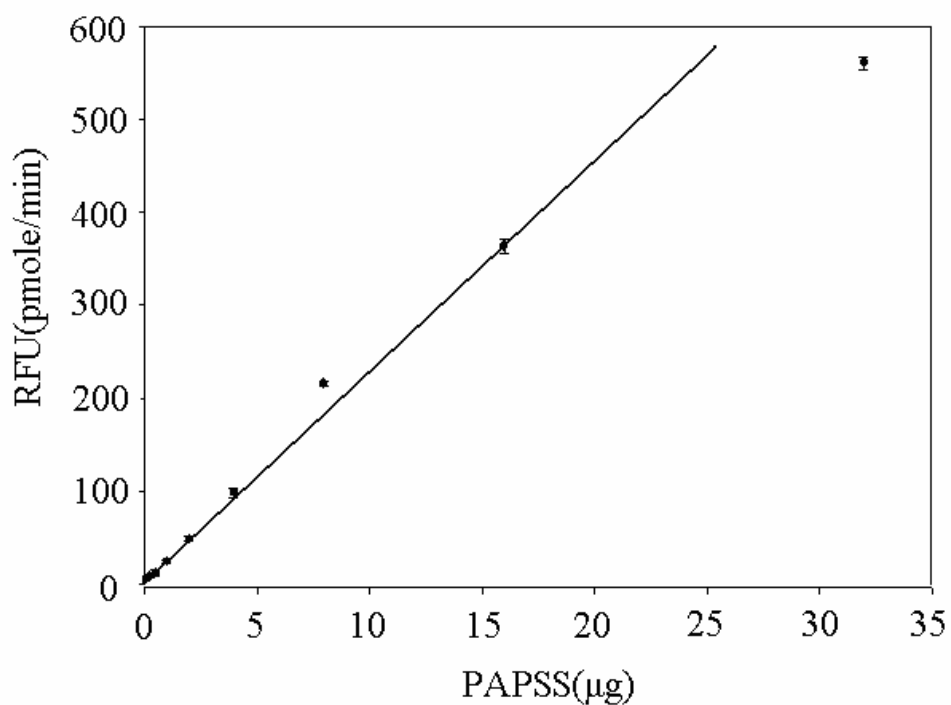


Figure 4. Standard curve of *h*PAPSS at 37°C. Complete reaction of coupled-enzyme assay were run in a total volume of 1000 µl at 37°C. Detailed procedures were described under *Coupled-enzyme assay for PAPSS* in *Materials and Methods*. Each point was determined from triplicate assay data and standard error was obtained by SigmaPlot 2001, V7.0 (SPSS Inc., Chicago, IL).

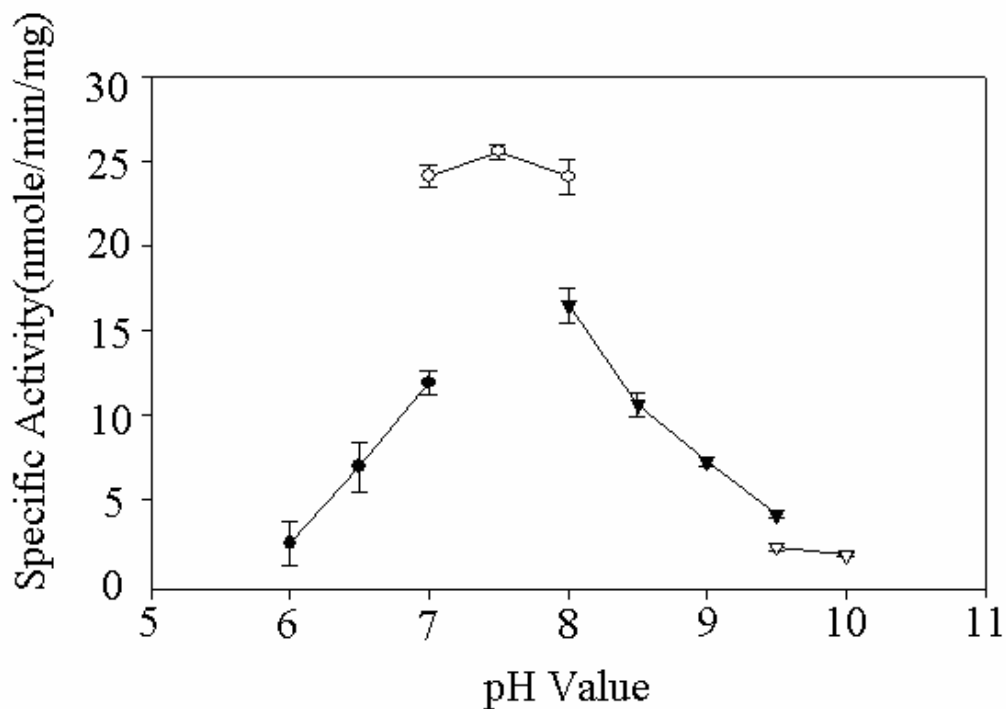


Figure 5. pH profile of recombinant hPAPSS. The enzymatic assays were carried out with about 2 μg recombinant hPAPSS under the condition of *Coupled-enzyme assay for AST* as described in *Materials and Methods* using different buffer systems (100 mM MES at pH 6.0 to 7.0, potassium phosphate at pH 7.0 to 8.0, Tris-base at pH 8.0 to 9.5, and glycine at pH 9.5 to 10.0). Each point was determined from triplicate assay data and standard error was obtained by SigmaPlot 2001, V7.0 (SPSS Inc., Chicago, IL).

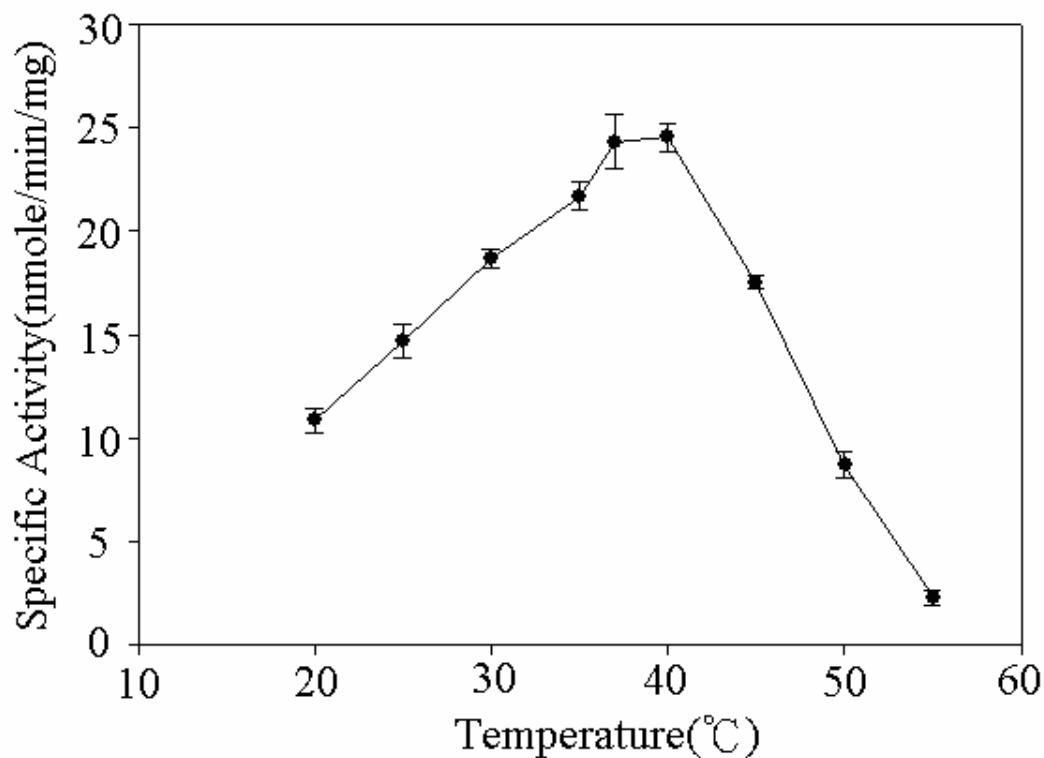


Figure 6. Temperature effect on the activity of recombinant *h*PAPSS. The enzymatic assays were carried out with about 2 μ g recombinant *h*DHEA-ST under the condition of coupled-enzyme assay for *h*PAPSS at pH 7.0 in different temperature. Each point was determined from triplicate assay data and standard error was obtained by SigmaPlot 2001, V7.0 (SPSS Inc., Chicago, IL).

Michaelis-Menten

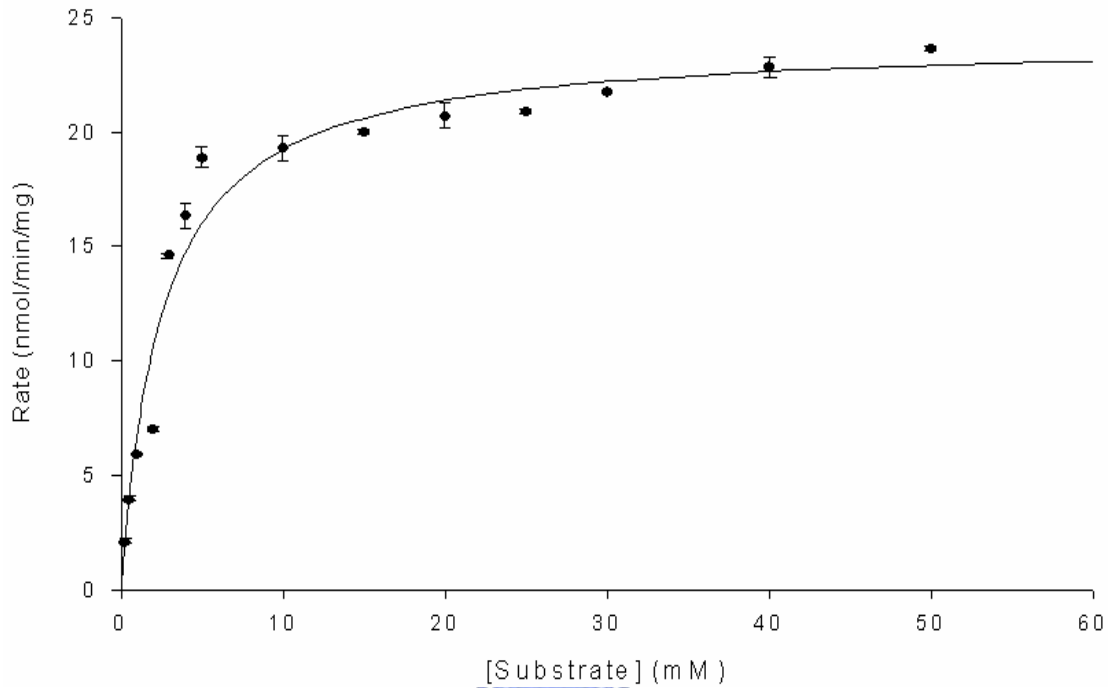


Figure 7. Concentration of ATP on *h*PAPSS activity. The enzymatic assays were carried out with about 2 μ g recombinant *h*PAPSS under the condition of coupled-enzyme assay for *h*PAPSS at pH 7.0 and 37°C in different concentration of ATP. Each point was determined from triplicate assay data and standard error was obtained by SigmaPlot 2001, V7.0 (SPSS Inc., Chicago, IL).

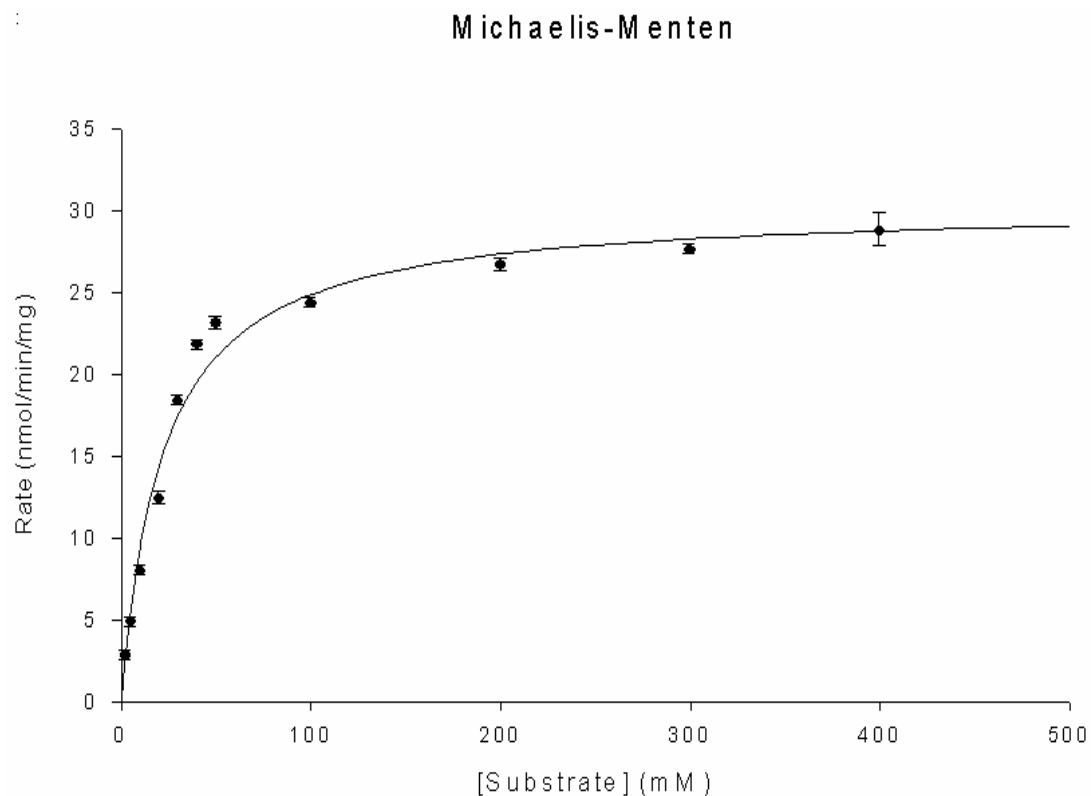


Figure 8. Concentration of sodium sulfate on *h*PAPSS activity. The enzymatic assays were carried out with about 2 μ g recombinant *h*PAPSS under the condition of coupled-enzyme assay for *h*PAPSS at pH 7.0 and 37°C in different concentration of sodium sulfate. Each point was determined from triplicate assay data and standard error was obtained by SigmaPlot 2001, V7.0 (SPSS Inc., Chicago, IL).

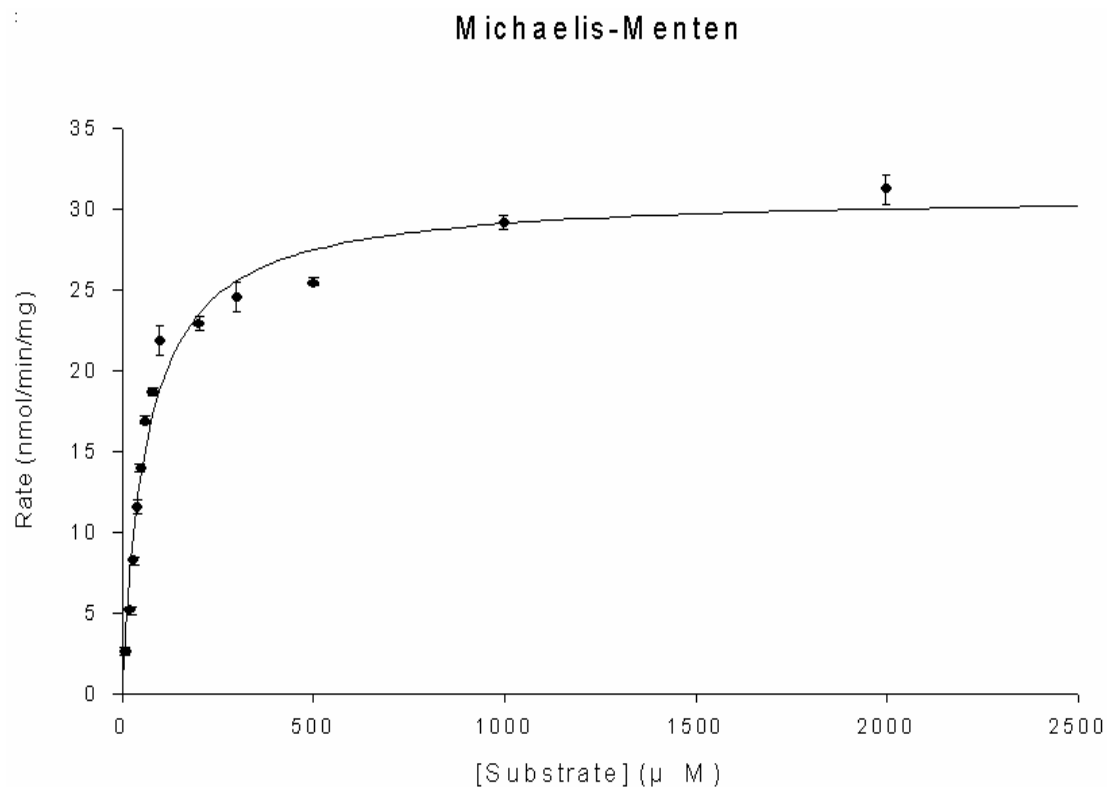


Figure 9. Concentration of APS on *h*PAPSS activity. The enzymatic assays were carried out with about 2 μ g recombinant *h*PAPSS under the condition of coupled-enzyme assay for *h*PAPSS at pH 7.0 and 37°C in different concentration of APS. Each point was determined from triplicate assay data and standard error was obtained by SigmaPlot 2001, V7.0 (SPSS Inc., Chicago, IL).

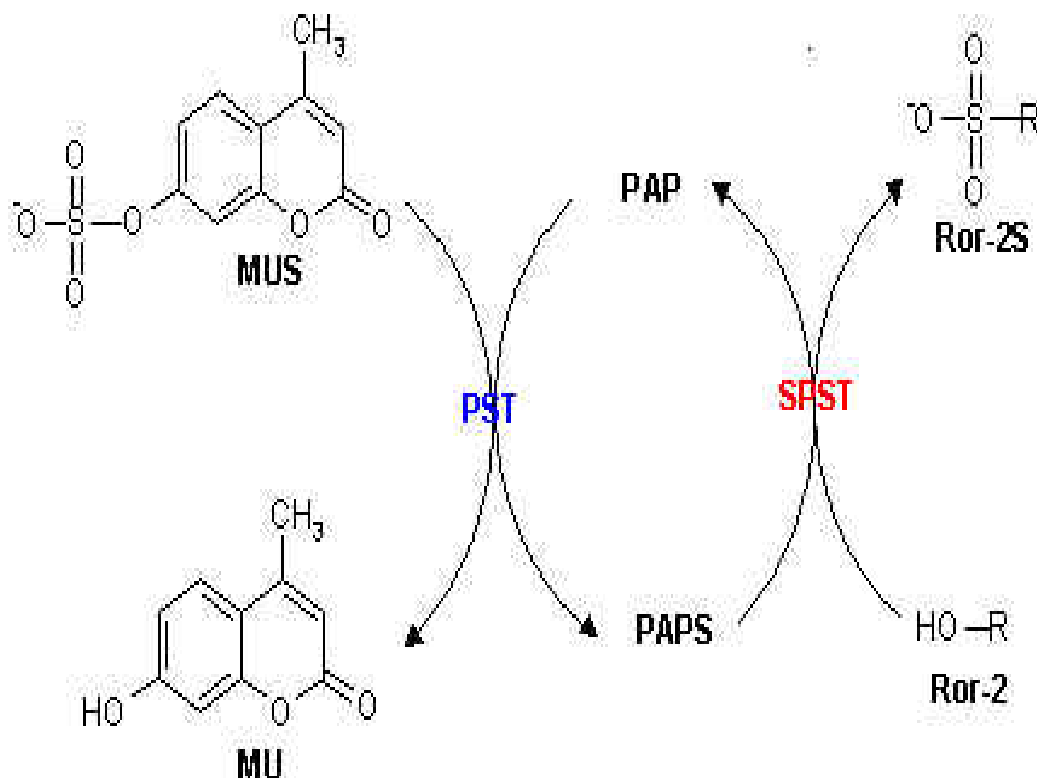


Figure 10. Scheme for the determination of protein sulfotransferase. This assay was based on the regeneration of PAPS from PAP catalyzed by a recombinant PST (K65ER68G) using MUS as the sulfuryl group donor. In coupled-enzyme assay, PST represented an auxiliary enzyme, and the product, MU, was used as 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.

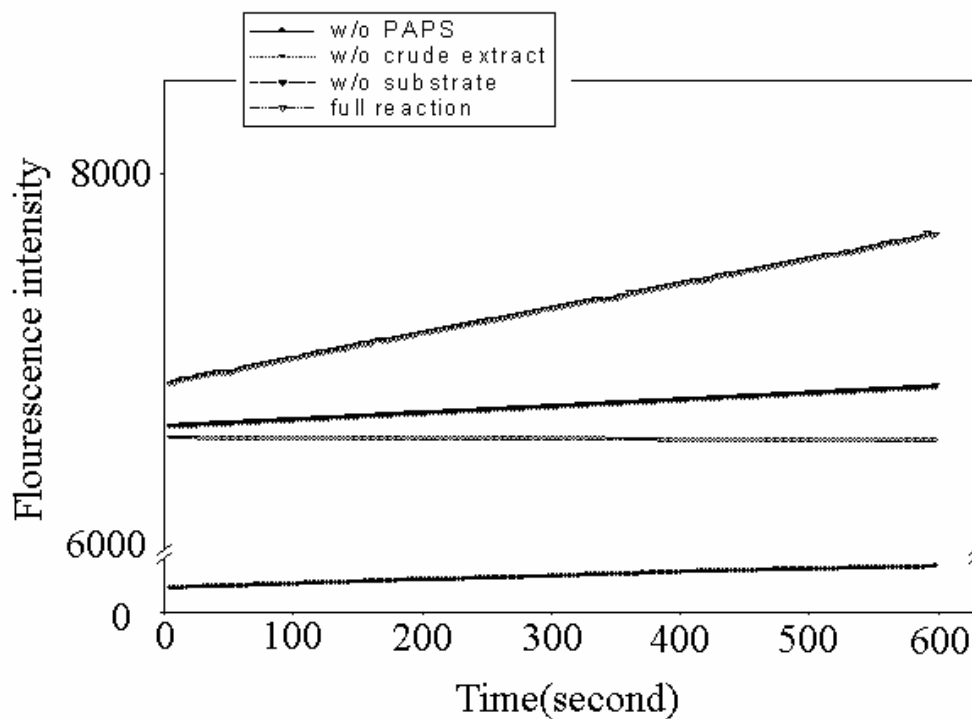


Figure 11. Progress curves of the coupled-enzyme assay for protein sulfotransferase. Complete reaction(▽) and control reactions without PAPS (●), substrate (▼) and crude extract (○) of coupled-enzyme assay for protein sulfotransferase were conducted at pH 7.0, 37°C. Detailed procedures were described under *Coupled-enzyme assay for protein sulfotransferase* in *Materials and Methods*.

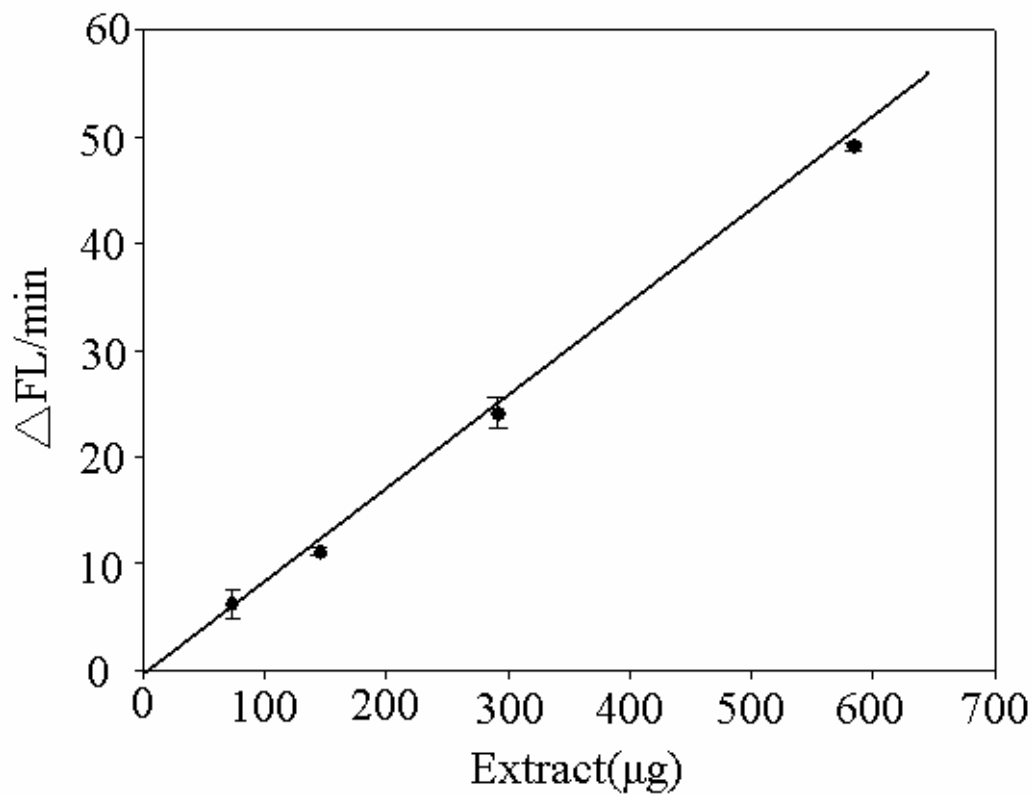


Figure 12. Effective range of fluorometric assay for protein sulfotransferase.

Complete reaction of coupled-enzyme assay were run in a total volume of 1000 μ l 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).

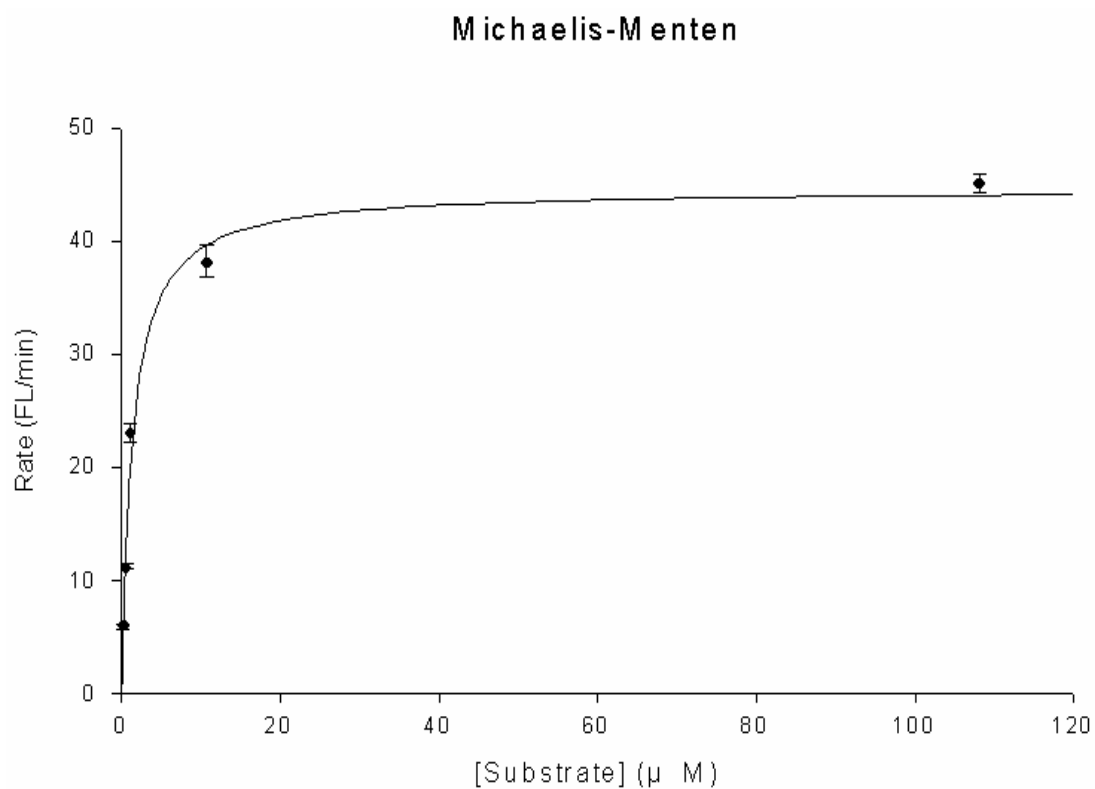


Figure 13. **Concentration of peptide on protein sulfotransferase activity.** The enzymatic assays were carried out with about 150 μ g rat crude extract under the condition of coupled-enzyme assay for protein sulfotransferase at pH 7.0 and 37°C in different concentration of peptide. Each point was determined from triplicate assay data and standard error was obtained by SigmaPlot 2001, V7.0 (SPSS Inc., Chicago, IL).

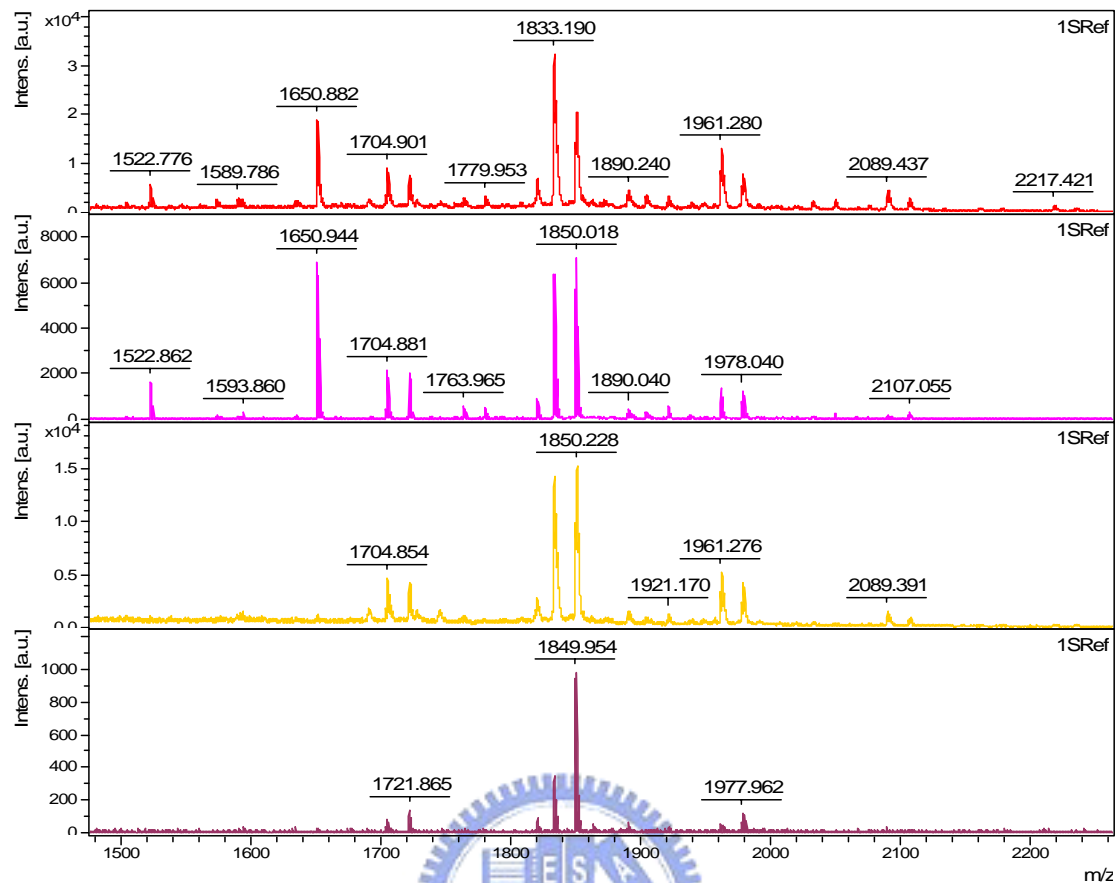
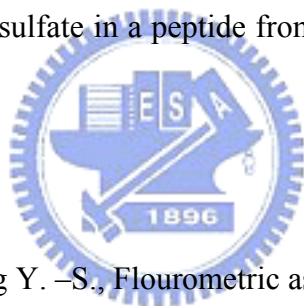


Figure 14. MALDI-TOF spectrum analysis. spectrum 1 and spectrum 2 are the peptide treated after coupled-enzyme assay, and spectrum 3 and spectrum 4 represent the untreated peptide.

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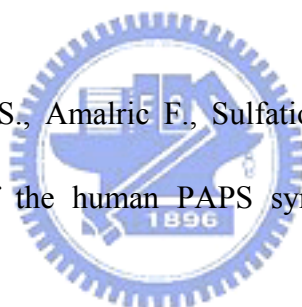
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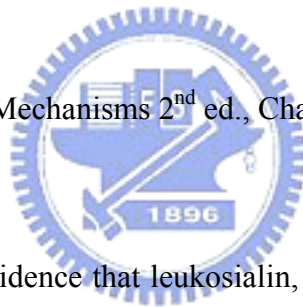
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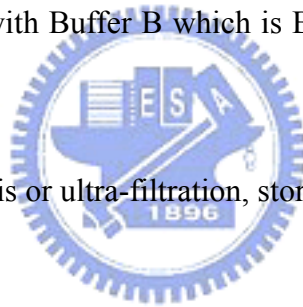
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Appendix

Enzyme purification (rat phenol sulfotransferase)

This experiment uses DEAE-Sephacel Column(Pharmacia, Hong Kong).

1. Load the sample into equilibrated DEAE-Sephacel Column, and wash the column with three column volumes of Buffer A (10mM Tris-Base at pH 7.4 、125mM Sucrose 、10% Glycerol 、1mM PMSF(2-propanol) 、1mM EDTA 、1mM Dithiothreitol) to elute non-bound protein
2. Elute the bound protein with Buffer B which is Buffer A containing 0.2 M NaCl and collect wash fraction.
3. Remove the salt by dialysis or ultra-filtration, store the protein at -80°C.



Enzyme purification (human PAPS synthetase)

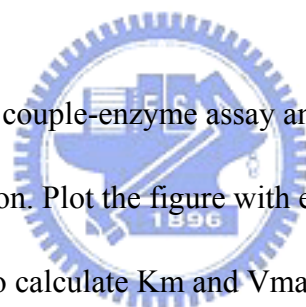
This experiment uses Hitrap Chelating HP (Pharmacia, Hong Kong).The following step is modified from affinity handbook of Pharmacia.

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. Prepare column by washing with distilled water and loading with 0.5 column volume of NiSO₄. After that, washing with 5 column volumes of distilled water again.
3. Equilibrate the column with 10 column volumes of binding buffer.

4. Apply sample at a flow rate 5 ml/min. Collect the flow-through fraction. A pump is more suitable for application of sample volumes greater than 15 ml.
5. Wash with 10 column volumes of binding buffer. Collect wash fraction.
6. Elute with 5 column volumes of elution buffer. Collect eluted fractions in small fractions such as 1 ml to avoid dilution of the eluate.
7. 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.

Estimate Km and Vmax

This experiment performed couple-enzyme assay and enzyme transformation with different substrate concentration. Plot the figure with enzyme activity and substrate concentration, use computer to calculate Km and Vmax.



Equation for calculating protein concentration

Rat phenol sulfotransferase (*rPST*)

$$\epsilon = (5690 \times 7 + 1280 \times 14 + 120 \times 5) \text{ M}^{-1} \text{ cm}^{-1} = 58350 \text{ M}^{-1} \text{ cm}^{-1}$$

M. W.: 33904

$$\epsilon = 58350 \text{ M}^{-1} \text{ cm}^{-1} \div 33904 = 1.7 \text{ (mg/mL)}^{-1} \text{ cm}^{-1}$$

Human PAPS synthetase (*hPAPSS*)

$$\epsilon = (5690 \times 11 + 1280 \times 22 + 120 \times 14) \text{ M}^{-1} \text{ cm}^{-1} = 92430 \text{ M}^{-1} \text{ cm}^{-1}$$

M. W.: 70833

$$\epsilon = 92430 \text{ M}^{-1}\text{cm}^{-1} \div 70833 = 1.3 \text{ (mg/mL)}^{-1}\text{cm}^{-1}$$



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