國立交通大學

生物科技學系

碩士論文

從原核表現人類酪氨酸亞硫酸基轉移酶

探討其催化活性與功能

Prokaryotic Expression, Purification and Characterization

of Human Tyrosylprotein Sulfotransferase 2 (TPST2)

研究生：陳柏翰

指導教授：楊裕雄 教授

中華民國九十七年七月

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碩 士 論 文

A Thesis
Submitted to Department of Biological Science and Technology
National Chiao Tung University
in Fulfillment of the Requirements
for the Degree of
Master of Science
in
Biologic Science and Technology
July 2007
Hsinchu, Taiwan, Republic of China

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酪氨酸亞硫酸基轉移酶負責催化許多分泌性蛋白質和膜上蛋白質之亞硫酸基化。酪氨酸亞硫酸基轉移酶已經在許多哺乳動物和低階的生物，如線蟲和果蠅中被發現，但在酵母菌與原核生物中卻沒有發現到。在大部分的研究中，酪氨酸亞硫酸基轉移酶的來源通常是由天然物與細胞培養方式來製備，為了能夠更加了解酪氨酸亞硫酸基轉移酶的催化特性，我們成功地開發出新的表現系統，從大腸桿菌中純化出人類酪氨酸亞硫酸基轉移酶-2，利用 Mass 指紋圖譜分析所得到結果確實為人類酪氨酸亞硫酸基轉移酶-2。藉由同位素標定的方式，探討重組的酪氨酸亞硫酸基轉移酶一些基本性質；包括催化是否隨著時間呈線性增加，以及不同的溫度、pH 值與金屬離子的濃度對酵素活性之影響，我們也針對此酵素對於 polyEAY 做酵素動力學試驗。未來我們將使用這一套系統去表現突變的酪氨酸亞硫酸基轉移酶，並尋找負責催化活性的胺基酸與一些尚未解決反應機制的問題。

Prokaryotic Expression, Purification and Characterization of Human Tyrosylprotein Sulfotransferase 2 (TPST2)

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ABSTRACT

Tyrosylprotein sulfotransferase (TPST) is responsible for the sulfation of a variety of secretory and membrane proteins. TPSTs have been found in mammalian and metazoans such as *Caenorhabditis elegans* and *Drosophila melanogaster*, but not in yeast and prokaryotes. TPSTs have been studied only from natural materials and eukaryotic cells following partial purification. To be able to fully characterize the biochemical properties of TPSTs in depth, we successfully overexpressed and purified human TPST2 in *E. coli*, and then confirmed by Mass fingerprinting identification. The properties of recombinant TPST2 were characterized using by isotope-based analysis. The optimal temperature, pH profile, and concentration-dependent of $MnCl_2$ for human TPST2 were determined. The kinetic constants of TPST2 using polyEAY as substrate were also determined. Furthermore, the important amino acids responsible for the activity of TPST and its mechanism of actions will be studied.

Acknowledgement

首先誠摯的感謝指導教授楊裕雄博士，悉心的教導使我得以一窺鍊金術領域的深奧，不時的討論並指點我正確的方向，使我在這些年中獲益匪淺。老師對學問的嚴謹更是我輩學習的典範。

本論文的完成另外亦得感謝實驗室大力協助，高中好友與父母的支持、張程翔學長在 isotope 實驗上的幫忙，以及呂平江教授、汪宏達教授與林照雄教授對我研究上的指教評點。因爲有你們的幫忙與教導下，使得本論文能夠更完整而嚴謹。

兩年裡的日子，實驗室裡共同的生活點滴，學術上的討論、言不及義的閒扯、讓人又愛又怕的宵夜、趕作業的革命情感、因爲實驗太晚而睡倒在實驗室(囧學長亂取"燈塔"稱號)，感謝大晃學長、Rich 學長、小米學姐、漢平學長、陸宜學長與青辰學長的耐心指導下，不厭其煩的指出我研究中的缺失，且總能在我迷惘時爲我解惑。也感謝 LEPE 所有實驗室同仁，還有很愛虧我的家煒= =(讓我箭箭重傷 XD 囧) ，還有唱歌好聽的咏馨與小 apple 當然也不能忘記，你們的幫忙及搞笑我銘感在心。

有你們的陪伴讓兩年的研究生活變得絢麗多彩。

而好友……(很多名)的陪伴更是令人感動，在此一併致謝。

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Abbreviations

Abbreviation and Symbol	Full name
ε	Absorption (exitinction) coefficient
A_{280}	Absorption at 280 nm
A_{600}	Absorption at 600 nm
k_{cat}	Turnover number
kDa	Kilodaton
K_m	Michaelis constant
MALDI-TOF	Matrix-assisted laser desorption inonzation time of flight
MES	2-[N-morpholino] ethanesulfonic acid
PAGE	Polyacrylamide gel electrophoresis
PAP	Adenosine 3',5'-diphosphate
PAPS	3'-phosphoadenosine 5'-phosphosulfate
polyEAY	polypeptiede (Glu: Ala : Tyr = 6 :3 :1)
SDS	Sodium dodecyl sulfate
SULT	Sulfotransferase
V_{max}	Maximum velocity

1. Literatures on tyrosine O-sulfation

1.1 Post-translational modification in the regulation of protein functions.

The post-translational modifications of proteins can regulate the protein functions and protein activities by causing the changes of the protein structure or the affinity of dynamic interaction between proteins and compounds. (**Fig. 1**) (Seo *et al*. 2004) A series of are involved in the signaling pathway from membrane to nucleus in response to external stimuli. (Zhao Y *et al*. 2008; Guo *et al*. 2007)

Some common and important post-translational modifications include sulfation, phosphorylation, acetylation, methylation, glycosylation, and ubiquitination. “For the discovery of ubiquitin-mediated protein degradation” was awarded 2004 Nobel Prize in chemistry. Protein sulfation has drawn a significant amount of attention recently. For instance, CXCR4 and CCR5 include modifications of the extracellular domains (N terminal, ECI, ECII, and ECIII) or the intracellular loops. Modifications of the extracellular domains include N-linked and O-linked glycosylation and tyrosine sulfation. The intracellular loops of the receptors may undergo palmitoylation, phosphorylation, and ubiquitination. These three types of modifications play major roles in receptor turnover and will be discussed in relation to receptor endocytosis and recycling.

In previous literatures, post-translational modification was identified by

radioisotope labeling, western analysis with antibody against specific modification, mutagenesis of modification sites and mass spectrometry. Mass spectrometry is a powerful tool to map modification sites and species. MS measures mass-to-charge ratio (m/z), yielding the molecular weight and the fragmentation pattern of peptides derived from proteins. (Mann *et al*. 2003)

1.2 Sulfotransferases

Sulfate-containing biomolecules were identified in 1876.(Baumann *et al*. 1876) Sulfotransferases(STs) catalyze the transfer of sulfonated group (SO^{2-}) from a donor, PAPS, to an acceptor which has alcohol, thiol, amine, or phenol as functional groups. There are two classes of STs: cytosolic sulfotransferases and membrane-associated sulfotransferases. Cytosolic sulfotransferases mediated small chemical compounds (Steroids, xenobiotics, dietary carcinogens, and neurotransmitters) to regulate hormone homeostasis, and metabolic detoxications/activation of xenobiotics. Membrane-associated sulfotransferases catalyze carbohydrates and proteins (Heparan, glycoproteins, oligopeptide, and proteins). Many of which have been implicated recently in crucial biological processes, for instances, modulating receptor binding, signaling processes, and intercellular communications.

1.3 Tyrosylprotein sulfotranferases

Tyrosylprotein sulfotransferases (TPSTs) are type II transmembrane proteins with a short N-terminal cytoplasmic domain, a single about 17-residue transmembrane domain (red), and a luminal catalytic domain.(**Fig. 8**)(Baeuerle *et al*. 1987; Lee *et al*. 1985) TPSTs are localized in the Golgi lumen. In most species, TPSTs are classified as TPST-1 and TPST-2, but *D. melanogaster* and C. elegan have only a single TPST gene, TPST-2 and TPST-A, respectively. Furthermore, no tyrosine-sulfated proteins, TPST activity, or putative TPST orthologs have been described in prokaryotes or in yeast. (Moore *et al*. 2003) Human TPST-1 and human TPST-2 share 68% sequence identity. (**Fig. 8**)

A recent gene-knockout researches revealed that mice deficient in TPST-1 had reduced body mass and increased postimplantation fetal death (Ouyang *et al*. 2002) and that mice deficient in TPST-2 had caused male infertility. (Borghei *et al*. 2006) Sulfation is similar to phosphorylation, but protein sulfation in signaling pathway was less studied as compared to phosphorylation. The Golgi localization and the luminal active site orientation of TPST-1 and -2 predict that tyrosine *O*-sulfation occurs only on proteins that transit the *trans*-Golgi network.(**Fig. 2**) PAPS is synthesized by the action of one of two PAPS synthases in the cytosol. TPSTs used PAPS, as the donor of sulfonate group, to catalyze the protein that transit trans-Golgi network. The sulfonate protein affects lots of protein-protein interactions, in hemostasis, leukocyte

adhesion, inflammatory response, chemokine receptor, and bioactive peptides. The following are the details about protein-protein interactions through protein sulfation.

1.3.1 Tyrosine sulfation of chemokine receptors

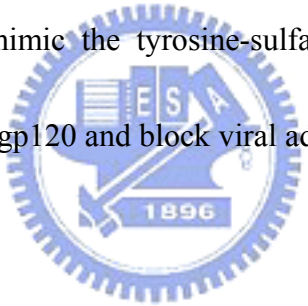
Chemokines are involved in leukocyte trafficking, angiogenesis, angiostasis, battling viral infections, and the host immune response to cancer (Zlotnik *et al*. 1999). In last decades, the sulfation of chemokine receptors is found to play important role to affect enzyme activity, protein lifespan and the binding affinity between proteins.(**Table 2**)

To days, sulfated tyrosines have been experimentally confirmed in only five human chemokine receptors: CXCR3, CXCR4, CCR2b, CCR5, and CX3CR1. (Farzan *et al*. 1999; Farzan *et al*. 2002; Preobrazhensky *et al*. 2000; Fong *et al*. 2002; Colvin *et al*. 2006) Many tyrosine sulfation was often found in the same groups of protein and peptides. For instance, CCR5 and CXCR4 have tyrosine sulfated. CCR5 carries four tyrosine residues in the amino-terminal region. (Farzan *et al*. 1999) Incubation of CCR5-expressing Cf2Th cells with sodium chlorate ($NaClO_3$)[a], a global inhibitor of the sulfation (Farzan *et al*. 1999), decreased binding of MIP-1 d MIP-1—two natural chemokine ligands for CCR5. Sulfonate tyrosine14 of CCR5 seems to play a particularly important role in binding to the chemokines MIP-1(Chapman *et al* 2004).

[a] Sodium chlorate is a specific inhibitor of the ATP sulfurylase activity of PAPS

synthetase, and a global inhibitor of the sulfation. (Farzan *et al*. 1999)

In addition to binding native chemokines, CCR5 also serves as a co-receptor for HIV-1 gp120, composing together with CD4 to mediate attachment of the virus and its subsequent infections.(**Fig. 3**) (Kuhmann *et al*. 2004; , Chapman *et al*. 2004) Mutation of the four sulfotyrosine residues in CCR5 to phenylalanine inhibits HIV infection by 50–75% in cultured cells, depending on the HIV isolate tested (Farzan *et al*. 1999). This information suggests that inhibitors of tyrosine sulfation might serve as anti-HIV herapeutics, as long as disruption of the modification is not globally toxic. Alternatively, peptides that mimic the tyrosine-sulfated sequence of CCR5 might compete for binding to HIV-1 gp120 and block viral adhesion.



1.3.2 Tyrosine sulfation of hemostasis

Tyrosine sulfation is also involved in hemostasis. This modification is crucial in the interaction between many plasma proteins such as fibronectin and fibrin (Suiko *et al*. 1988), hirudin and thrombin (Stone *et al*. 1986), coagulation factor VIII, and von Willebrand factor (VWF)(Leyte *et al*. 1991) and glycoprotein (GP) Ibα with both VWF and thrombin(Marchese *et al*. 1995; Ward *et al*. 1996; Fredrickson *et al*. 1998; Dong *et al*. 2001; Murata *et al*. 1991). The interaction between the platelet membrane protein (GP) Ibα and VWF is essential for initiation of hemostasis. For

instance, in previous studies, they found many sulfated tyrosine residues, located within this region at positions 276, 278, and 279, in the platelet membrane protein (GP) Ibα . (**Fig. 4**) (Marchese *et al*. 1995; Ward *et al*. 1996; Fredrickson *et al*. 1998) The region has been demonstrated that how sulfated tyrosine residues affect both the conformation of the amino-terminal region of GPIbα and the electrostatic interaction with VWF in the presence of both ristocetin and botrocetin.(Lee *et al*. 1983)

1.3.3 Tyrosine sulfation mediate leukocyte adhesions and inflammatory responses

The inflammatory response results from producing and releasing of chemical agents by cells in the infected, injured or diseased tissue. The chemical agents induced the leukocyte into the site of the inflammatory. In the processes, leukocytes roll upon, adhere to and transmigrate between endothelial cells at sites of inflammation. Tyrosine sulfation near the amino terminus of PSGL-1 plays an essential role in P-selectin binding.(**Fig. 5**) (Pouyani *et al*. 1995; Sako *et al*. 1995; Wilkins 1995)

1.4 Application of antibody to researches of tyrosylproteins.

Antibody is a useful tool to discovery lots of things, including staining tissue, immunoprecipitation, immunoblotting, immunoaffinity purification, tagging proteins and the epitope mapping. (Kasinathan *et al*. 2005; William *et al*. 1997; Ramaprasad *et al*. 1998; Hoffhines *et al*. 2006; United States Patent 5716836)

Antibodies in researches of tyrosine O-sulfation were widely used. I am interested in the epitope mapping of tyrosine O-sulfation. In 1992, united states patent, patent number:5716836, Masahito suiko was successfully produced the anti-sulfated tyrosine antibody specific for sulfated tyrosine but not to unsulfated tyrosine.(United States Patent 5716836), the antibody of anti-sulfated tyrosine cound be not purchased but polyclonal anti-TPST1 anibody, abnova (H00008460-A01) and polyclonal anti-TPST2 antibody, abcam (ab59958), could. If we could buy the anti-sulfated tyrosine antibody, we would use proteomics tools to discovery lots of the sulfations of proteins, and how to affect biological functions in cells. In previous studies, most of these used antibody to purification enzymes, location in cells, and detection the quantity of protein expressions.(Ramaprasad *et al.* 1998; Kasinathan *et al.* 2005). More interacted proteins could be found out and then to deduce the functions and pathways by using the antibody to help to facilitating the technique of protein-protein interaction such as far western blotting (Wu *et al.* 2007) (**Fig. 6**), but previous researches don't study in these aspects.

1.5 Predication the sulfation of protein by bioinformation tools

The post-translational modifications (PTMs) modulate all aspects of cellular life. Many PTMs have been discovered including protein kinases, sulfation, acetylation, methylation, amidation….etc.(Table1) PTMs affect lots of functions

thought protein modification in physiology. How to predict PTMs sites from a protein sequence is very important. Previous researches used Position-Specific-Scoring-Matrix (PSSM) to compare the distributions of amino acids around the query tyrosine sites with those of known sulfated and non-sulfated tyrosine sites in Swiss-Prot.(Liu *et al*. 2008) Each query tyrosine site was assigned a score measuring the similarity of the amino acids around the tyrosine to those around either the known sulfated tyrosine sites or those around non-sulfated tyrosine sites. The site of tyrosine o-sulfation took high score. (Nicholas *et al*. 1999; Lin *et al*. 2003) The softwares, Sulfonator(http://ca.expasy.org/tools/sulfinator/) and SulfoSite (http://sulfosite.mbc.nctu.edu.tw/) , are often used to predict sulfation sites. The figure 1 as the example, Justin Liu *et al*. screened all human chemokine receptors and found that many tyrosines predicted to be sulfated were in a region localized to the N-terminus of their receptor.(**Table 3.**)(Farzan *et al*. 1999) The following tyrosines are known to be sulfated: Y12 and Y21 of CXCR4; Y26 of CCR2; Y3, Y10, Y14, and Y15 of CCR5; and Y14 of CX3CR1. CCR8 is known to be tyrosine sulfated in the mouse, probably in both Y14 and Y15. These tyrosine sulfation sites were frequently completely conserved in predictions.

Previous researches also discoveried some conditions to increase the chances of the tyrosine sulfation. There is the presence of negatively charged acidic amino acid

residues around the site of tyrosine o-sulfation.(Baeuerle *et al*.1986;Liu *et al*. 2008) The secondary and tertiary protein structure promotes to expose adjacent tyrosines to TPSTs. (Huttner *et al*. 1984) The bioinformatics tool for prediction of specific post-translational modifications will be useful in discovering these important protein modifications.

Prokaryotic Expression, Purification and Characterization of Human Tyrosylprotein Sulfotransferase 2 (TPST2)

2.1 Introduction

Tyrosine *O*-sulfation is a common post-translational modification of protein in lots of mammalian cells. Lee and Huttner directly demonstrated tyrosine *O*-sulfation is catalyzed by tyrosylprotein sulfotransferases (TPSTs, EC 2.8.2.20) (Wilkins *et al*. 1995). So far only two distinct human TPSTs were indentified, TPST1 and TPST2. They are in similar size (370–377 residues) and share 67% identity in primary sequence. TPSTs are type II transmembrane topology with a short N-terminal cytoplasmic domain and a luminal catalytic domain. It transfers the sulfonate group (SO_3^{-1}) from 3'-phosphoadenosine-5'-phosphosulfate (PAPS), the only known sulfonate donor (Robbins P, 1956), onto a tyrosyl residue within target proteins as shown in **Fig. 7**. The sulfation plays an important role in many biological mechanisms described as followed: 1) Modulation of receptor binding (Preobrazhensky *et al*., 2000; Farzan *et al*., 2002; Wilkins *et al*., 1995); 2) Intercellular communication (Rossi *et al*., 2000); 3) Signaling processes (Goettsch *et al*., 2002).

To this day, tyrosine *O*-sulfation affects lots of critical pathology. In the previous research, the experiment of TPST double knockouts mice had discoveried

that TPST-1 and TPST-2 both affected the functions of thyroid.(Westmuckett *et al*. 2008) The absence of TPST1 and TPST2 caused euthyroid and primary hypothyroidism, respectively. Furthermore, the mouse lungs of TPST double knockouts failed to expand at birth so that the mouse died in asphyxia during the early postnatal period (Westmuckett *et al*. 2008). Tyrosine *O*-sulfation also come into notice about HIV infections and inflammations in recent years. Sulfonated CCR5 renders gp120 of HIV-1 interacts with the sulfonated CCR5/CD4 complex, and then leads to the entry of the virus into the host cell. (Choe *et al*. 2003) The tyrosine *O*-sulfation near the amino terminus of PSGL-1 also plays an essential role in P-selectin binding.(Kehoe *et al*, 2000) Sulfation enhances the affinity binding interaction so that sulfonated PSGL-1 helps rolling leukocyte adheres to endothelial cells at sites of inflammation.

According to the analyses of known peptides, some acidic and basic amino acid around the tyrosine from +5 residues to -5 residues affect the catalytic activity of tyrosine *O*-sulfation in its substrates. (Mishiro *et al*. 2006; Bundgaard *et al*. 1997; Lin *et al*. 1992) Substrate specificity and mechanism have not been well-known in the past years, so that developing the powerful and quick method of the purification to obtain adequate TPST is necessary. Furthermore, the crystal structure and antibody preparation are also urged to facilitate the study in advanced.

Previous studies investigated the characterization of TPSTs, whose enzyme source were purified from crude and partially purified preparations from a variety of mammalian cells neither in yeast nor prokaryote **(Table 4)**. Expresses desired enzyme from prokaryotic system will be convenient, stable, time-and-effort-saving, and acquire lots of the enzyme quantities. Firstly we set up the prokaryotic expression system to express human TPST2, and further study the characterizations of recombinant TPST2. In the future, we will facilitate this platform to discover the important amino acid regulating PAPS binding, substrate specifity, catalysis, and enzymatic mechanism.

2.2 Materials

The human TPST2 clone (accession No. is AAH17509) was obtained from GenDiscovery Biotechnology, Inc. *PfuTurbo* DNA polymerase was purchased from Stratagene, and T4 DNA ligase, BanHI, XhoI restriction endonucleases were purchased from New England Biolabs. Oligonucleotide primers were synthesized by Mission Biotech Co., Ltd. (Taiwan). MES, NaOH, Trizma base, NaCl, Triton X-100 and imidazole were products of Sigma (St. Louis, MO, USA). HisTrap fastflow sepharose, HiTrap Q fast flow, and glutathione S-transferase sepharose fast flow were obtained from GE Healthcare. Cellulose thin-layer chromatography plates were obtained from MERCK, Ltd. All other reagents were of the highest grades commercially available.

2.3 Experimental procedures

Protein sequence alignment and prediction of transmembrane domain of TPST1 and TPST2—The sequence pairwise alignment was performed by ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html) and sorted shading by BOXSHADE server (http://www.ch.embnet.org/software/BOX_form.html). The *black* background indicated identity to each other and the *gray* one meant conserved substitutions. The residue colored in *red* is the predicted transmembrane domain calculated by PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/psiform.html) ranged from residue 6 to 28 both for human TPST1 and TPST2.

Cloning of cytosolic domain of TPST2—The human TPST2 incorporated into the vector pOTB7 originally was subcloned into pET-43a (**Appendix 2**). The potential cytosolic domains of TPST was amplified by PCR through specific primers designed to contain BamHI restriction site in the forward direction was 5'-tgaaggatccctagagtgccgggcggtgctggc-3', and the reverse one contained XhoI restriction site was 5'-gccactcgagtcacgagcttcctaagtgggagg-3'. cDNA fragments were inserted into the BamHI/XhoI doubly-restriction sites and then confirmed using an ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA) following the standard protocol.

Cloning of substrate peptides—The specific primers were designed to produce the self-annealed cDNA encoding the N-terminal region of PSGL-1 (ATEYEYLDYDFL), CCR5 (DYQVSSPIYDINFYTSE), and Ror2 (MCRNKQKASASTPQRRQLMASPSQDMEMPLINQHKQAKLKEISLSAVRFM E), respectively. The self-annealed oligonucleotides were subcloned into BamHI–XhoI restriction site of pGEX-4T1 and transformed into BL21 (DE3) to express further.

Protein expression and purification of human TPST2 and its substrates—A single colony of BL21 (DE3) consisted of TPST2 or substrates (PSGL-1, and CCR5) plasmid was used to inoculate in the LB broth with ampicillin as the antibiotic at 37 °C. Growth was continued to an OD_{A600} of 0.4–0.6 and then induced with 0.4 mM isopropyl-thio-β-$_D$-galactoside (IPTG) for 16 hours incubation at 20 °C. The cultures were centrifuged at 14000×g for 20 minutes, and the pellet was sonicated in IMAC5 buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 5 mM imidazole, and 10% glycerol) for TPST2 and GST lysis buffer (20 mM Tris–HCl, pH 8.0, 150 mM NaCl, and 10% glycerol) for substrates. Further the HisTrap sepharose charged with $NiSO_4$ was facilitated to the TPST2 purification. GSTrap for Substrates were purified by GSTrap sepharose following the digestion against the bovine thrombin at 4 °C for 16 hours

and then applied to HiTrap Q chromatography. A homogeneous protein **(Figs. 9 and 16)** was obtained as determined by SDS-polyacrylamide gel electrophoresis (38).

Protein identification by LC-MS-MS spectrometry—For MS analysis, protein spots were excised from the gel and digested with trypsin according to published procedures (Shevchenko *et al*. 1996). The protein spots were sent to Academia Sinica, Core Facilities for Proteomics and Structural Biology Research, for Mass Spectrometric analysis. Proteins were identified by searching the protein databases NCBInr using MASCOT (http://www.matrixscience.com). To denote a protein as unambiguously identified, the Mowse scoring algorithms were sued. **(Fig. 10)**

TPST enzymatic activity assay—For the determination of TPST activity from bacterial expression, we detected radiation of ^{35}S using [^{35}S]PAPS as donor and transferred sulfate group to substrate, such as PSGL-1 peptides, polyEAY, or CCR5 peptides. The complete assay mixture consisted of sulfate acceptor (TPST substrate), 40 μM polyEAY peptides, 50 mM MES at pH 6.0, 25 mM $MnCl_2$, 50mM NaF,0.5 μM [^{35}S]PAPS (15Ci/mmole), and 4 μg human TPST2 in a final volume of 15 μl. Catalytic reactions were initiated by the addition of the enzyme and incubated for 30 minutes at 30 °C. The reactions were terminated by heating at 95 °C for 2 minutes.

The supernatant was collected and analyzed by spotting 1 μl aliquot of the reaction mixture on a cellulose thin-layer chromatography (TLC) plate and developed with n-butanol/pyridine/88%formic acid/water (4:2:2:4; by volume) as the solvent system. The dried plate was exposed with Kodak BioMax MR films which provided the optimal resolution for ^{35}S autoradiography.

Time-dependent TPST enzymatic activity assay—This activity of TPST2 was measured as described above. Each assay consisted of 50 mM MES at pH6.0, 25 mM $MnCl_2$, 50 mM NaF, 0.5 μM [^{35}S]PAPS (15Ci/mmole), 40 μM polyEAY, 0.5% Triton-X100 and 4 μg human TPST2 in a final volume of 10 μl. Data shown here is the result of a typical experiment performed in triplicate.

pH profile of TPST enzymatic activity—Each assay contained 50 mM MES at desired pH (5.5, 6.0, 6.5, 7), 25 mM $MnCl_2$, 50 mM NaF, 0.5 μM [^{35}S]PAPS (15Ci/mmole), 40 μM polyEAY, and 4 μg human TPST2 in a final volume of 10 μl. Data shown here are the result of a typical experiment performed in triplicate. This experiment was repeated three times with similar results.

Temperaturen effect on TPST activity—This experiment was performed described

above under various temperatures (25, 30, 35, 37, 40, 45, 50 °C). Data shown here is the result of a typical experiment performed in triplicate. and repeated three times with similar results.

$MnCl_2$ dependence of TPST enzyme assay—Various concentrations of $MnCl_2$ were examined in the enzyme assay. Data shown here is the result of a typical experiment performed in triplicate.

Kinetic constants determinations of TPST—Measurements of kinetic constants were performed by varying the polyEAY concentration while keeping the PAP at a fixed and near saturating concentrations. The apparent K_m and V_{max} were determined using nonlinear regression by SigmaPlot 2001. V7.0 and Enzyme module, V1.1.

2.4 Result

Expression of recombinant human TPST2 in prokaryote expression system.

The human TPST2 is localized in the membrane of Golgi apparatus network and the transmembrane domain is shown as **Fig 8**. In our experiment, the catalytic domain of TPST2 from residue 29 to 377 was incorporated into various expression vectors. Various expression vectors incorporated human TPST2 cDNA in the open reading frame were examined (data not shown). Most of the expression vector could not prevent TPST2 from inclusion body. pET-43a (**Appendix 2**), a vector of expressing proteins was purchased from Novagen, was competent to express recombinant human TPST2 in *E. coli* with the reducing amount of inclusion body. The prokaryotic expression of human TPST2 was optimized to reach the maximal soluble amount and purified to nearly homogeneity **(Fig. 9)**. A band on the SDS-PAGE of 100 kDa protein composed of NUS-Tag fusion protein (60 kDa) and TPST protein (40 kDa) upon treatment in coomassie blue R350. The spots excised from SDS-PAGE were analyzed by LC-MS/MS. **(Fig. 10)** There were many peptides (colored in *red*) come after trypsin digestion and they indicated the peptide sequences of human TPST2. The alignment of these peptide sequences showing homology to human TPST2 with high scores of confidences.

Time-dependence of human TPST2-catalyzed tyrosine *O*-sulfation

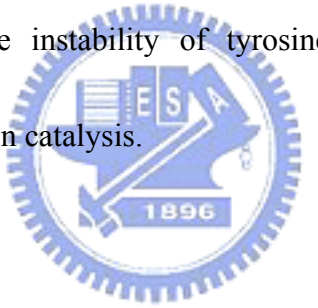
polyEAY is a substrate of TPST2 with higher specificity activity than other endogenous substrates as described in the previous reports. Firstly the time dependence of the activity of the human TPST2 with polyEAY as substrate was examined. The concentrations of PAPS and polyEAY, were both saturated in the reactions. The tyrosine *O*-sulfation of polyEAY increased linearly with the incubation time as shown in the **Fig. 11**. The slope in this reaction was not appreciably reduced up to 120 mins.

Temperature effect on TPST activity

So far the catalytic activity of human TPST2 in different temperatures was not studied clear in the past. The experiment demonstrated that human TPST2 exhibited the activity at 25 °C was three times than that at 40 °C (**Fig. 12**). This catalytic reation was almost quenched under the treatment of 45 °C for 30 mins. The human TPST2 might denature and thus lost the fuction of tyrosine *O*-sulfation catalysis. The activity at 37 °C (body temperature) was also investigated and it was apparently lower than the activity at 25 °C. It might be attributed to the reason as decribed above.

pH profile of TPST

pH affects the electricity of amino acid and further contributes to the substrate binding affinity, enzymatic catalysis, and protein conformational structure. The pH profiles of the recombinant human TPST2 were determined by measuring the activity at various pH values. The pH optimum was ranged from 5.5 to 6.0 within the error tolerance as showed in **Fig. 13**. The catalytic activity of human TPST2 appreciably decreased from pH 6.0 to 6.5 and was nearly nondetectable at pH 7. pH values higher than 6.0 might result in the instability of tyrosine *O*-sulfated peptide, or the dysfunction of human TPST2 in catalysis.



Metal ion effect on TPST2

It is known to require exogenous metal ions for activity with Mn^{2+} and Mg^{2+} to activate the highest activity of TPSTs (Mishiro *et al*. 2006). The data indicated that whether there is metal ion in enzyme catalysis or not, it is not necessary for human TPST2 (**Fig. 14**). The concentration of Mn^{2+} at 25 mM performed the maximal activity and 2.5 folds higher than the absence of Mn^{2+}.

Kinetics of TPST2 utilized polyEAY as substrate

Previous studies indicated that tyrosine sulfation was studied in the subcellular fractions containing the enzyme activity.(William *et al*. 1997; Sane *et al*. 1993; Lin *et al*. 1990) The most widely used sulfonate acceptor was EAY as a positive control. The kinetic constants toward polyEAY, the synthetic polypeptides0 composed of Glu, Ala, and Tyr in the ratio 6:3:1, demonstrated that K_m was 10.6 μM and V_{max} was 4.8 pmole/min/mg (**Fig. 15**). It revealed that the heterologous expression of human TPST2 was active in the catalysis and performed the similar kinetic constants compared to the previous studies. (Sane *et al*. 1993)

2.5 Discussion

Tyrosine *O*-sulfation is firstly discovered in 1954. (Bettelheim, F.R.1954) Tyrosylprotein sulfotransferase (TPST) is demonstrated to catalyze tyrosine *O*-sulfation by Lee and Huttner in 1983. In opposition to the researches of kinases, the ones of TPST are extremely few. It may be attributed to some characteristics of TPST, such as the difficulty to purify the homogenous and ample amount of TPST. TPST is reported to be labile and is hard to purify during the process of purification (Ouyang *et al*. 1998). In the previous studies, the enzyme source came from the nature materials or mammalian cell lines, and further purified through affinity column whose beads conjugated with its substrate or antibody.(Ramaprasad *et al.* 1998; Kasinathan *et al.* 2005) In this research, the heterologous expression system utilizing *E. coli* as host to purify the human TPST2 was optimized with high recovery. In the process of the purifications, the inclusion body and contamination of chaperonin 60 (GroEL) resulted in the difficulties to overcome.

The formation of inclusion body includes solubility limitation, protein size, type of promoter, and improper disulfide formation. (Hartley *et al*. 1988; Marston *et al*. 1986) The choice of vector and expression host can significantly increase the activity and amount of target protein present in the soluble fraction. In the previous study, the truncated form of human TPST2 comprising the catalytic domain was

secreted from stably transfected Chinese hamster ovary (CHO) cells (Ouyang *et al*.1998). According to the topological analysis of primary sequence of TPST2, the N-terminal transmembrane domain in TPST2 was truncated to prevent hydrophobic domain from interfering in this study. Moreover, the fusion protein, Nus•Tag, on the expression vector was utilized to enhance the solubility of target proteins (Davis *et al*. 1999; Harrison *et al*. 2000). Furthermore, the contamination of chaperonin 60 (GroEL) was found to co-elute with TPST2 in this study. Common features of chaperone action are transient interaction with non-native species in the prevention of aggregation and promotion of correct folding and assembly (Young *et al*. 2004; Bukau *et al*. 2006; Anken *et al*. 2005). The existence of the GroEL represented that TPST was not easy to fold or not fold into the correct stage. This interaction between TPST2 and GroEL was interfered through Triton X-100 to be the competence of the hydrophobic force and separate each other. This purification procedure in the study is simple, straightforward, and can produce great quantities and homogeneous sources of TPST2 (**Fig. 9**).

The homogeneous human TPST2 was measured further to understand the characterizations and mechanism of action. The catalytic activity of zebrafish TPST with N-terminal PSGL-1 peptide as substrate indicated the optimal activity ranged from 28 to 37 °C (Mishiro *et al*. 2004). The temperature profile of human TPST2 with

polyEAY as substrate in this study was different from the catalytic activity of zebrafish TPST (**Fig. 12**). The activity of TPST2 decreased with the increasing temperature and approached to inactive while the temperature was higher than 45 °C. Furthermore, previous research revealed that the TPST under the treatment of detergent possessed the half life of 48 hours at 4 °C (Niehrs *et al*. 1990). The catalytic specificity of human TPST2 is unknow in this studies.

Previous studies had revealed that TPST1 and TPST2 are localized in the Golgi apparatus and the catalytic domain is situated in the lumen, which is an acidic environment (Baeuerle *et al* 1987; Lee *et al*.1985). The recombinant human TPST2 indicated that the activity is adaptable under acidic environment and become labile while the pH value was higher than 6.0 (**Fig. 13**). It might be resulted from the influence of the affinity of PAPS or substrate binding sites. The information from previous studies also indicated that the optimal pH of human TPST2 expressed from *E. coli* was the same as that from 293T cells, but was different from human saliva.(Mishiro *et al*. 2006; Kasinathan *et al*. 2005) It might infer that human TPST2 possessed the isoforms so that resulting in the different optimal pH towards the catalytic activity.

Membrane lysates of Golgi apparatus have revealed the stimulatory effects of Mn^{2+} on the activity of TPST.(Mishiro *et al.* 2006; Kasinathan *et al.* 2005) The

catalytic activity of human TPST from salivary and PC12 cells is stimulated by the divalent cations, such as Mn^{2+}, Ca^{2+} and Mg^{2+}, and is inhibited by EDTA.(Kasinathan *et al.* 2005) On the contrary, tyrosine *O*-sulfation of the endogenous membrane proteins in A431 cell is not inhibited by EDTA.(Liu *et al*. 1986) The stimulatory effect and mechanism of metal inons, however, was still not clear so far. When $MnCl_2$ was up to 40 mM in the reaction mixture, the enzyme activity was apparently decreased (**Fig. 14**). High concentration of Mn^{2+} might affect the structure and render the protein denaturation. The appropriate concentration of Mn^{2+} might also stabilize the sulfonate groups while catalysis and further to reduce the activation energy. It could be comparable to the role of Mg^{2+} in the kinase catalysis.

According to the previous studies, the K_m of platelet TPST for polyEAY as substrate was 3.7 μM and the V_{max} was 0.09 pmol/min (Sane *et al*. 1993). In our study, the kinetic constants indicated that the K_m and V_{max} were 10.5 μM and 4.8 pmole/min/mg, respectively (**Fig. 15**). This difference might result from that the polyEAY is synthetized with distinct ratio of components (Glu, Ala, and Tyr), and the various composition of the sequences also led to the different catalytic efficiency.

According to these characterizations of recombinant human TPST2, the NusA protein fused TPST2 expressed from *E. coli* was similar to that either from natual materials or eukaryotic expression. The NusA protein obviously did not affect the

catalytic activity of human TPST2 and render the high solubility to facilitate TPST folding.

In summary, we first purified TPST from prokaryote systems (*E. coli*) with catalytic activity. By means of this purification procedure, the time-and-effort-saving, inexpensive, high quality and quantity platform was established to express and purify homogenous human TPST2 with only one chromatography step for further biochemical characterization. The catalytic mechanism of substrate specificity, for example PSGL-1(**Fig. 16**), metal ion effect, and the regulatory residues will be examined. Furthermore, the crystal structure and antibody will be pursued to study in advanced for either the physiological or pathological functions and regulations.

PTM type	ΔMass[a] (Da)	Stability[b]	Function and notes
Phosphorylation			Reversible, activation/inactivation of enzyme activity, modulation of molecular interactions, signaling
pTyr	+80	+++	
pSer, pThr	+80	+/++	
Acetylation	+42	+++	Protein stability, protection of N terminus. Regulation of protein–DNA interactions (histones)
Methylation	+14	+++	Regulation of gene expression
Acylation, fatty acid modification			Cellular localization and targeting signals, membrane tethering, mediator of protein–protein interactions
Farnesyl	+204	+++	
Myristoyl	+210	+++	
Palmitoyl	+238	+/++	
etc.			
Glycosylation			
N-linked	>800	+/++	Excreted proteins, cell–cell recognition/signaling
O-linked	203, >800	+/++	O-GlcNAc, reversible, regulatory functions
GPI anchor	>1,000	++	Glycosylphosphatidylinositol (GPI) anchor. Membrane tethering of enzymes and receptors, mainly to outer leaflet of plasma membrane
Hydroxyproline	+16	+++	Protein stability and protein–ligand interactions
Sulfation (sTyr)	+80	+	Modulator of protein–protein and receptor–ligand interactions
Disulfide bond formation	–2	++	Intra- and intermolecular crosslink, protein stability
Deamidation	+1	+++	Possible regulator of protein–ligand and protein–protein interactions, also a common chemical artifact
Pyroglutamic acid	–17	+++	Protein stability, blocked N terminus
Ubiquitination	>1,000	+/++	Destruction signal. After tryptic digestion, ubiquitination site is modified with the Gly-Gly dipeptide
Nitration of tyrosine	+45	+/++	Oxidative damage during inflammation

[a]A more comprehensive list of PTM Δmass values can be found at: http://www.abrf.org/index.cfm/dm.home
[b]Stability: + labile in tandem mass spectrometry, ++ moderately stable; +++ stable.

Table 1[a]. Some common and important post-translational modifications.

[a] Adapted from Mann *et al*. 2003.

Table 2. Specific effects of tyrosine O-sulfation[a]

Effect	Chemokine Receptor	Details
Increased binding of chemokines	CMV US28	Viral receptor CMV US28: Increased binding to CCL3*, CCL4†, CCL5‡, CX3CL1§
Increased binding of chemokines	CXCR3	Receptor CXCR3: Increased binding to CXCL9‖, CXCL10**, CXCL11††
	CXCR4	Receptor CXCR4: Increased binding to CXCL12‡‡
	CCR2b	Receptor CCR2b: Increased binding to CCL2§§
	CCR5	Receptor CCR5: Increased binding to CCL3*, CCL4†, and CCL5‡
Increased binding to chemokine/ adhesion molecules	CX3CR1	Receptor CX3CR1: Increased firm adhesion to CX3CL1§
	CXCR3	Receptor CXCR3: Increases chemotaxis after binding to CXCL9‖, CXCL10**, and CXCL11††
Increased signaling/ Downstream events	CCR2b	Receptor CCR2b: Increased Ca^{++} influx and chemotaxis after binding to CCL2§§
	CX3CR1	Receptor CX3CR1: Increased Ca^{++} influx after binding to CXC3L1§

* CCL3/MIP-1α
† CCL4/MIP-1β
‡ CCL5/RANTES.
§ CX3CL1/Fractalkine.
‖ CXCL9/MIG.
** CXCL10/IP-10.
†† CXCL11/I-TAC.
‡‡ CXCL12/SDF-1.
§§ CCL2/MCP-1.

[a] Adapted from Liu *et al*. 2008

Receptor Name	N-terminus
CXCR1	MSN----------I----------TDPQM-WDFDDLNFTGMPPAD--ED SPCML
CXCR2	MED----------F----------NME---------SDSFEDFWKGEDLSN----YSYSSTLPPFL--LDAAPCEP
CXCR3	MVL----------EVSDHQVLNDAEVAALLE----------NFS-SS D ---GENESDSCCT-SPPCPQ
CXCR4	MEG----------ISIYTSDN TEEMGS----------G-D- ----------DSM-KEPCFR
CXCR5	MNY----------PLTLEMDLENLEDLFWEL----------DRLD----NY-----NDTSL--VENHLCPA
CXCR6	MAE----------H----------D HED----- -G-F-------SSFN--DSSQEEH
CCR1	MET----------P----------NTTED DTTTEF-D ---------GD---ATPCQK
CCR2	MLS----------T--------SRSRFIRNTNESGEEVTTFFD ---------D-Y-GAPCHK
CCR3	MTT----------S----------LDTVETFGTTSY----- ----------DDV-GLLCEK
CCR4	MNP----------T---------DIADTTLDESIYSNY--YL-Y----------ESI-PKPCTK
CCR5	MDY----------Q----------VSSPIYDIN----- ----------DDV-GLLCEK
CCR6	MSG----------ESMNFSDVFDSSEDYFVSVNT--SY-----Y--------SVDSE-MLLCSL
CCR7	MDLGKPMKSVLVVALLVIFQVCLCQDEVTDDYI----------GD--NTT----VDY---------TL--FESLCSK
CCR8	MDY----------T----------LDLSVTTVTD ----YY---------PD-IFSSPCDA
CCR9	MTP----------TDFTSPIPNMADD GSESTS----------SMED- VNFN----------F-T-DFYCEK
CCR10	MGT----------E----------ATEQVSW--GHYSGDEEDA -------SAEP-L-PELCYK
CX3C1	MDQ----------F----------PESVTENFE ----------DDL-AEACYI
XCR1	MES----------S----------GNPESTTFFY-----Y----------DLQ-SQPCEN

Table 3[a]. Conservation of tyrosine sulfation sites in human chemokine receptors. Sulfation sites with scores of 2.5 or higher are in black. Sites with intermediate scores between 1.5 and 2.5 are in gray.

[a] Adapted from Liu ,*et al*. 2008

Table 4. TPSTs purified from many source.

Source	Sulfotransferase species	Type	Substrate	Reference
Mammalian cell	rat TPST	3Y1 & SRA-3Y1	3Y1 & SRA-3Y1	Liu M.C., et al. *J. Biochem*. 1987
	human TPST-HPC4	CHO-K1 cell		
	human TPST-HPC4	293T cell	PSGL-1	Ouyang Y., et al. *Proc Natl Acad Sci*. 1998
	mouse TPST	293T cell		
	human TPST-1 & 2	HEK293T cell	PSGL-1;CCR5	Seibert C.,et al. *Proc Natl Acad Sci*. 2002
	human TPST-1 & 2	293T cell embroynic kidney cell	PSGL-1	Mishiro E.,et al. *J. Biochem*.2006
Animal	rat (TPST)	rat liver	EAY	Rens-Domiano S.,et al. *J Biol. Chem*. 1989
	bovine (TPST)	bovine adrenal medulla	CCK; Sgi	Niehrs C,et al. *J Biol. Chem*. 1990
	Human (TPST)	human liver	EAY	Lin W.H.,et al. *Biochem. Pharmacol*. 1990
	bovine (TPST)	bovine adrenal medulla	(PKG or others)	Niehrs C,et al. EMBO J. 1990
	rat Salivary Glands (TPST)	Rat Salivary Glands	EAY	Kasinathan C,et al. Gen. Pharmacol. 1995
	rat Salivary Glands (TPST)	Rat Submandibular Salivary Glands	EAY	William S.,et al. Arch. Biochem. Biophys. 1997
	rat liver	rat liver	PSGL-1	Ouyang Y. et al.Proc Natl Acad Sci. 1998
	Rat Liver (TPST)	Rat Liver	EAY	Ramaprasad P.,et al. Gen Pharmacol. 1998
	rat TPST	rat liver	EAY	Kasinathan C.,et al. Alcohol. 1998
	Human Saliva (TPST)	Human Saliva	Statherin; EAY	Kasinathan C.,et al. Int. J. Biol. Sci. 2007

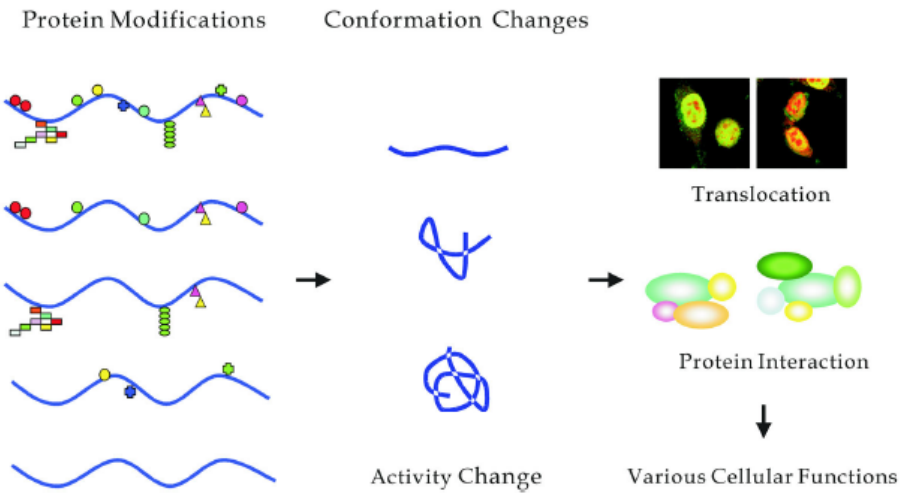


Figure 1[a] Schematic representation of protein modifications related to the regulation of biological processes.

[a] Adapted from Seo *et al*. 2004

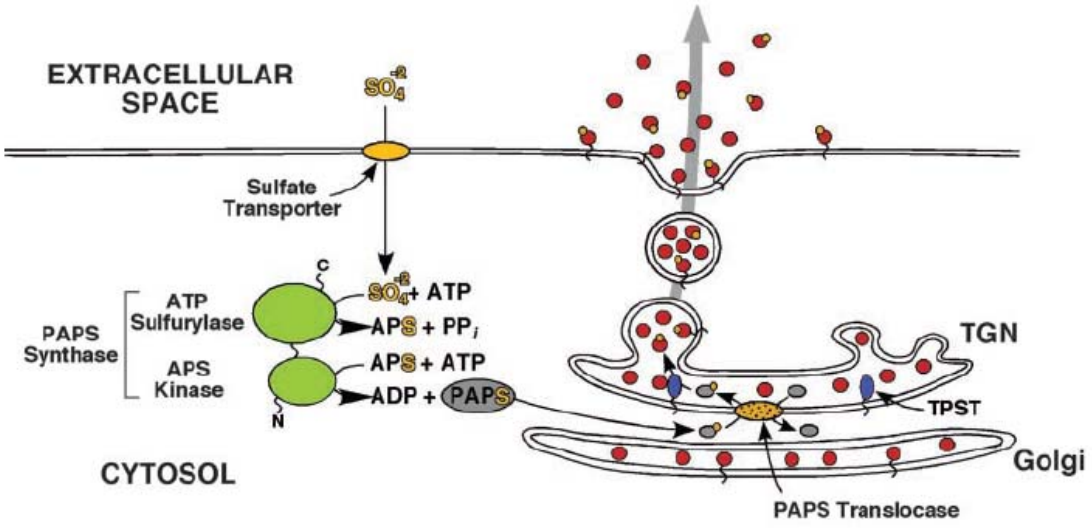


Figure 2[a]. Sulfate activation and tyrosine *O*-sulfation.

[a] Adapted from Moore *et al*. 2003

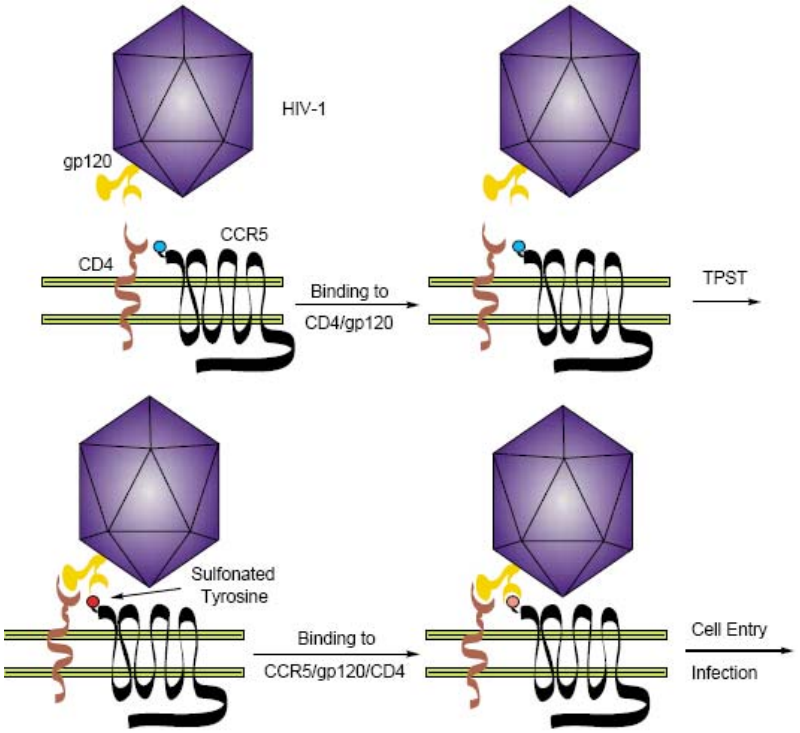


Figure 3[a]. Schematic representation of cell entry by HIV-1 following sulfation of CCR5 by a tyrosylprotein sulfotransferase.

[a] Adapted from Chapman *et al*. 2004.

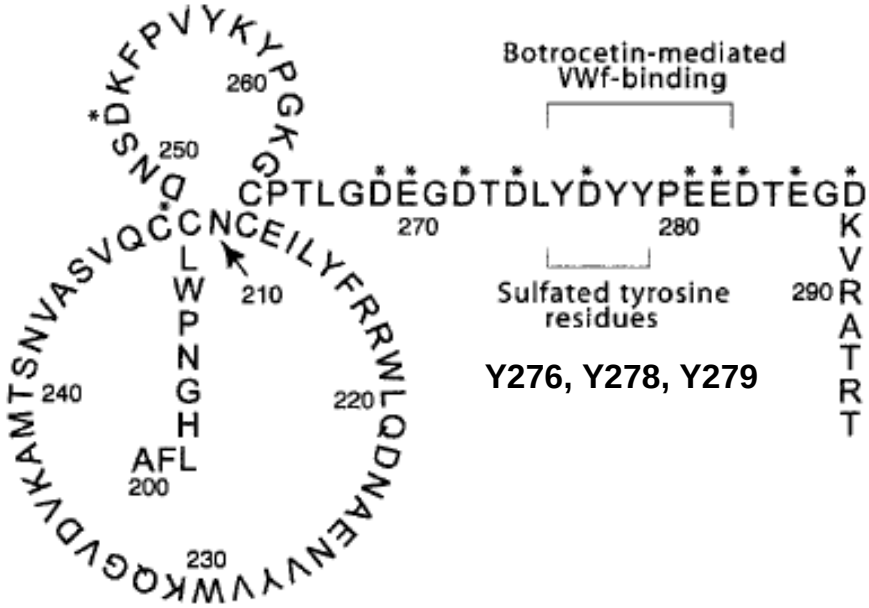


Figure 4[a] GPIbα from amino acid 200-294

[a] Adapted from Murata *et al*. 1991

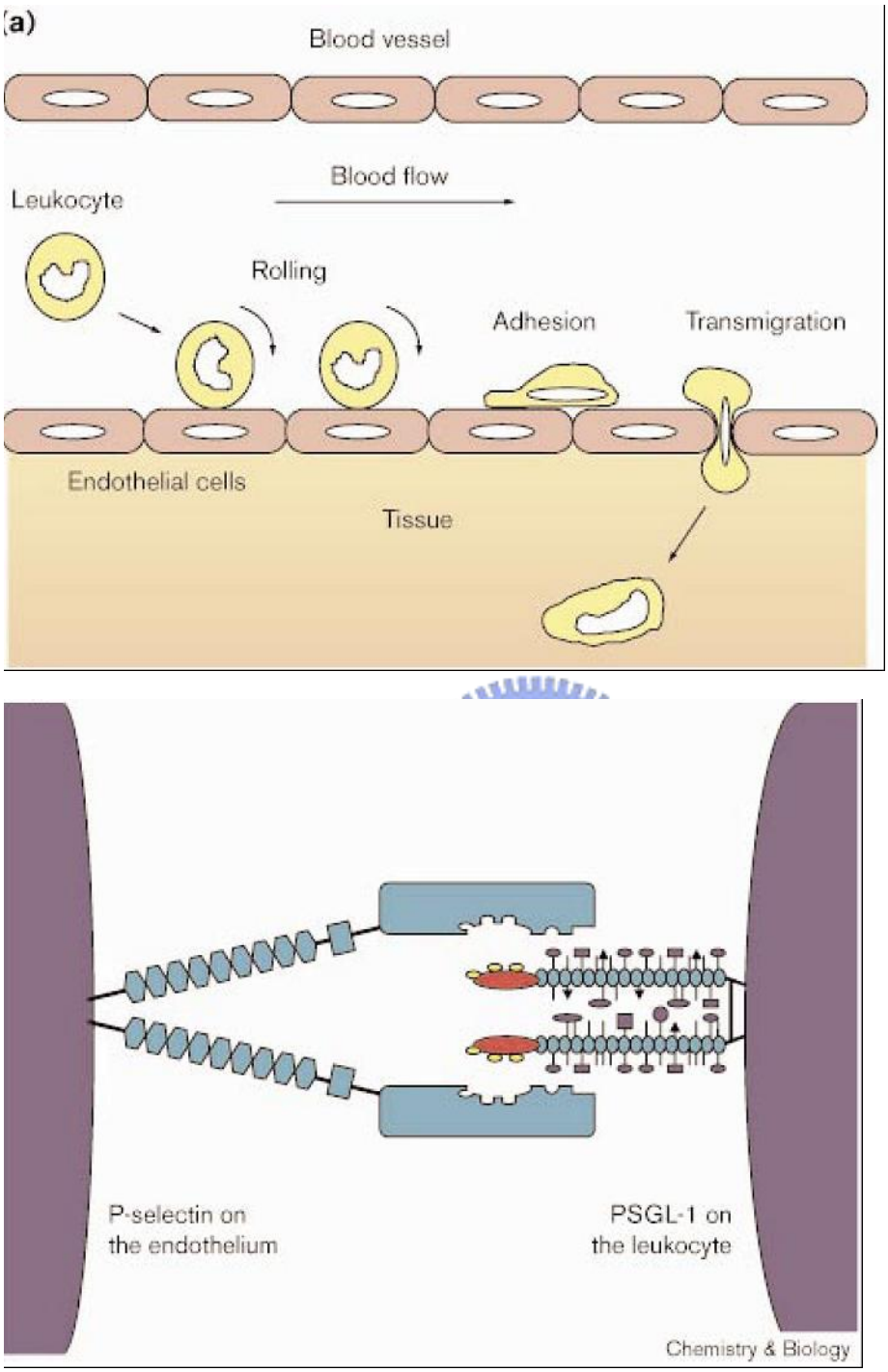


Figure 5 [a]. Tyrosine sulfation plays an important role in the immune response.

[a] Adapted from Kehoe *et al*. 2000

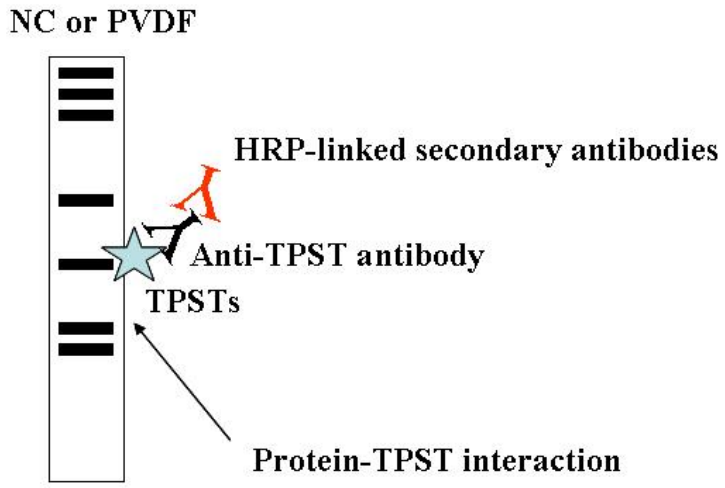


Figure 6. Graphical presentation of the far-Western immunoblot technique.
☆ = TPSTs, Y = Anti-TPST antibody, Y = HRP-linked secondary antibodie

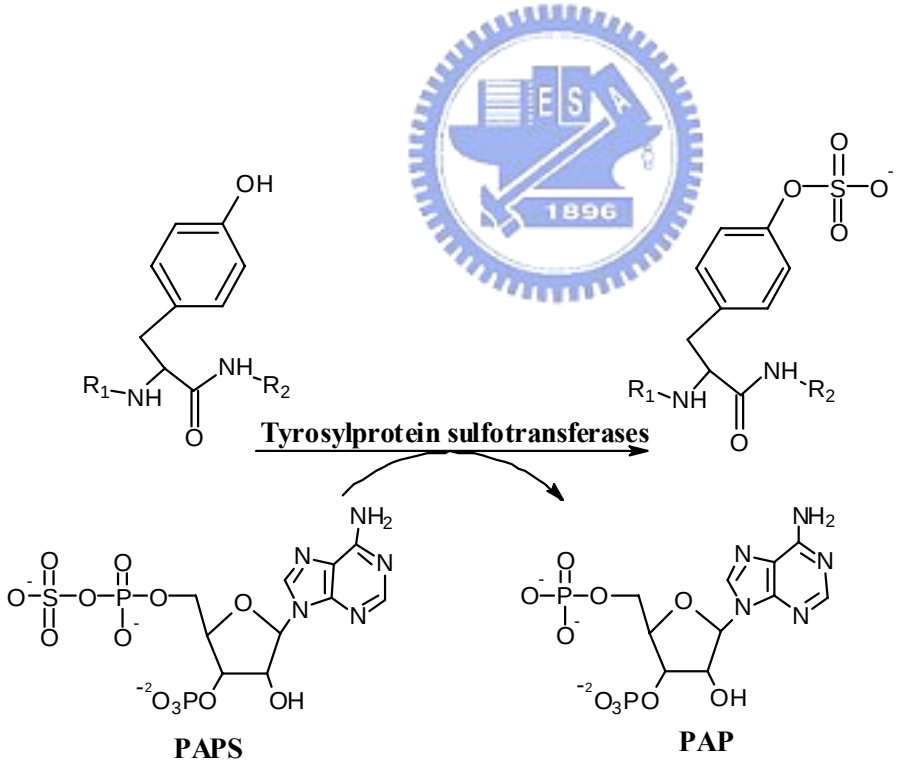


Figure 7. The catalyzed reaction of tyrosylprotein sulfotransferase.

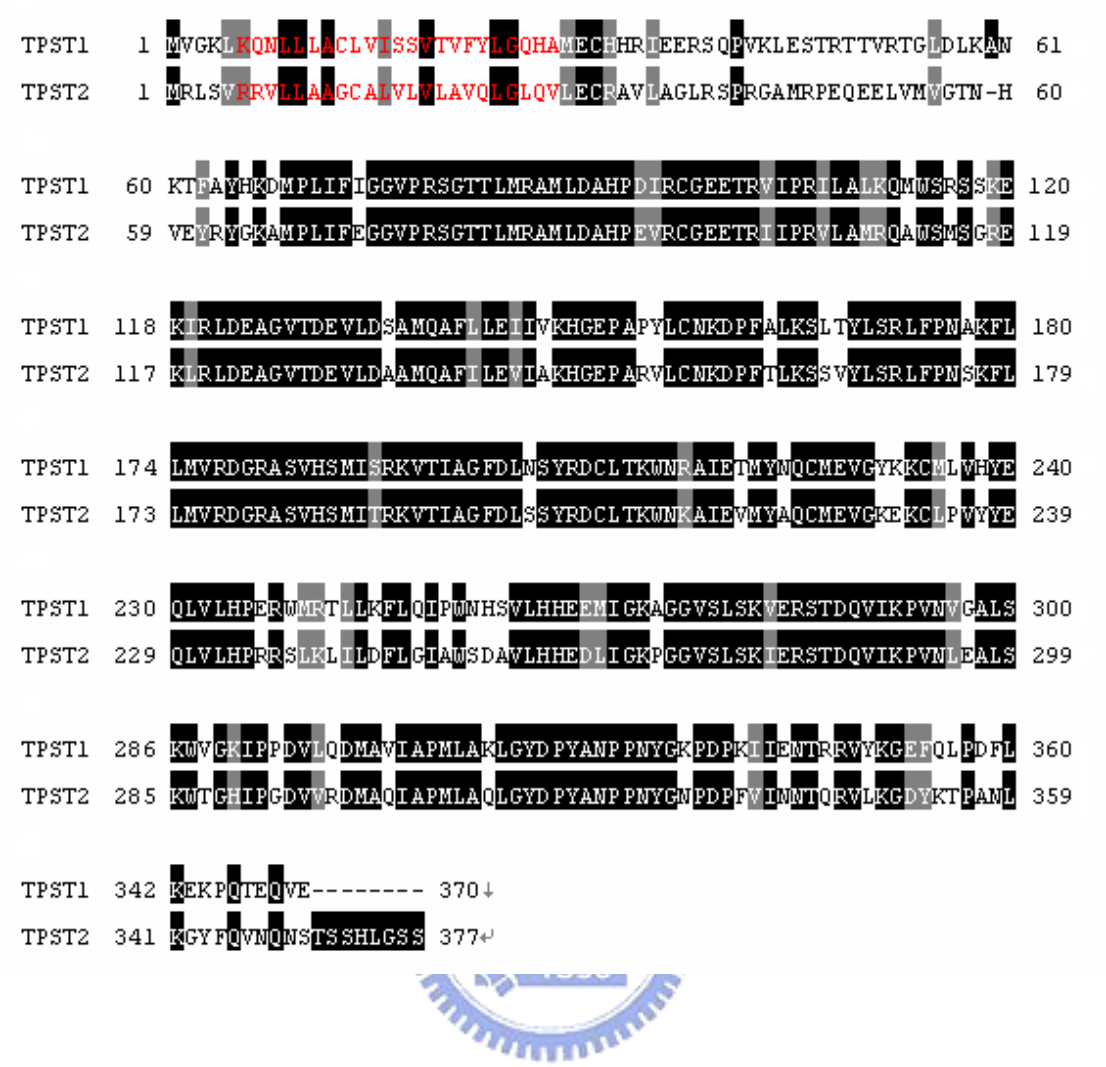


Figure 8. Sequence alignment and transmembrane domain analysis of tyrosyl protein sulfotransferase. The sequence pairwise alignment is performed by ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html) and sorted shading by BOXSHADE server (http://www.ch.embnet.org/software/BOX_form.html). The *black* background indicated identity to each other and the *gray* one meant conserved substitutions. The residue colored in *red* is the predicted transmembrane domain calculated by PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/psiform.html) ranged from residue 6 to 28 both for human TPST1 and TPST2.

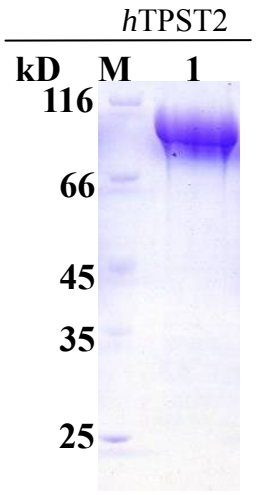
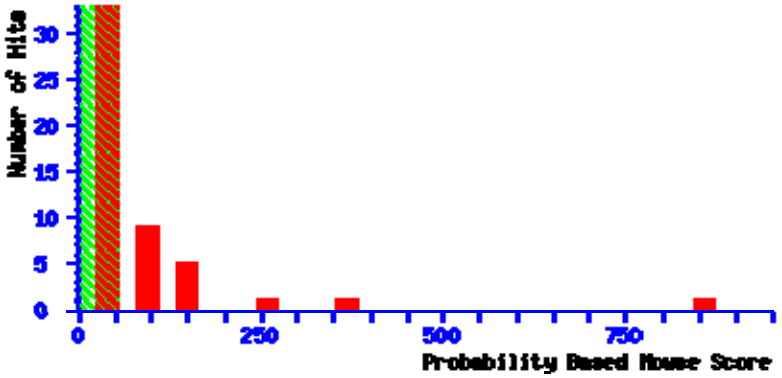


Figure 9. Prokaryotic expression and purification of human *h*TPST2. The prokaryotic expression of human TPST2 was optimized to reach the maximal soluble amount and purified to near homogeneity. Lane 1 was purified from HisTrap chromatography followed the procedure as described in the "Experimental procedures." Lane M was protein marker consisted of β-galactossidase (116.0 kDa), bovine serum albumin (66.2 kDa), ovalbumin (44 kDa), lactate dehydrogenase (35 kDa), and restriction endonuclease Bsp98I (25 kDa).

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  1 MRLSVRRVLL AAGCALVLVL AVQLGQQVLE CRAVLAGLRS PRGAMRPEQE
 51 ELVMVGTNHV EYRYGKAMPL IFVGGVPRSG TTLMRAMLDA HPEVRCGEET
101 RIIPRVLAMR QAWSKSGREK LRLDEAGVTD EVLDAAMQAF ILEVIAKHGE
151 PARVLCNKDP FTLKSSVYLS RLFPNSKFLL MVRDGRASVH SMITRKVTIA
201 GFDLSSYRDC LTKWNKAIEV MYAQCMEVGK DKCLPVYYEQ LVLHPRRSLK
251 LILDFLGIAW SDAVLHHEDL IGKPGGVSLS KIERSTDQVI KPVNLEALSK
301 WTGHIPGDVV RDMAQIAPML AQLGYDPYAN PPNYGNPDPI VVNNTQRVLK
351 GDYKTPANLK GYFQVNQNST SSHLGSS
```

Figure 10. The protein of LC-MS-MS fingerprinting analysis was identified for human TPST2. The excised spot from SDS-PAGE was identified for human TPST2 by LC-MS-MS. The sequence (*red*) by mass fingerprinting was mapped to the protein sequence with high confidence. (The result was particularly descrided in **appendix**)

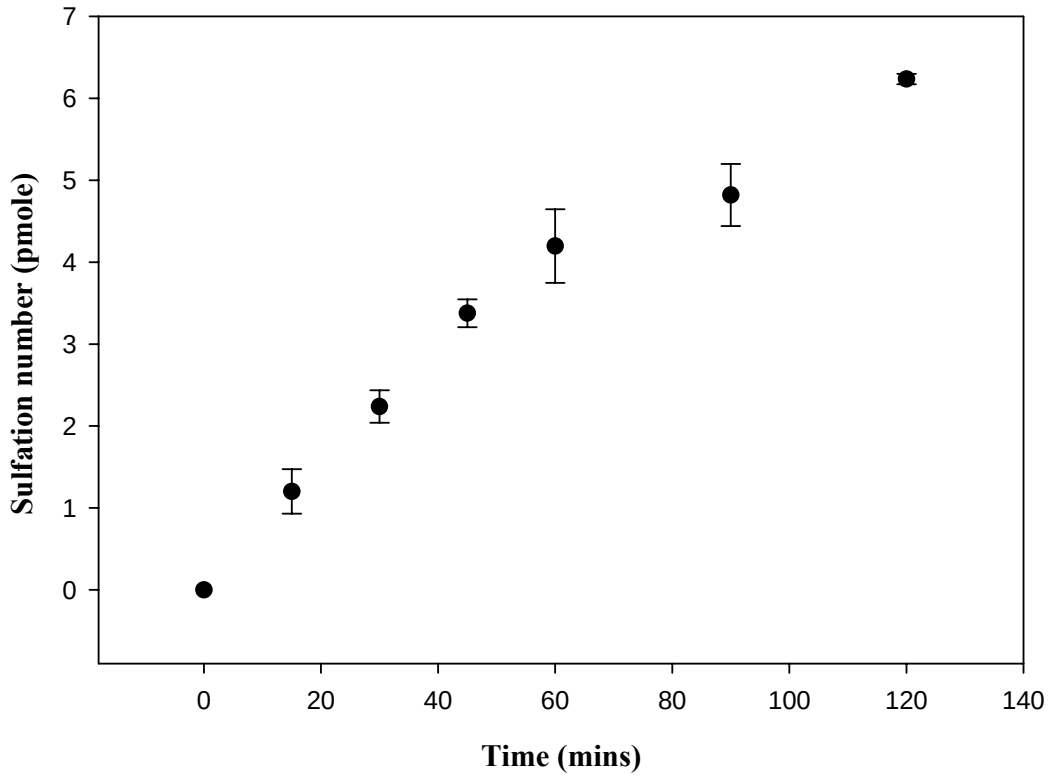

Figure 11. Reaction of polyEAY sulfation as a function of time. This experiment was performed as described under the "Experimental procedures." Each assay consisted of 50 mM MES at pH 6.0, 25 mM $MnCl_2$, 50 mM NaF, 0.5 μM [^{35}S]PAPS (15Ci/mmole), 40 μM TSH-R signaling polyEAY, 0.5 % Triton-X100 and 4 μg human TPST2 in a final volume of 10 μl. Data shown here is the result of a typical experiment performed in triplicate.

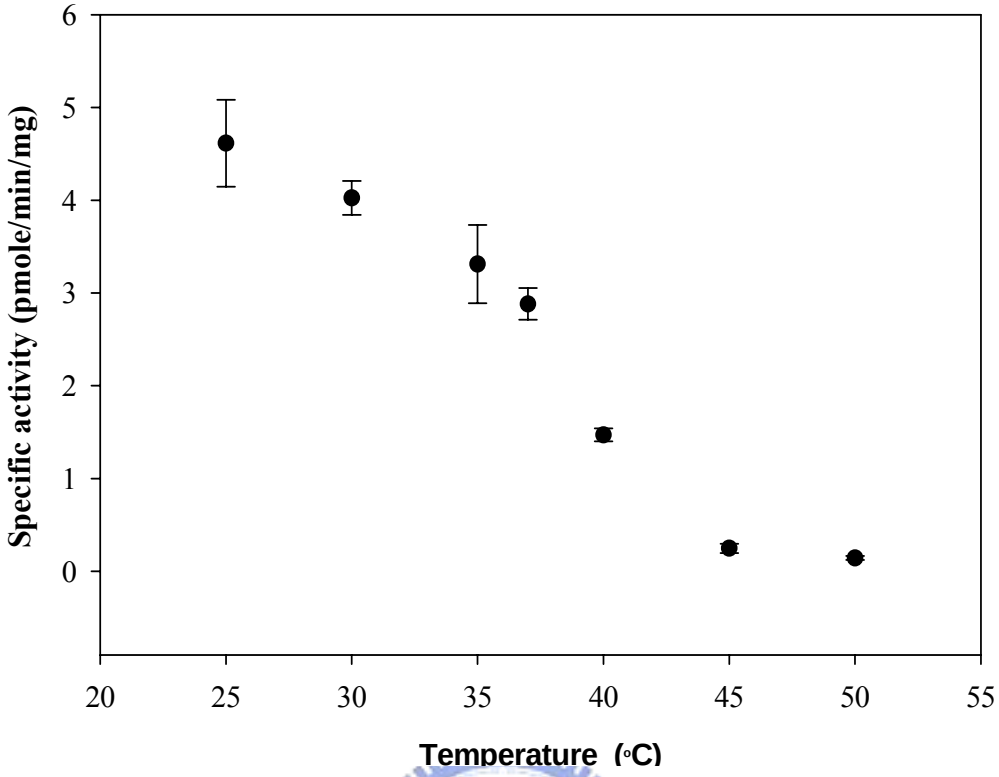

Figure 12. Effect of Temperature on *h*TPST2 activity. This experiment was performed as described under the "Experimental procedures." Using different temperature in the same mixture resulted in changes of the catalytic activity of human TPST2. Data shown here is the result of a typical experiment performed in triplicate.

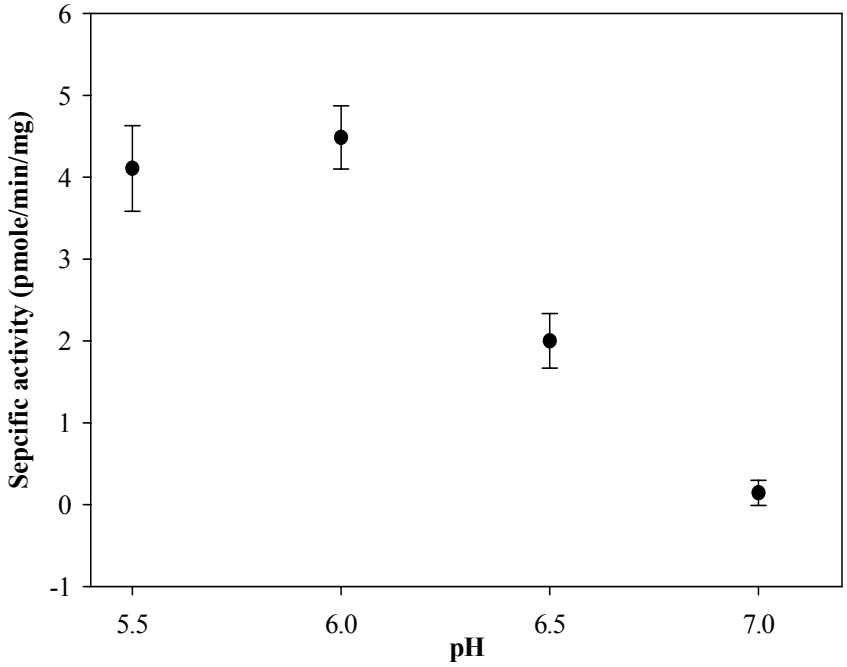

Figure 13. Different pH affects the catalytic activity of human TPST2. The experiment was performed as described under the "Experimental procedures." The result indicated pH affects of tyrosine O-sulfation in human TPST2 enzyme assay. The data was performed in triplicate.

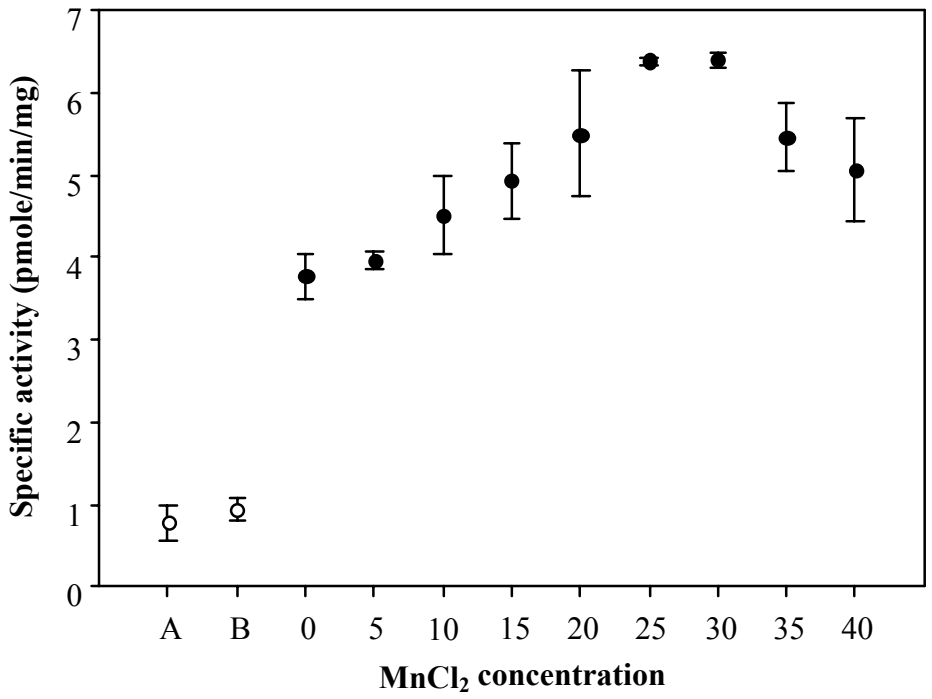

Figure 14. Effect of $MnCl_2$ on *h*TPST2 activity. This experiment was performed as described under the "Experimental procedures.".Data shown here was the result of a typical experiment performed in triplicate. This experiment was repeated three times with similar results. Point A indicated the total reaction without polyEAY and $MnCl_2$, and point B meant the total reaction without human TPST2 and MnC_2.

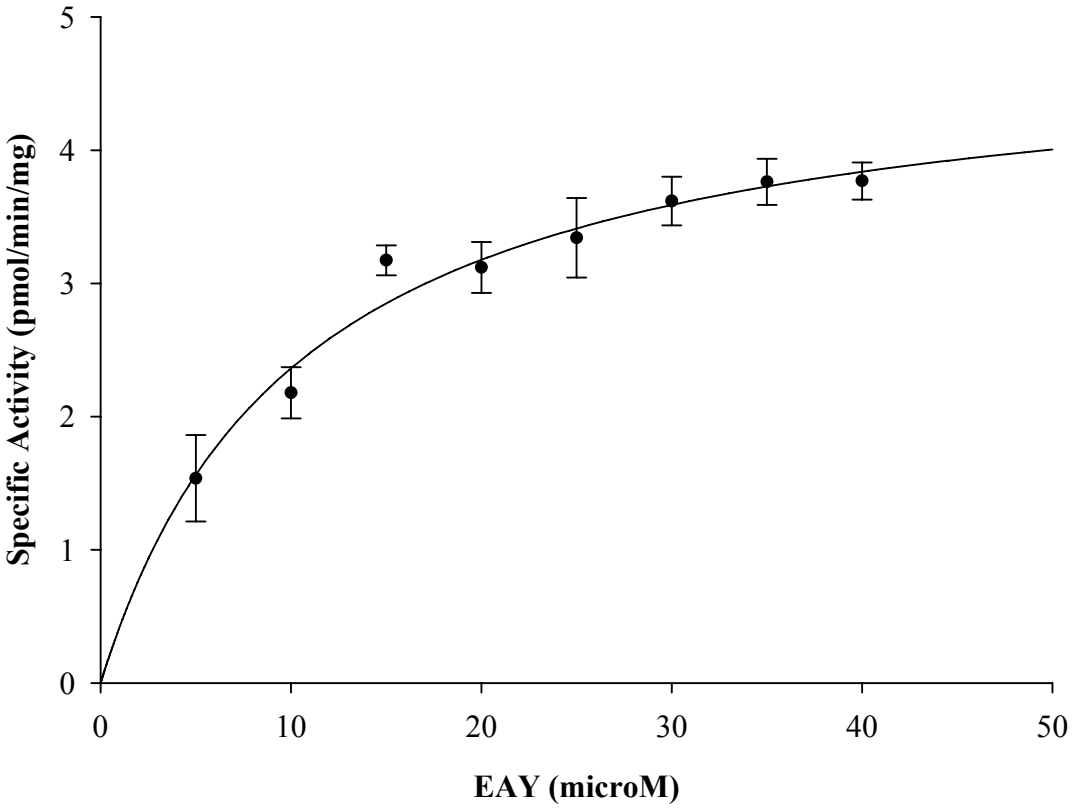

Figure 15. Kinetics of human TPST2 for polyEAY as substrate. Each assay consisted of 50 mM MES at pH 6.0, 25 mM $MnCl_2$, 50 mM NaF, 0.5 μM [^{35}S]PAPS (15Ci/mmole), various concentrations of polyEAY, and 4 μg human TPST2 in a final volume of 10 μl. Data shown here was the result of a typical experiment performed in triplicate. The data indicated the K_m was 10.5 ± 2.1 μM and V_{max} was 4.8 ± 0.3 pmole/min/mg. This experiment was repeated three times with similar results.

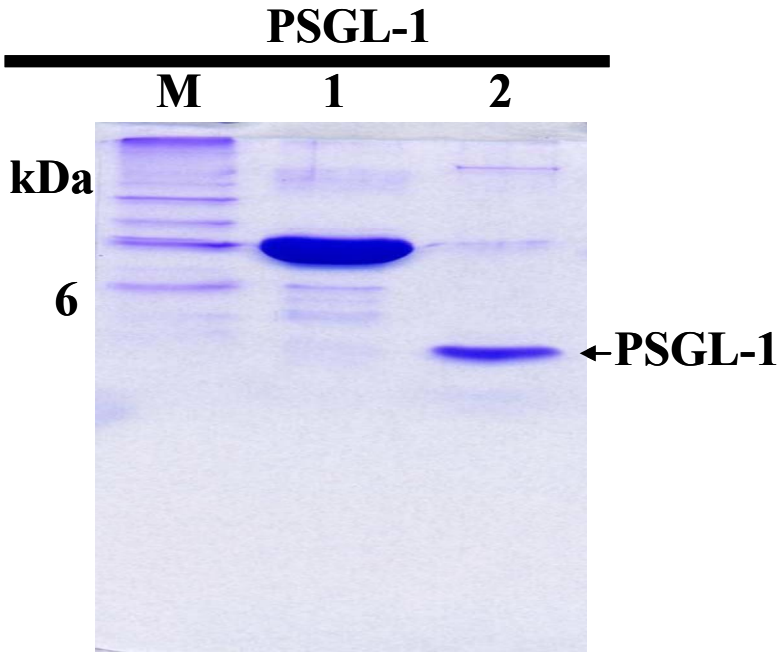

Figure 16. The SDS-PAGE of PSGL-1 peptide purification. Lane M was protein marker consisted of β-galactossidase (116.0 kDa), bovine serum albumin (66.2 kDa), ovalbumin (44 kDa), lactate dehydrogenase (35 kDa), and restriction endonuclease Bsp98I (25 kDa). Lane 1 was the elution of GST-fused PSGL-1. Lane 2 was PSGL-1 peptide purified from GSTTrap chromatography after the thrombin digestion.

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Appendix 1

Mascot Search Results

```
User            : BHC
Email           : J730703@YAHOO.COM.TW
Search title    :
MS data file    : C:\Documents and Settings\LEPE\桌面\MI704625.pkl
Database        : MSDB 20060831 (3239079 sequences; 1079594700 residues)
Timestamp       : 4 Aug 2008 at 18:06:03 GMT
Protein hits    : FJEC          transcription termination-antitermination factor nusA - Escheri
                  AAG41947      AF304164 NID: - Homo sapiens
                  Q4R863_MACFA  Testis cDNA clone: QtsA-13311, similar to human tyrosylprotein
                  Q4VAQ2_HUMAN  Keratin 2A (Epidermal ichthyosis bullosa of Siemens).- Homo sap
                  BAC29428      AK036436 NID: - Mus musculus
                  KRHU0         keratin 10, type I, cytoskeletal - human
                  K2C1_MOUSE    Keratin, type II cytoskeletal 1 (Cytokeratin-1) (CK-1) (Keratir
                  Q2C7T3_9GAMM  Transcription elongation factor NusA.- Photobacterium sp. SKA34
                  Q6IG03_RAT    Type II keratin Kb36.- Rattus norvegicus (Rat).
                  Q7VLI1_HAEDU  Transcription termination-antitermination factor nusA, N utiliz
                  Q8D2X5_WIGBR  NusA protein.- Wigglesworthia glossinidia brevipalpis.
                  Q3TTY5_MOUSE  10 days neonate skin cDNA, RIKEN full-length enriched library,
                  BAA19418      AB001594 NID: - Homo sapiens
                  TRPGTR        trypsin (EC 3.4.21.4) precursor - pig (tentative sequence)
                  Q1V532_VIBAL  Transcription elongation factor NusA.- Vibrio alginolyticus 12G
                  AAH03630      BC003630 NID: - Homo sapiens
                  Q1ZCR5_9GAMM  Transcription elongation factor NusA.- Psychromonas sp. CNPT3.
                  Q32MB2_HUMAN  K6IRS3 protein.- Homo sapiens (Human).
                  Q163L6_ROSDE  Oxidoreductase, short chain dehydrogenase/reductase family (EC
```

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 53 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

Error: try setting browser cache to automatic.

Peptide Summary Report

Format As [Peptide Summary] Help

Significance threshold p< [0.05] Max. number of hits [AUTO]

Standard scoring ◉ MudPIT scoring ○ Ions score or expect cut-off [0] Show sub-sets [0]

Show pop-ups ◉ Suppress pop-ups ○ Sort unassigned [Decreasing Score] Require bold red

[Select All] [Select None] [Search Selected] ☐ **Error tolerant**

1. FJEC **Mass:** 54837 **Score:** 858 **Queries matched:** 25

transcription termination-antitermination factor nusA - Escherichia coli (strain K-12)

2. AAG41947 **Mass:** 66027 **Score:** 352 **Queries matched:** 8

AF304164 NID: - Homo sapiens

3. Q4R863_MACFA **Mass:** 41823 **Score:** 254 **Queries matched:** 6

Testis cDNA clone: QtsA-13311, similar to human tyrosylprotein sulfotransferase 2),.- Macaca fascicularis (Crab eating macaque) (Cynomolgus monkey)

4. Q4VAQ2_HUMAN **Mass:** 65393 **Score:** 176 **Queries matched:** 3
Keratin 2A (Epidermal ichthyosis bullosa of Siemens).- Homo sapiens (Human).
5. BAC29428 **Mass:** 42056 **Score:** 165 **Queries matched:** 4
AK036436 NID: - Mus musculus
6. KRHU0 **Mass:** 59492 **Score:** 147 **Queries matched:** 3
keratin 10, type I, cytoskeletal - human
7. K2C1_MOUSE **Mass:** 65052 **Score:** 144 **Queries matched:** 3
Keratin, type II cytoskeletal 1 (Cytokeratin-1) (CK-1) (Keratin-1) (K1)
8. Q2C7T3_9GAMM **Mass:** 54979 **Score:** 122 **Queries matched:** 5
Transcription elongation factor NusA.- Photobacterium sp. SKA34.
9. Q6IG03_RAT **Score:** 119 **Queries matched:** 2
Type II keratin Kb36.- Rattus norvegicus (Rat).
10. Q7VLI1_HAEDU **Mass:** 55131 **Score:** 103 **Queries matched:** 3
Transcription termination-antitermination factor nusA, N utilization substance protein A
11. Q8D2X5_WIGBR **Mass:** 55614 **Score:** 101 **Queries matched:** 4
NusA protein.- Wigglesworthia glossinidia brevipalpis.
12. Q3TTY5_MOUSE **Mass:** 70880 **Score:** 89 **Queries matched:** 2
10 days neonate skin cDNA, RIKEN full-length enriched library, clone:4732404G19 product:
13. BAA19418 **Mass:** 25913 **Score:** 86 **Queries matched:** 2
AB001594 NID: - Homo sapiens
14. TRPGTR **Mass:** 24394 **Score:** 85 **Queries matched:** 2
trypsin (EC 3.4.21.4) precursor - pig (tentative sequence)
15. Q1V532_VIBAL **Mass:** 54909 **Score:** 69 **Queries matched:** 2
Transcription elongation factor NusA.- Vibrio alginolyticus 12G01.
16. AAH03630 **Score:** 68 **Queries matched:** 2
BC003630 NID: - Homo sapiens
17. Q1ZCR5_9GAMM **Mass:** 55527 **Score:** 68 **Queries matched:** 2
Transcription elongation factor NusA.- Psychromonas sp. CNPT3.
18. Q32MB2_HUMAN **Score:** 65 **Queries matched:** 2
K6IRS3 protein.- Homo sapiens (Human).
19. Q163L6_ROSDE **Mass:** 25428 **Score:** 56 **Queries matched:** 1
Oxidoreductase, short chain dehydrogenase/reductase family (EC 1.1.1.47).- Roseobacter

Appendix 2

pET-43.1a-c(+) Vector

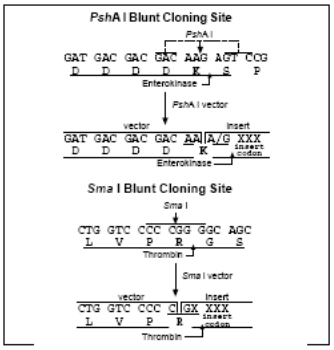
TB288 09/00

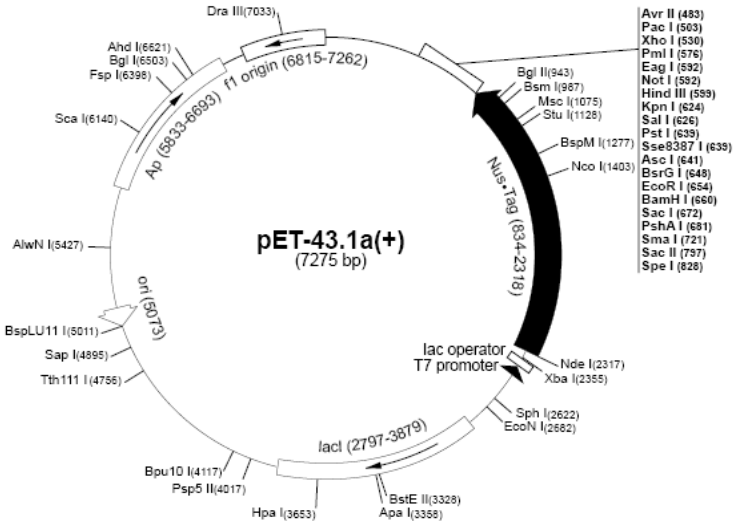
	Cat. No.
pET-43.1a(+) DNA	70939-3
pET-43.1b(+) DNA	70940-3
pET-43.1c(+) DNA	70941-3

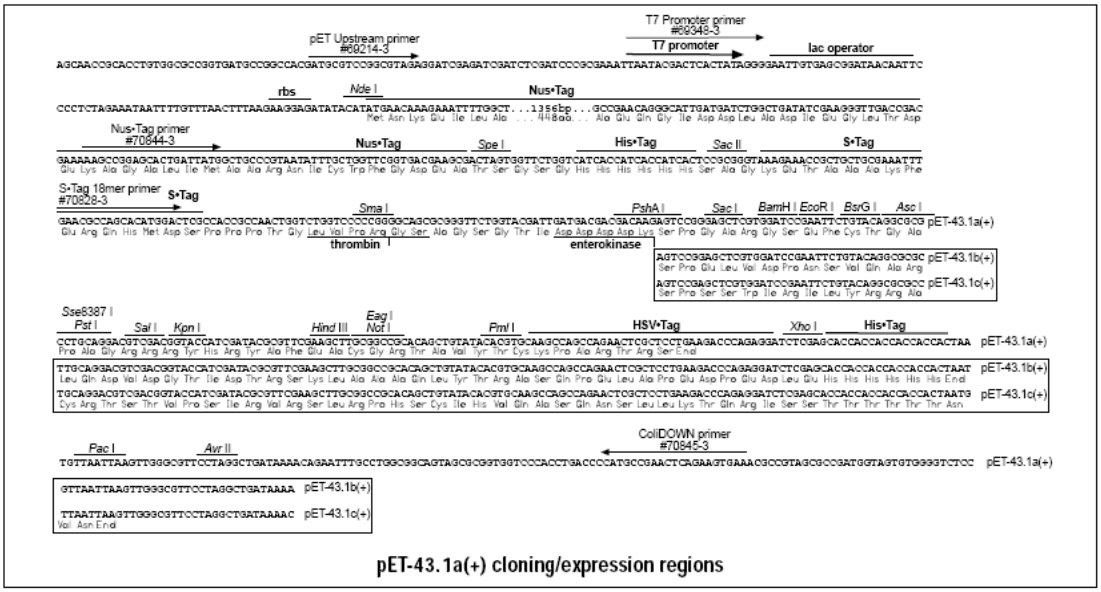
pET-43.1a(+) sequence landmarks

T7 promoter	2390-2406
T7 transcription start	2390
Nus•Tag™ coding sequence	834–2318
His•Tag® coding sequence	801–818
S•Tag™ coding sequence	747–791
Multiple cloning sites (*Sma* I-*Xho* I)	721–686
HSV•Tag® coding sequence	537–572
His•Tag® coding sequence	513–530
T7 terminator	27–73
*lac*I coding sequence	2797–3879
pBR322 origin	5073
bla (Ap) coding sequence	5833–6693
f1 origin	6815–7262

The pET-43.1 series of vectors are designed for cloning and high-level expression of peptide sequences fused with the 491 aa Nus•Tag™ protein. Unique sites are shown on the circle map. Note that the sequence is numbered by the pBR322 convention, so the T7 expression region is reversed on the circle map. The cloning/expression region of the coding strand transcribed by T7 RNA polymerase is shown below. The f1 origin is oriented so that infection with helper phage will produce virions containing single stranded DNA that corresponds to the coding strand. Therefore, single stranded sequencing should be performed using the ColiDOWN primer (cat. no. 70845-3). Vector encoded sequence can be completely removed when cloning into the *Psh*A I or *Sma* I sites (as shown below) by cleaving the Nus•Tag fusion protein with enterokinase or thrombin, respectively.







pET-43.1a(+) cloning/expression regions