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碩 士 論 文

黑腹果蠅蛋白質酪氨酸亞硫酸化酵素之鑑定與分析

Identification and Characterization of the Enzyme Responsible for Protein Tyrosine Sulfation in *Drosophila melanogaster*

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摘要

蛋白質酪氨酸的亞硫酸化為許多特定分泌性蛋白質或膜蛋白質很重要的後修飾。酪氨酸亞硫酸基轉移酶(tyrosylprotein sulfotransferase)負責的酪氨酸亞硫酸化，在細胞間的蛋白質與蛋白質交互作用，和許多重要的生物功能反應擔任關鍵的調節作用，其中包含人類免疫缺陷病毒(HIV)感染入侵、發炎反應、凝血機制、不孕...等等。然而這些生理與病理的機制都還是不清楚的。利用已經被解碼的染色體序列和生物資訊系統分析的協助，搜尋表達序列標記(EST)的資料庫，發現有一段基因可能負責黑腹果蠅體內酪氨酸亞硫酸化的反應。本研究將具有酵素活性的黑腹果蠅酪氨酸亞硫酸基轉移酶(*Dm*TPST)表達在大腸桿菌BL21(DE3)pLysS寄主細胞，純化出高產率均質的酵素，並且探討其特性；利用polyEAY當作受質，定義出黑腹果蠅酪氨酸亞硫酸基轉移酶理想的最佳反應狀況。最後，藉由基因重組的酪氨酸亞硫酸基轉移酶催化了drosulfokinin的亞硫酸化，證明能夠進一步的解釋黑腹果蠅蛋白質酪氨酸的亞硫酸化未知的機制與功能。

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ABSTRACT

Protein tyrosine sulfation, catalyzed by tyrosylprotein sulfotransferase (TPST), is one of the most common post-translational modifications towards secretory and transmembrane proteins. Protein tyrosine sulfation is a key modulator of extracellular protein-protein interactions and responsible for various important biological functions including HIV entry, inflammation, coagulation, and sterility. These physiological and pathological mechanisms, however, are not clear. With the assistances of decoded genome sequences and bioinformatic analysis, a promising gene competent for catalysis of protein tyrosine sulfation in *Drosophila melanogaster* was discovered by searching the expressed sequence tag (EST) database. Enzymatically active *Drosophila melanogaster* TPST (*Dm*TPST) was first cloned, expressed in *Escherichia coli* BL21(DE3)pLysS host cells and purified to homogeneity in high yield. The homogeneous *Dm*TPST was characterized through radioactive assay with polyEAY as substrate and its optimal reaction conditions were determined. Finally, the drosulfokinin sulfation catalyzed by recombinant *Dm*TPST was firstly demonstrated, which provided direct link to tyrosine sulfation in *Drosophila melanogaster* and further opportunity to decipher the obscure mechanisms and functions of protein sulfation.

Acknowledgement

不知不覺的碩士班就過完了。當初剛考來交大時，還很擔心碩士的生活有沒有辦法很順利，畢竟來到了一個新的環境，而且碩士生活不像大學生一樣只需要拼命唸書就行，所以深怕無法步入軌道，但是在楊裕雄老師的實驗室中，從一剛開始的新生訓練開始，一步一步的將我帶入了研究的領域當中，在我碩士研究的期間，要感謝的人實在好多，真是多虧了這些夥伴在精神上或是實質上的幫助，才讓我順利的拿到碩士畢業證書。首先要感謝的就是我的老闆楊裕雄教授，看到老師總是帶著笑容亦師亦友的跟我們分享許多實驗或生活中的經驗，並且提供我們一個良好的研究環境，開啟我的研究生涯並且讓我了解生為一個研究生應該具備的觀念。再來是已經畢業離開 LEPE 實驗室的學長姐們：小米學姊，謝謝妳當初在實驗上的教導，讓我了解到實驗操作中嚴謹的態度。秀華學姊，坐在我隔壁，跟我說了實驗室的一些守則跟大家的個性，讓我融入其中。音汝學姊，當初要出國時妳就像是導遊一樣，規劃的非常周到，讓我們可以好好參加研討會又可以觀光。再來是生電組的組員：呈允學長，一直要到了你快畢業時的這幾個月才跟你比較熟，歡迎你以後時常回來跟我們敘敘舊。淵仁學長，一直耳聞你興趣非常廣泛，能文能武，以會有機會再跟你請教。小志學長，非常謝謝你時常在 meeting 時從不同的觀點給我不同的意見，讓我了解我報告中的缺失。俊瑋，你非常的厲害，學習能力很強，但你現在正在休學中，也期待你可以回來，繼續你未完成的學業。欣怡，也恭喜你要跟我們一起畢業囉，以後也歡迎回來敘舊。曉萍，未來生電組的棟樑，生電組的一姊就由妳接手，把生電組帶向另一個境界吧。還有介於生電組與酵素組之間的第三類組，就成為整合組吧，這組唯一的成員奇叡，接下來你可要好好的我們兩組的研究架起一道橋樑吧。接下來就是我們酵素組的成員：首

先是我這組的組長，陸宜學長，非常感謝你在我們這組的貢獻，把我們這組照顧的這麼好，你不管在研究或是各方面都是略懂一點，但在我看來似乎只是謙虛而已，在接下來的日子裡，我還有很多要跟你學習的地方，希望你能夠多多包含囉。普普學長，也是一位非常有趣的學長，非常感謝你在實驗上給的意見，我也覺得你懂的東西非常多，之後也希望你能夠多多的教導。小胖學長，謝謝你當初帶我們做新生訓練，其實我知道你都是為新生好，所以才會比較嚴苛，但這一切都是等到我跟你比較熟後才體會的，有你在我們這組增加了許多的樂趣，往後也請你多多照顧，一起弄好酵素組吧。咏馨，從學姊變成同學，第一次看到妳時，就覺得妳很厲害，畢竟也在 LEPE 待久了，所以很多實驗室的狀況妳都知道，接下來也要一起好好的把酵素組的研究給延伸下去。資翔，也恭喜你要跟我們一起畢業了，也非常感謝你延伸我的研究，雖然你沒有時間可以完成後續發表的工作，但我好好好的整合你的研究，到時一起發表出去。芝綺，身為酵素組，但做的研究也是有跨到整合的部份，未來也希望妳能夠好好的把我們這組的研究給應用到生電組中吧。還有 Sonia，很感謝妳幫我們處理實驗室帳務上的問題，讓我們省去許多的時間。接下來就是我的同袍們：文燦與康寧，總是跟我在一起喇賽，雖然沒在同一組，但是你們總是可以給我一些實驗上的意見，而且也謝謝你們幫忙我處理實驗室的一些大小事，接下來也希望文燦可以找到好工作，當個傑出學長贊助 LEPE；康寧可以趕緊畢業，也期待妳健身有成囉。而且這一年來，實驗室的氣氛越來越好，實在是很喜歡這種感覺，希望以後可以一直維持下去，畢業的各位，也多多回來關照與敘舊，讓新生們了解你們的豐功偉業。接下來要感謝各位參加我碩士論文口試的老師們：彭惠玲、汪宏達、簡慶德老師們，感謝你們再我碩士口試中給的意見，讓我知道當中的缺失，讓我可以好好的修改我的碩士論文，

使論文更加的完整。最後要感謝的就是我的家人：爸爸跟媽媽，總是擔心我們學業狀況，也擔心未來的出入，也給我許多自主權，讓我沒有經濟的壓力，可以順利的唸完碩士。阿公跟阿嬤，年紀都很大了，寧願省吃儉用也要給我們這些孫子們好的照顧。姊姊跟妹妹，也很謝謝妳們給我的關心與照顧囉。致謝就寫到這邊，希望曾經幫助過我的大家都可以平安、順心，謝謝！

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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
CCK	Cholecystokinin
CCR5	Chemokine (C-C motif) receptor 5
CD4	Cluster of differentiation 4
D. melanogaster	*Drosophila melanogaster*
gp120	Glycoprotein 120
HIV	Human immunodeficiency virus
k_{cat}	Turnover number
kDa	Kilodaton
K_m	Michaelis constant
MALDI-TOFt	Matrix-assisted laser desorption ionization time of flight
MES	2-[N-morpholino] ethanesulfonic acid
PAGE	Polyacrylamide gel electrophoresis
PAP	Adenosine 3',5'-diphosphate
PAPS	3'-phosphoadenosine 5'-phosphosulfate
polyEAY	polypeptide(Glu : Ala : Tyr = 6 : 3 : 1)
PSGL-1	P-selectin glycoprotein ligand-1
PTM	Post-translational modification
SDS	Sodium dodecyl sulfate
STs	Sulfotransferases
TPST	Tyrosylprotein sulfotransferase
V_{max}	Maximum velocity

Introduction

1.1 Post-translational modifications

Post-translational modifications (PTMs) are one of the most important biological in both prokaryote and eukaryote proteins that can regulate the protein functions and activities by causing the changes of the protein structure or the affinity of dynamic interaction between proteins and compounds. (**Appendix 1**) (Seo *et al*. 2004) Some common and important post-translational modifications include acetylation, acylation, glycosylation, methylation, phosphorylation, ubiquitination, and sulfation (**Appendix 2**). These modifications can have both structural and regulatory functions, which modulate the properties of proteins by proteolytic cleavage or by the addition of a modifying group to amino acid, which may involve proteins' activity state, localization, turnover, and interaction with other proteins. Sulfation and phosphorylation are similar in modifying group, mass altered, and molecular interaction. (Mann *et al*., 2003).

1.2 Sulfotransferases

Sulfonation reactions are usually classified by the acceptor group involved in sulfoconjugation, for instance, *O*-sulfonation (ester), *N*-sulfonation (amide), and *S*-sulfonation (thioester). *O*-Sulfonation is dominant in cellular sulfation reaction which includes an alcohol group and can occur with diverse, relatively small

endogenous compounds such as catecholamines, steroids, thyroid hormones, and vitamins, and macromolecules such as glycosaminoglycans, proteoglycans, proteins, and galactoglycerolipids. *N*-Sulfonation is a crucial reaction in the modification of carbohydrate chains in macromolecules such as heparin, heparan sulfate proteoglycans, and also involved in the metabolism of xenobiotics. (Strott, 2002). Sulfate-containing biomolecules were identified in 1876 (Baumann *et al*. 1876), but the mechanism of sulfation remains unknown until the active 3'-phosphoadenosine 5'-phosphosulfated (PAPS) was isolated. Sulfotransferases(STs) use PAPS as sulfate group (SO^{3-}) donor, to catalyze the sulfuryl group into a variety of amine and hydroxyl substrates (**Appendix 3**). STs can be basically divided into two classes: cytosolic STs and membrane-associated STs. Cytosolic STs are soluble proteins located in cytoplasm, and mediated small chemical compounds including steroids, xenobiotics, dietary carcinogens, and neurotransmitters. They are involved in detoxification, hormone regulation, and drug metabolism. Membrane-associated STs are membrane anchored proteins located in the *trans*-Golgi network (TGN), which implied that they are involved in the post-translational modification of larger biomolecules including carbohydrates and protein such as heparan, glycoproteins, and oligopeptide. They are mainly involved in molecular-recognition events and biochemical signaling pathways (Chapman *et al*., 2004).

1.3 Tyrosylprotein sulfotranferases

Tyrosine *O*-sulfation of protein was first discovered in bovine fibrinopeptide B by Bettelheim in 1954 (Bettelheim, 1954). However, limited information was known about tyrosylprotein sulfation until 1982, when Huttner directly identified that this PTM was mediated by tyrosylprotein sulfotransferase (TPST), an enzyme that catalyzes the transfer of a sulfuryl group from PAPS to the hydroxyl group of tyrosine residue in the protein/peptide (**Fig. 1 step B**) (Moore, 2003). Furthermore, Huttner proved that TPST was membrane-bound and located in the *trans*-Golgi network (**Appendix 4**) (Baeuerle and Huttner, 1987), and also characterized and purified TPST from bovine adrenal medulla (Niehrs and Huttner, 1990). It is now known that TPST is a widespread enzyme in multicellular eukaryotic organisms throughout the plant and animal kingdoms, and can be detected in most tissues and cell types from humans and rats.(Mishiro *et al.*, 2006) (Nishimura and Naito, 2007). TPST 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. 2**)(Baeuerle *et al*. 1987; Lee *et al*. 1985). In most species, TPST have two isoenzyme: TPST-1 and TPST-2, but *D. melanogaster* have only a single TPST gene. The TPST-1 and TPST-2 share 65-68% sequence identity. Furthermore, tyrosine-sulfated proteins, TPST activity or putative TPST orthologs have not been described in prokaryotes or in yeast. (Moore *et al*. 2003). The SwissPort Group

developed a software, called Sulfinator (http://ca.expasy.org/tools/sulfinator) (Monigatti *et al*., 2002), which predicts possible proteins that can process tyrosine sulfation and also its tyrosine sulfation site. It has been estimated that up to approximately 1% of all tyrosine residues in eukaryotic cells are predicted to undergo tyrosine sulfation, but only a few hundred proteins have been identify presently (Seibert and Sakmar, 2008).

1.4 Biological functions of protein tyrosine sulfation

TPSTs catalyze the sulfation of tyrosine residues within specific peptide sequences, which have also been implicated in several crucial physiological and pathological mechanisms. Current thinking holds that this PTM serves as a key modulator of protein–protein interactions of secreted and membrane-bound proteins (John W Kehoe *et al*., 2000). Tyrosine sulfation has been implicated in intracellular trafficking and proteolytic processing of secreted proteins, and a key modulator of extracellular protein-protein interactions, which includes hormonal regulation, hemostasis, inflammation and infectious diseases (Seibert and Sakmar, 2008).

1.4.1 Chemokine receptor

Chemokine are small, secreted proteins that exert many biological functions

through G-protein-coupled receptors, including leukocyte trafficking, angiogenesis, angiostasis, viral infections, and host immune response to cancer (Zlotnik *et al*., 1999). Several chemokine receptors (CXCR3, CXCR4, CCR2b, CCR5, and CX3CR1) have been shown to undergo tyrosine sulfation (Farzan *et al*. 1999; Farzan *et al*. 2002; Preobrazhensky *et al*. 2000; Fong *et al*. 2002; Colvin *et al*. 2006). Currently, the most popular topic on the study of tyrosine sulfation focuses on CCR5 due to its involvement of HIV-1 entry. The chemokine receptor CCR5 is post-translationally modified by sulfation of its N-terminal tyrosines. Sulfated tyrosines contribute to the binding of MIP-1α, MIP-1β, and HIV-1 gp120/CD4 complexes and to facilitator HIV-1 to enter cells expressing CCR5 and CD4 (**Appendix 5**). The N terminus of CCR5 contains four tyrosines at positions 3, 10, 14, and 15(Samson et al., 1996), and modified stepwise at positions 14 or 15, followed by position 10 and finally the tyrosine residue at position 3 (Sasaki *et al*., 2007). Mutation of the four sulfotyrosine residues in CCR5 to phenylalanine and chlorate inhibits HIV infection by 50–75%. This information suggests that inhibiting tyrosine sulfation of CCR5 may provide a basis for the design of therapeutic agents aimed at blocking HIV-1 cellular entry.

1.4.2 Leukocyte adhesion and inflammatory response

P-selectin glycoprotein ligand-1 (PSGL-1) is a glycoprotein found on leukocyte

cell and endothelial cells that binds to P-selectin. In immune response, the leukocytes need to reach the inflammation site through passage of the blood circulation, the then roll upon, adhere to, and finally transmigrate between the endothelial cells and infective site (**Appendix 6a**). The extreme amino terminus of PSGL-1 carries three tyrosine sulfation sites. These sulfate esters, and specific glycans on PSGL-1, are key binding determinants for P-selectin (**Appendix 6b**). The binding between PSGL-1 of leukocyte and P-selectin of endothelial cells is essential for leukocyte adhesion in this inflammatory response (Kehoe and Bertozzi, 2000; Pouyani and Seed, 1995). Treatment of PSGL-1 with a bacterial arylsulfatase releases sulfate from tyrosine reduced the binding ability to P-selectin (Wilkins *et al*., 1995), and the results were also supported by point mutagenesis of tyrosine (Sako *et al*., 1995). According to result, TPST has become a therapeutic target for autoimmune diseases caused by chronic inflammation, such as rheumatoid arthritis and multiple sclerosis (Hsu *et al*., 2005).

1.4.3 Hemostasis and anticoagulation

The biological function of tyrosine sulfation is also involved in hemostasis. This modification is crucial in the interaction between many plasma proteins such as hirudin and thrombin (Stone *et al*. 1986), fibronectin and fibrin (Suiko *et al*. 1988), 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). Moreover, the complete mechanism of platelet attachment is accomplished by vWF that bridges subendothelial collagen and platelet membrane protein GP Ibα. The binding between vWF and n GP Ibα is dependent upon the sulfation of three tyrosine residues (Tyr276, 278, 279). In anticoagulation, hirudin is a potent anticoagulant protein secreted in the salivary gland of the leech. When Tyr63 has been sulfated, the tyrosine sulfation of hirudin has a 10-fold higher affinity for thrombin than unsulfated form, which prevents coagulation by inhibit thrombin (Stone and Hofsteenge, 1986).

1.5 Bottlenecks of protein sulfation research

In the last five decades of studies on this topic, many questions remain unknown about TPSTs and protein sulfation. The bottlenecks of studying TPSTs include the difficulty of characterizing TPST due to the lack of source of homogenous protein samples. It is hard to develop a fast and accurate assay for quantitative kinetics analysis, because sulfation detect limit in pico-mole level. Moreover, tyrosine *O*-sulfate may instability on the tyrosine residue of TPST substrate, which makes it difficult to detect or isolate sulfated proteins and peptides. Previous research on protein sulfation had focused on few TPST substrates as described above, therefore the understandings of TPST's roles are restricted by the biological regulations and

pathways of those few substrates. Because of tools and methods are undeveloped in protein sulfation, limited information available.

1.6 Tyrosylprotein sulfotransferase in *Drosophila melanogaster*

Most vertebrates (such as rat, cow, chicken, zebrafish, African clawed frog) and invertebrates (such as *Anopheles gambiae* (mosquito), and *Caenorhabditis elegans)* have two TPSTs. It is interesting to note that *Drosophila melanogaster* is so far the only species that was discovered to contain a single TPST gene (Moore, 2003). Therefore, *D. melanogaster* is a good model to study TPST, which a complete elimination of protein sulfation modification can be reached by a simple knockout or knockdown of a single gene. About 75% of known human disease genes have a recognizable match in the genetic code of fruit flies (Reiter et al., 2001), and 50% of fly protein sequences have mammalian analogues. Many advantages of using *D. melanogaster* as a study model include the short generation time and easy growth. Therefore, *D. melanogaster* is a suitable model to study protein tyrosine sulfation by using complete genetic tools to understand physiological and pathological mechanisms. The completion of genomic database is helpful for protein identification and its function. According to speculated that there are approximately up to 1% tyrosines of total proteins in an organism that are sulfated, but *D. melanogaster*

published less substrate, such as Drosulfakinin, Vitellogenin, and Glutactin (Nichols R. *et al.* 1988; Baeuerle P.A. *et al*. 1985; Olson P.F. *et al*. 1990).

1.7 Contribution from this study

This was the first research focused on the identification, cloning, expression and characterization of *Dm*TPST at protein level. Following the expression of *Dm*TPST in a prokaryotic system, the desired tyrosine sulfated proteins were further produced *in vitro* in a PAPS generating system. The homogeneous *Dm*TPST was characterized through the PAPS generating system with polyEAY as a substrate. Large quantity of homogeneous *Dm*TPST was obtained that facilitated further studies and applications in protein tyrosine sulfation. Optimal reaction conditions for *Dm*TPST catalysis and pH profile were determined. Finally, an endogenous compound of *D. melanogaster*, drosulfakinin, was demonstrated to serve as substrate of recombinant *Dm*TPST. The results indicated that recombinant *Dm*TPST can further decipher the ignorant mechanisms and functions of protein tyrosine sulfation in *Drosophila melanogaster*.

2. Materials

Adenosine 5'-triphosphate (ATP), tris[hydroxymethyl]aminomethane (Tris), 2-[N-morpholino]ethanesulfonic acid (MES), poly-(Glu6, Ala3, Tyr1) (EAY: M_r 33KDa), inorganic pyrophosphatase, and imidazole were purchased from Sigma (St. Louis, MO, USA). Potassium phosphate (dibasic), glycine, and sodium dodecyl sulfate (SDS) were obtained from J. T. Baker (Phillipsburg, NJ, USA). Sodium [^{35}S]sulfate (1050-1600 Ci/mmol) of 99.0% radiochemical purity was purchased from PerkinElmer (Boston, MA, USA). *Taq* polymerase, T4 DNA ligase, and reagents for PCR were obtained from New England Biolabs (Beverly, MA, USA). *Pfu* DNA polymerase was purchased from Stratagene (La Jolla, CA, USA). Expression vector and BL21(DE3) pLysS competent cells were purchased from Novagen (Madison, WI, USA). HisTrap sepharose was obtained from GE Healthcare (Uppsala, Sweden). Cellulose thin-layer chromatography (TLC) plates were products of Merck (Whitehouse Station, NJ, USA). All other chemicals were of the highest purity commercially available.

3. Experimental procedures

3.1 Prediction of transmembrane domain of *Dm*TPST

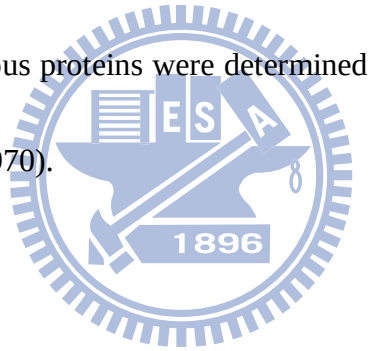
The transmembrane region and orientation of *Dm*TPST were predicted on the website—PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/psiform.html) (Jones, 2007). Only scores of hydrophobicity above 0 were considered significantly to be the potential transmembrane region.

3.2 Cloning of *Dm*TPST

The *Drosophila melanogaster* TPST cDNA was subcloned into pET-43a vector. The potential catalytic domain of TPSTs predicted above was amplified by PCR with specific primers designed to contain BamHI in the forward direction (5'-TGAAGAATTCGACGCCCCCAACGAGCTCTCCTC-3') and EcoR1 restriction sites in the reverse one contain XhoI restriction (5'-TGCCCTCGAGCTCTCCCACAGCATTCGATTGGC-3'). cDNA fragments were inserted into the EcoRI/XhoI double-restriction sites and then confirmed using an ABI Prism, 346 DNA sequencer (Applied Biosystems, Foster City, CA) following the standard protocol.

3.3 Protein expression and purification of *Dm*TPST

A single colony of BL21(DE3)pLysS consisted of *Dm*TPST plasmid was used to inoculate in the LB broth with ampicillin as the antibiotic at 37℃. Growth was continued to an ODA600 of 0.4–0.6 and then induced with 1 mM isopropyl-thio-β -D-galactoside (IPTG) for 24-hr incubation at 20°C. The cultures were centrifuged at 14000g for 20 minutes, and the pellet was sonicated in IMAC5 buffer (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 5 mM imidazole, and 10% glycerol) for *Dm*TPST. Further the HisTrap sepharose charged with $NiSO_4$ was utilized to the *Dm*TPST purification. The homogeneous proteins were determined by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970).



3.4 Mass analysis

The in-gel digestion of interested proteins was performed by the conventional protocol. MALDI-TOF was carried out to study the identification of excised proteins. The PMF data was analyzed by MASCOT based on the NCBInr database.

3.5 A assay of *Dm*TPST enzymatic activity

For the determination of *Dm*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 polyEAY. The couple-enzyme (human PAPS synthetase 1 and *Dm*TPST) radioactive assay was newly established for the measurement of *Dm*TPST activity. The complete assay mixture contained the following components: sulfate acceptor *Dm*TPST substrate (polyEAY), 4 mM inorganic $Na_2[^{35}S]SO_4$, 5 mM 2-mercaptoethanol, 1 mM $MgCl_2$, 50mM MES (pH6.5), 5 μg recombinant human PAPS synthetase 1 (*h*PAPSS1), *Dm*TPST, and 0.5 U pyrophosphatease in a final volume of 20 μl. Assays were initiated by the addition of the *h*PAPSS1 and incubated for 15 min at 37℃ followed by the addition of *Dm*TPST incubation for 45 min at 37℃. The reactions were terminated by heating at 95℃ for 2 min. 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/formic acid/water (5:4:1:3; by volume) as the solvent system. The dried plate was exposed with Kodak BioMax MR film which provide the optimal resolution for ^{35}S autoradiography.

3.6 Using enzymatic activity assay characterize *Dm*TPST

According to TPST enzymatic activity assay control *Dm*TPST amounts (from 0.5 g to 20 g) and, pH profile (from pH5.5 to pH8.5), reaction time (from 15mins to 120 mins). Finally, calculated the kinetics when polyEAY as substrate. The

*Dm*TPST enzymatic activity of different substrates was changed from polyEAY to drosulfakinin proceeding TPST enzymatic activity assay.

4. Results

4.1 Expression of recombinant *D. melanogaster* TPST in prokaryote expression system.

The expression vector, pET-43a, harboring *Dm*TPST cDNA was competent to express recombinant TPST in *E. coli*. The prokaryotic expression of TPST was optimized to reach the maximal soluble amount and purified to nearly homogeneity (**Fig. 3**). A 96-kDa protein showed on the SDS-PAGE was composed of NusA-tag fusion protein (60 kDa) and *Dm*TPST (36 kDa) upon treatment in coomassie blue R250. The spot excised from SDS-PAGE was analyzed by LC-MS/MS (**Fig. 4**). Two peptides (colored in red) come after trypsin digestion the alignment of these peptide sequences showed homology to *Dm*TPST with high scores of confidences. The purification table revealed the purification-related information of *Dm*TPST **(Table. 1**).

4.2 Sulfation of polyEAY using a PAPSS and TPST coupled system

Autoradiography on the cellulose TLC plate demonstrated that the *Dm*TPST activity could be determined under the enzymatic activity assay condition **(Fig. 5).** [^{35}S]-labeled EAY produced only in the presence of *Dm*TPST and complete PAPS regenerating system (lane 4). The [^{35}S]-labeled polyEAY could not be produced in the

absence of PAPSS (lane 1), polyEAY (lane 2) or *Dm*TPST (lane 3), respectively. These results indicated that PAPS produced through PAPSS catalytic reactions could be used for the sulfation of polyEAY catalyzed by *Dm*TPST.

4.3 Determination of the effective range and time course of *Dm*TPST enzyme catalysis

The linearly effective range of *Dm*TPST amount in the standard assay ranged within 5 μg as shown in **Fig. 6**. Accordingly, 4 μg *Dm*TPST was used in further experiments as standard assay. The time dependence of *Dm*TPST activity with polyEAY as substrate was examined. The concentrations of sulfate and polyEAY, were both saturated in the reactions. The tyrosine *O*-sulfation of polyEAY increased linearly with the incubation time from 15 to 120 minutes as shown in **Fig. 7**.

4.4 pH profile of *Dm*TPST

pH affects the electricity of amino acid and further contributes to the substrate binding affinity, enzymatic catalysis, and protein conformational structure. The pH profile of the recombinant *Dm*TPST was determined by measuring the activity at various pH values. The pH values from 5.5 to 8.5 were shown in **Fig. 8**. The optimal pH was 6.5 that showed the highest catalytic activity of *Dm*TPST.

4.5 Kinetics of *Dm*TPST with polyEAY as substrate

The kinetic constants toward polyEAY, which is synthetic polypeptides composed of Glu, Ala, and Tyr in the ratio 6:3:1, demonstrated that K_m and V_{max} was 11.5 μM and 4.5 nmole/min/mg, respectively (**Fig. 9**). It revealed that the expression of *Dm*TPST was active in the catalysis and performed the similar kinetic constants compared to the previous studies. **(Table 2.)**

4.6 Sulfation of endogenous substrate, drosulfakinin, by *Dm*TPST

Drosulfakinin, composed of 14 amino acid residues, is a known endogenous substrate in *Drosophila melanogaster* of *Dm*TPST. The result from **Fig. 10** revealed that the *Dm*TPST was competent to catalyze not only the synthetic peptide polyEAY (lane 4), but the endogenous substrate drosulfokinin. (lane 5).

5. Discussion

Tyrosine sulfation was discovered in 1950s in bovine fibrinogen (Bettelheim, 1954), and afterwards, the tyrosylprotein sulfotransferase (TPST) was identified to be responsible for this post-translational modification in 1982 (Huttner, 1982). Since the discovery of the tyrosine *O*-sulfation, little about the the enzyme mechanisms have been elucidated. This may be attributed to the lack of TPST related information, such as the difficulty of sourcing the homogeneous enzyme and ample amount of TPST, limited information of enzyme characteristics (kinetics), unstable sulfate groups on the substrate, and lack of sensitive detecting methods for the sulfated tyrosine. *Drosophila melanogaster* was chosen as the source of animal study due to easy growth, short generation span, solved genomic database, well-established transgenic tools, and more importantly, *D. melanogaster* only has a single *TPST* gene (Moore, 2003). The amino acid sequence of TPST in *D.melanogaster* shares 58% and 56% with human TPST1, and TPST2, respectively (**Fig. 2**). Approximately 75% of known human disease genes have a recognizable match in the genetic code of *D. melanogaster*, and 50% of *D. melanogaster* protein sequences have mammalian analogues (Reiter et al., 2001), which makes *D. melanogaster* an appropriate animal model for pathological studies on TPST.

According to the successful development of TPST expression in prokaryotic

system (Lu et al., unpublished), the NusA-fused *Dm*TPST was firstly obtained with maximal solubility and high purity (**Table 1 and** lane 2 in **Fig. 3**), and used for studying the enzymatic characterization. The purification yield of *Dm*TPST showed higher than that of *h*TPST2 and although the protein sequence of *Dm*TPST and *h*TPST2 has a similarity approximately 60%. The distinct characteristics between human and *D. melanogaster* TPST need to study further. The NusA-*Dm*TPST possessed high homogeneity in our study, however, the ratio of *Dm*TPST in this fusion protein was merely 35% and the total molecular weight was close to 100 kDa. The NusA protein obviously performed no interference with the enzymatic activity of *Dm*TPST and rendered high solubility to facilitate *Dm*TPST folding. Overall, this purification procedure of *Dm*TPST was simple with stable material source, great quantity, and homogeneous *Dm*TPST in this study.

By the facilitation of coupled enzyme reaction (*h*PAPSS1 and *Dm*TPST), the productive rate of tyrosine sulfation was faster than that of the conventional reaction which utilized PAPS directly as sulfate donor as shown in **Fig. 1** (Liu et al., unpublished). The approach avoided the contamination of PAPS from PAP (Rens-Domiano and Roth, 1989; Miller and Waechter, 1979). PAPS is extremely costly and it tends to hydrolyze easily to form PAP, a known competitive inhibitor of sulfotransferases (Lin and Yang, 1998). In this experimental design, *h*PAPSS1

generated saturated PAPS from inorganic sulfate, and this scheme could obviously prevent the background from the hydrolysis of PAPS. Moreover, the production of protein tyrosine sulfation by this method was extremely efficient than previous studies and it might potentially apply to spectrometric analysis in additional to radioactive assay (Liu et al., unpublished; Mishiro et al., 2006).

In this study, *Dm*TPST properties including the *Dm*TPST amount (**Fig. 6**), time dependence of the activities of *Dm*TPST (**Fig. 7)**, pH profile (**Fig. 8)**, and kinetic parameters of *Dm*TPST (**Fig. 9),** were examined. The optimal *Dm*TPST dosage and reaction time was 5 μg and 2 hours, respectively, which located in the linear range. In the pH-dependent experiment, *Dm*TPST displayed an optimal activity at pH 6.5 (**Fig. 8**), which was similar to that of TPST in human liver and rat submandibular salivary glands (Lin and Roth, 1990; William et al., 1997). The result of **Fig. 8** also indicated that potassium phosphate was inhibitory to the *Dm*TPST catalyzed sulfation of polyEAY.

According to the previous studies, the K_m values for of recombinant *h*TPST1 and *h*TPST2 obtained from cell culture (293T cells) using PSGL-1 as substrate was 9.67 and 26.9 mM, respectively; the V_{max} *h*TPST1 and *h*TPST2 was 3.95 and 71.4 pmol/min/mg (the k_{cat} was thus 1.67×10^{-4} and 2.99×10^{-3} min^{-1} for each other) (Mishiro et al., 2006). As coupled to the PAPS generating system using synthetic

substrate polyEAY, the K_m and V_{max} was individually 3.4 mM and 176 pmol/min/mg; the k_{cat} was thus 7.0×10^{-3}.(Liu et al., unpublished)(**Table. 2**). In this study, the K_m, V_{max}, and k_{cat} of *Dm*TPST towards polyEAy as substrates was 11.5 mM, 4.5 nmol/min/mg, and 1.6×10^{-1}, respectively (**Fig. 9**). Obviously, the K_m values were similar regardless of the TPST acquired from diverse sources, species and assayed by different methods. However, the distinct V_{max} measured from coupled-enzyme reaction was higher than that of previous method for approximately 10 folds, because the method made some modifications. Consequently the detection of polyEAY sulfate reached to nanomolarr range (weird) and contributed to the discovery of sulfated peptides in the future.

In the study, commercial polyEAY consisted of Glu, Ala, Tyr with random synthesis followed the ratio of 6:3:1. Consequently, standard substrate was urgent to be utilized in the assay. The *D.melanogaster* endogenous substrate, Drosulfokinin, was selected to analyze in the *Dm*TPST activity assay (**Fig. 10**). The data demonstrated that recombinant *Dm*TPST could not only catalyze synthetic peptide (polyEAY) but endogenous substrate (drosulfokinin). The recombinant *Dm*TPST will be used further in the aspects of substrate examination, substrate screening, and proteomic application.

In conclusion, we first purified *Dm*TPST from prokaryotic system and showed

the various purification characteristics as compared to human TPST. Furthermore, the combination of PAPS generating system facilitated to increase the catalytic rate of *Dm*TPST, and define *Dm*TPST optimal condition and kinetic parameters. This will be beneficial to not only the aspects of fundamental researches but apply to *Drosophila* protein sulfation in biological study.

References

1. Baeuerle, P. A., and Huttner, W. B. (1985) Tyrosine sulfation of yolk proteins 1, 2, and 3 in Drosophila melanogaster. *J. Biol. Chem.* 260:6434-6439.

2. Baeuerle P. A., and Huttner, W. B. (1987) Tyrosine sulfation is a trans-Golgi-specific protein modification. *J. Cell Biol.* **105**: 2655–2664.

3. Baumann. (1876) *Ber. Dtsch. Chem. Ges.* **9**:54-58.

4. Beisswanger, R., Corbeil, D., Vannier, C., Thiele, C., Dohrmann, U., Kellner, R., Ashman, K., Niehrs, C., and Huttner, W. B. (1998) Existence of distinct tyrosylprotein sulfotransferase genes: molecular characterization of tyrosylprotein sulfotransferase-2. *Proc. Natl. Acad. Sci. U. S. A.* 95:11134-11139.

5. Bettelheim, F. R. (1954) Tyrosine-O-sulfate in a peptide from fibrinogen. *J. Am. Chem. Soc.* 76:2838–2839.

6. Chapman E., Best M.D., Hanson S.R., and Wong C.H. (2004) Sulfotransferases: structure, mechanism, biological activity, inhibition, and synthetic utility. *Angew. Chem. Int. Ed. Engl.* **43**:3526-3548.

7. Colvin R.A., Campanella G.S., Manice L.A., and Luster A.D. (2006) CXCR3 requires tyrosine sulfation for ligand binding and a second extracellular loop arginine residue for ligand-induced chemotaxis. *Mol. Cell. Biol.* **26**:5838–5849.

8. Dong J., Ye P., Schade A.J., Gao S., Romo G.M., Turner N.T., McIntire L.V., and López J.A. (2001) Tyrosine sulfation of glycoprotein Ibα: role of electrostatic interactions in von Willebrand factor binding. *J. Biol. Chem.* **276**:16690-16694.

9. Farzan M., Mirzabekov T., Kolchinsky P., Wyatt R., Cayabyab M., Gerard N.P., Gerard C., Sodroski J., and Choe H. (1999) Tyrosine sulfation of the amino terminus of CCR5 facilitates HIV-1 entry. *Cell.* **96**:667–676.

10. Farzan, M., Babcock, G. J., Vasilieva, N., Wright, P. L., Kiprilov, E., Mirzabekov, T., and Choe, H. (2002) The role of post-translational modifications of the CXCR4 amino terminus in stromal-derived factor 1 alpha association and hiv-1 entry. *J. Biol. Chem.* 227:29484-29489.

11. Fong A.M., Alam S.M., Imai T., Haribabu B., and Patel D.D. (2002) CX3CR1 tyrosine sulfation enhances fractalkine-induced cell adhesion. *J. Biol. Chem.* **277**:19418–19423.

12. Fredrickson B.J., Dong J.F., McIntire L.V., and Lo´pez J.A. (1998) Shear-dependent rolling on von Willebrand factor of mammalian cells expressing the platelet glycoprotein Ib-IX-V complex. *Blood.* **92**:3684-3693.

13. Hsu, W., Rosenquist, G. L., Ansari, A. A., and Gershwin, M. E. (2005) Autoimmunity and tyrosine sulfation. *Autoimmun. Rev.* 4:429-435.

14. Huttner, W. B. (1982) Sulphation of tyrosine residues-a widespread modification of proteins. *Nature* 299:273-276.

15. Jones, D. T. (2007) Improving the accuracy of transmembrane protein topology prediction using evolutionary information. *Bioinformatics* 23:538-544.

16. Kehoe, J. W., and Bertozzi, C. R. (2000) Tyrosine sulfation: a modulator of extracellular protein-protein interactions. *Chem. Biol.* 7:R57-61.

17. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.

18. Lee, R. W., and Huttner, W. B. (1985) (Glu62, Ala30, Tyr8)n serves as high-affinity substrate for tyrosylprotein sulfotransferase: a Golgi enzyme. *Proc. Natl. Acad. Sci. U. S. A.* 82:6143-6147.

19. Leyte A., van Schijndel H.B., Niehrs C, Huttner W.B., Verbeet M.P., Mertens K., and van Mourik J.A. (1991) Sulfation of Tyr1680 of human blood coagulation factor VIII is essential for the interaction of factor VIII with von Willebrand factor. *J Biol Chem.* **266**:740-746.

20. Lin, E. S., and Yang, Y. S. (1998) Nucleotide binding and sulfation catalyzed by phenol sulfotransferase. *Biochem. Biophys. Res. Commun.* 271:818-822.

21. Lin, W. H., and Roth, J. A. (1990) Characterization of a tyrosylprotein sulfotransferase in human liver. *Biochem. Pharmacol.* 40:629-635.

22. Liu et al., unpublished.

23. Mann M, and Jensen O.N. (2003) Proteomic analysis of post-translational modifications. *Nat. Biotechnol.* **21**:255-261.

24. Marchese P., Murata M., Mazzucato M., Pradella P., De Marco L., Ware J., and Ruggeri Z.M. (1995) Identification of three tyrosine residues of glycoprotein Ibα with distinct roles in von Willebrand factor and α-thrombin binding. *J. Biol. Chem.* **270**:9571- 9578.

25. Mishiro, E., Sakakibara, Y., Liu, M. C., and Suiko, M. (2006) Differential enzymatic characteristics and tissue-specific expression of human TPST-1 and TPST-2. *J. Biochem.* 140:731-737.

26. Monigatti F, Gasteiger E, Bairoch A, and Jung E. (2002) The Sulfinator: predicting tyrosine sulfation sites in protein sequences. Bioinformatics. 18(5):769-70.

27. Monigatti, F., Hekking, B., and Steen, H. (2006) Protein sulfation analysis--A primer. *Biochim. Biophys. Acta* 1764:1904-1913.

28. Moore, K. L. (2003) The biology and enzymology of protein tyrosine O-sulfation. *J. Biol. Chem.* 278:24243-24246.

29. Murata M., Ware J., and Ruggeri Z.M., (1991) Site-directed mutagenesis of a soluble recombinant fragment of platelet glycoprotein Ibα demonstrating

negatively charged residues involved in von Willebrand factor binding. *J. Biol. Chem.* **266**:15474-15480.

30. Nichols R., Schneuwly S.A., Dixon J.E. (1988) Identification and characterization of a Drosophila homologue to the vertebrate neuropeptide cholecystokinin. J. Biol. Chem. 263:12167-12170.

31. Niehrs, C., and Huttner, W. B. (1990) Purification and characterization of tyrosylprotein sulfotransferase. *EMBO J.* 9:35-42.

32. Olson P.F., Fessler L.I., Nelson R.E., Sterne R.E., Campbell A.G., Fessler J.H. (1990) Glutactin, a novel Drosophila basement membrane-related glycoprotein with sequence similarity to serine esterases. *EMBO* J. 9:1219-1227.

33. Ouyang, Y., Lane, W. S., and Moore, K. L. (1998) Tyrosylprotein sulfotransferase: purification and molecular cloning of an enzyme that catalyzes tyrosine O-sulfation, a common posttranslational modification of eukaryotic proteins. *Proc. Natl. Acad. Sci. U. S. A.* 95:2896-2901.

34. Preobrazhensky A.A., Dragan S., Kawano T., Gavrilin M.A., G0lina I.V., Chakravarty L., and Kolattukudy P.E. (2000) Monocyte chemotactic protein-1 receptor CCR2B is a glycoprotein that has tyrosine sulfation in a conserved extracellular n-terminal region. *J Immunol* **165**:5295–5303.

35. Pouyani, T., and Seed, B. (1995) PSGL-1 recognition of P-selectin is controlled by a tyrosine sulfation consensus at the PSGL-1 amino terminus. *Cell.* **83**:333-343.

36. Reiter LT, Potocki L, Chien S, Gribskov M, Bier E. (2001) A systematic analysis of human disease-associated gene sequences in Drosophila melanogaster. Genome Res. 11(6):1114-25.

37. Sako D., Comess K.M., Barone K.M., Camphausen R.T., Cumming D.A., and Shaw G.D. (1995) A sulfated peptide segment at the amino terminus of PSGL-1 is critical for P-selectin binding. *Cell.* **83**, 323-331.

38. Seibert, C., and Sakmar, T. P. (2008) Toward a framework for sulfoproteomics: Synthesis and characterization of sulfotyrosine-containing peptides. *Biopolymers* 90:459-477.

39. Seo J., and Lee K.J. (2004) Post-translational modifications and their biological functions: proteomic analysis and systematic approaches. *J. Biochem. Mol. Biol.* **37**:35-44.

40. Stone S.R., and Hofsteenge J. (1986) Kinetics of the inhibition of thrombin by hirudin. *Biochemistry.* **25**:4622-4628.

41. Suiko M., and Liu M.C. (1988) Change in binding affinities of 3Y1 secreted fibronectin upon desulfation of tyrosine-O-sulfate. *Biochem. Biophys. Res. Commun.* **154**:1094-1098.

42. Ward C.M., Andrews R.K., Smith A.I., and Berndt M.C. (1996) Mocarhagin, a novel cobra venom metalloproteinase, cleaves the platelet von Willebrand factor receptor glycoprotein Ibα Identification of the sulphated tyrosine/anionic sequence Tyr-276–Glu-282 of glycoprotein Ibαas a binding site for von Willebrand factor and α-thrombin. *Biochemistry.* **35**:4929-4938.

43. Wilkins, P.P., Moore, K.L., McEver, R.P. and Cummings, R.D. (1995) Tyrosine sulfation of P-selectin glycoprotein ligand-1 is required for high affinity binding to P-selectin. *J. Biol. Chem.* **270**:22677-22680.

44. William S., Ramaprasad P., and Kasinathan C. (1997) Purification of tyrosylprotein sulfotransferase from rat submandibular salivary glands. *Arch. Biochem. Biophys.* **338**:90-96.

45. Zlotnik A., Morales J. and Hedrick J.A. (1999) Recent advances in chemokines and chemokine receptors. *Crit. Rev. Immunol.* **19**:1-47.

Tables

Table 1. Purification of NusA-*Dm*TPST from *E. coli.*

Step	Total Activity (pmole/min)	Total Protein (mg)	Specific Activity (pmole/min/mg)	Yield (%)	Purification fold
Crude extract	**37325**	**1420**	**26 . 3**	**100**	**1**
Ni-NTA column	**9720**	**12 . 4**	**783 . 8**	**26**	**29 . 8**

Table 2. Comparison of coupled enzyme assay-obtained kinetic characterization of *Dm*TPST with previous radiometric assay

Enzyme assay	Enzyme	Source	substrate	Kinetics			*References*
				V_{max} (pmol.min^{-1}.mg^{-1})	K_m (μM)	k_{cat} (min^{-1})	
Coupled-enzyme TPST assay	***Dm*TPST**	***E*. coli**	**polyEAY[a]**	**4459 ± 214**	**12 ± 2.5**	**1.6 × 10^{-1}**	**The present study**
	***h*TPST2**	***E*. coli**	**polyEAY[a]**	**176 ± 15**	**3.4 ± 1.2**	**7.0 × 10^{-3}**	**Liu et al., unpublished**
	***h*TPST2**	***E*. coli**	**PSGL-1[b]**	**3200 ± 170**	**24 ± 3.5**	**1.1 × 10^{-1}**	**Lu et al., unpublished**
Traditional PAP^{35}S assay	***h*TPST2**	***E*. coli**	**polyEAY[a]**	**4.8 ± 0.5**	**11 ± 3.0**	**4.8 × 10^{-4}**	**Liu et al., unpublished**
	***h*TPST1**	**293T cell**	**PSGL-1[b]**	**3.95**	**9.67**	**1.7 × 10^{-4}**	**Mishiro et al. (2006)**
	***h*TPST2**	**293T cell**	**PSGL-1[b]**	**71.43**	**26.89**	**3.0 × 10^{-3}**	**Mishiro et al. (2006)**

[a] polyEAY was synthesized followed the ratio of Glu : Ala : Tyr = 6 : 3 : 1.

[b] PSGL-1 was P-selectin glycoprotein ligand-1 N-terminal peptide (ATEYEYLDYDFL).

Figures

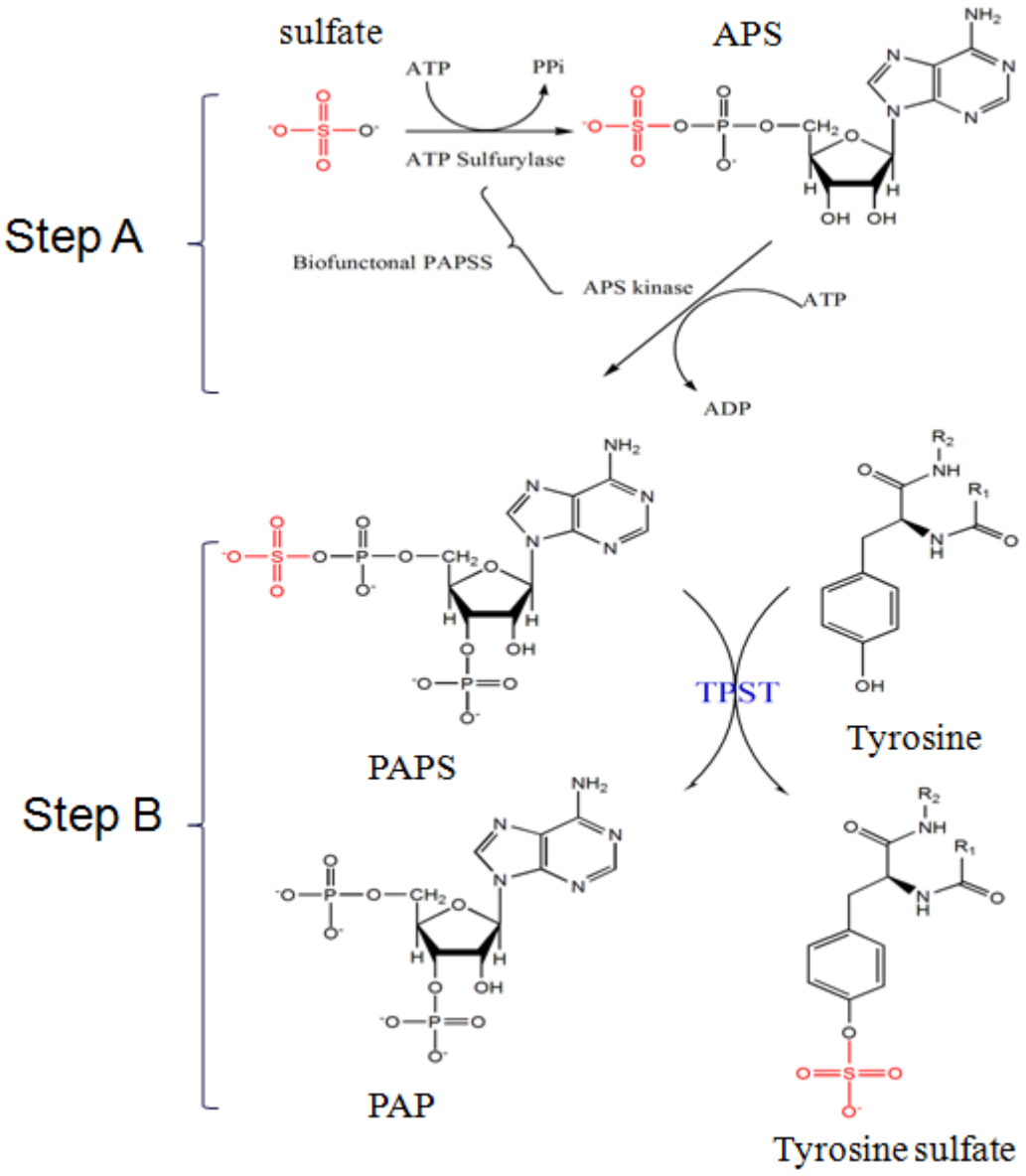


Figure 1. Scheme for the determination of TPST activity. Isotope-based analysis (^{35}S) was used for the *Dm*TPST assay using PAPS as the sulfuryl group donor. Biosynthesis of PAPS was catalyzed by PAPSS from ATP and SO_4^{2-} as shown in Step A. Step B showed the reaction catalyzed by TPST using tyrosylprotein as the sulfuryl group acceptor.

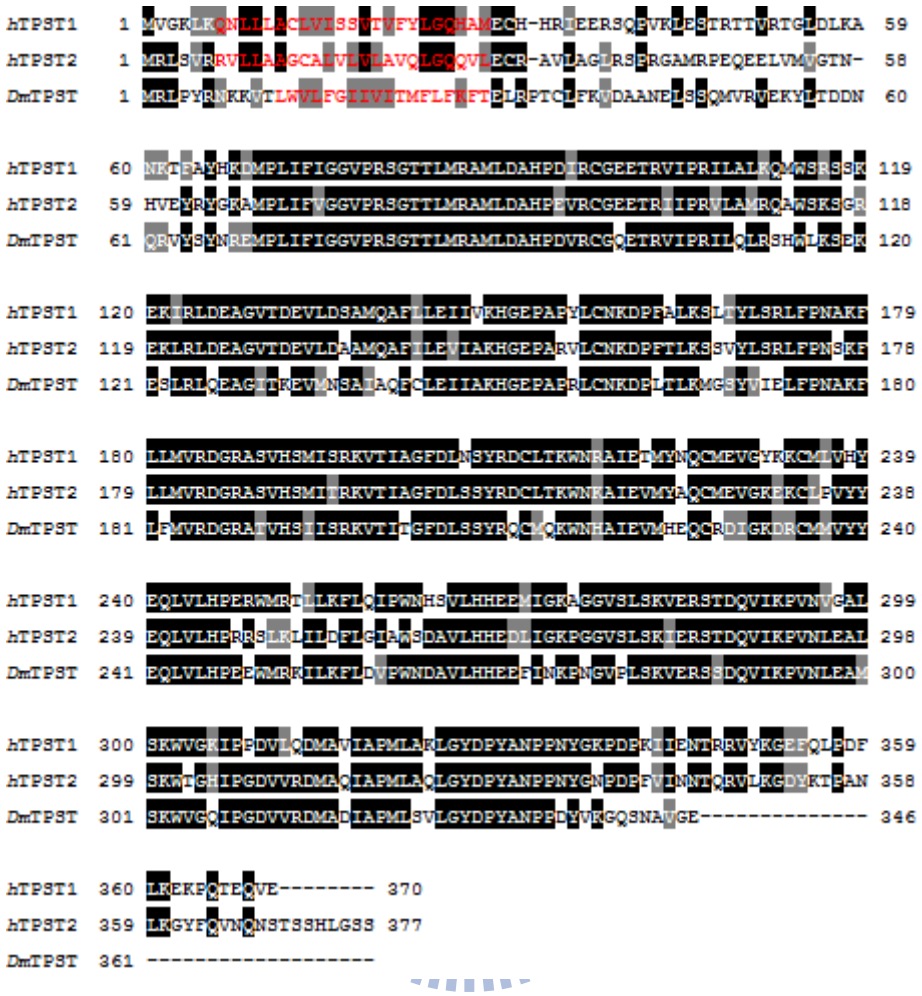


Figure 2. Bioinformatic analysis of protein sequence identity and transmembrane domain for human and *Drosophila melanogaster*.

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, and 12 to 28 for Drosophila melanogaster TPST, respectively. The pairwise sequence identity of these TPSTs for *Dm*TPST/*h*TPST-1 (58%), *Dm*TPST/*h*TPST-2 (56%), and *h*TPST-1/*h*TPST-2 (63%), respectively, was calculated.

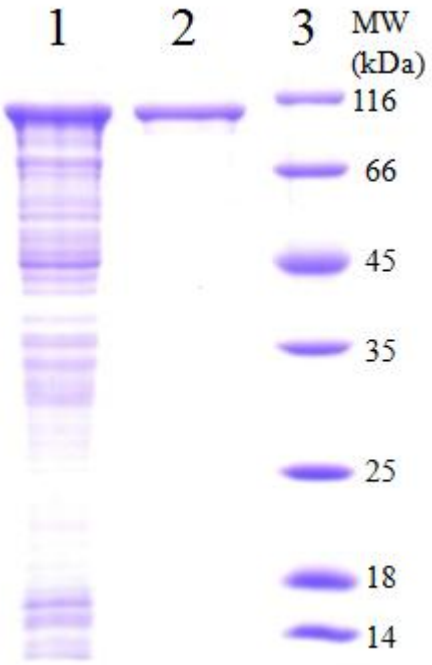


Figure 3. Purification of homogenous recombinant *Dm*TPST

The protein was expressed in BL21(DE3)pLysS cells and purified through His-Tag column. Lane 1 and lane 2 was crude extract and homogenous *Dm*TPST, respecticely, and lane 3 was standard protein marker.

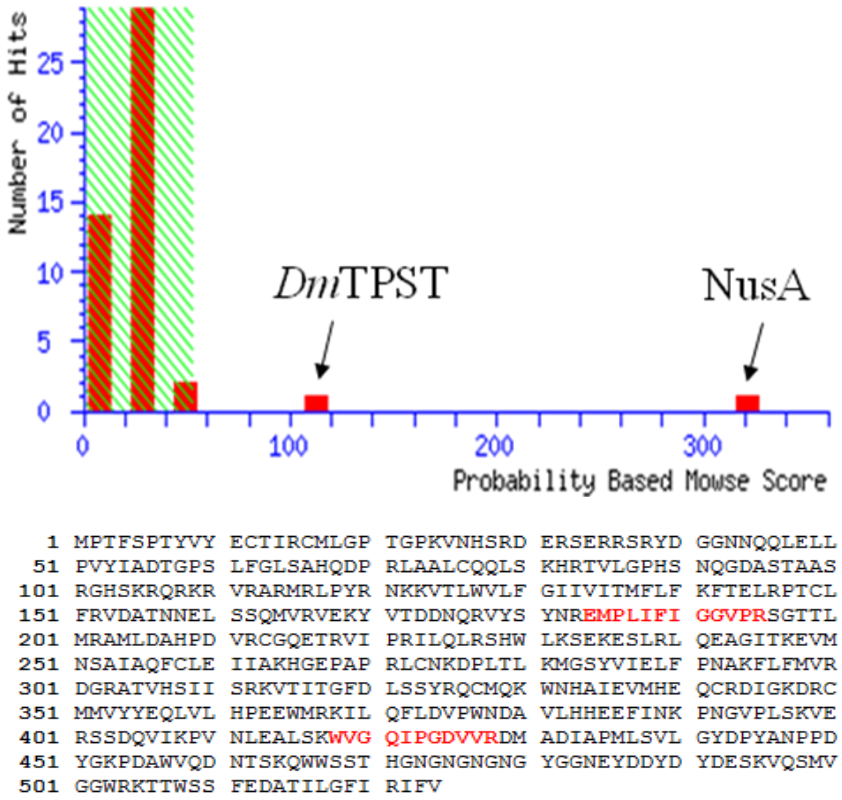


Figure 4. The protein of LC-MS-MS fingerprinting analysis was identified as *Dm*TPST.

The excised spot from SDS-PAGE was identified as *Dm*TPST by LC-MS-MS. The sequence (*red*) obtained from mass fingerprinting was mapped to the protein sequence with high confidence. The result was particularly described in **Appendix 8 and Appendix 9**.

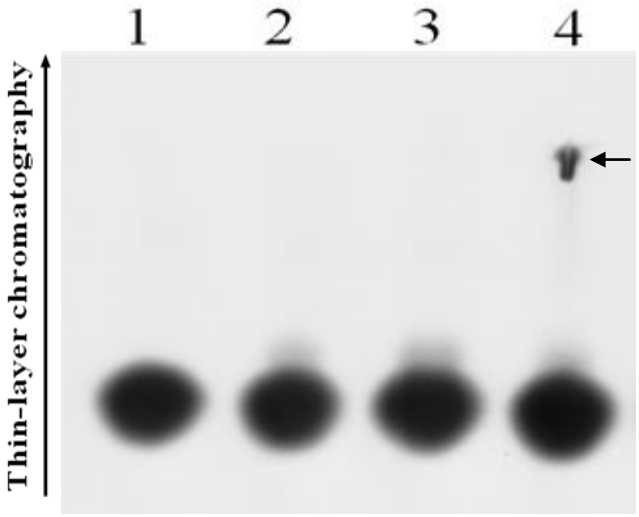


Figure 5. Autoradiography of [^{35}S]-labeled polyEAY catalyzed by *Dm*TPST.
Lanes 1 to 3 were controlled reactions by the presence of *Dm*TPST w/o PAPSS (lane 1), polyEAY (lane 2) and *Dm*TPST (lane 3), respectively. Lane 4 using polyEAY as substrate proceeding sulfation reaction. The arrowheads indicated the [^{35}S]sulfated polyEAY peptides and [^{35}S]sulfate.

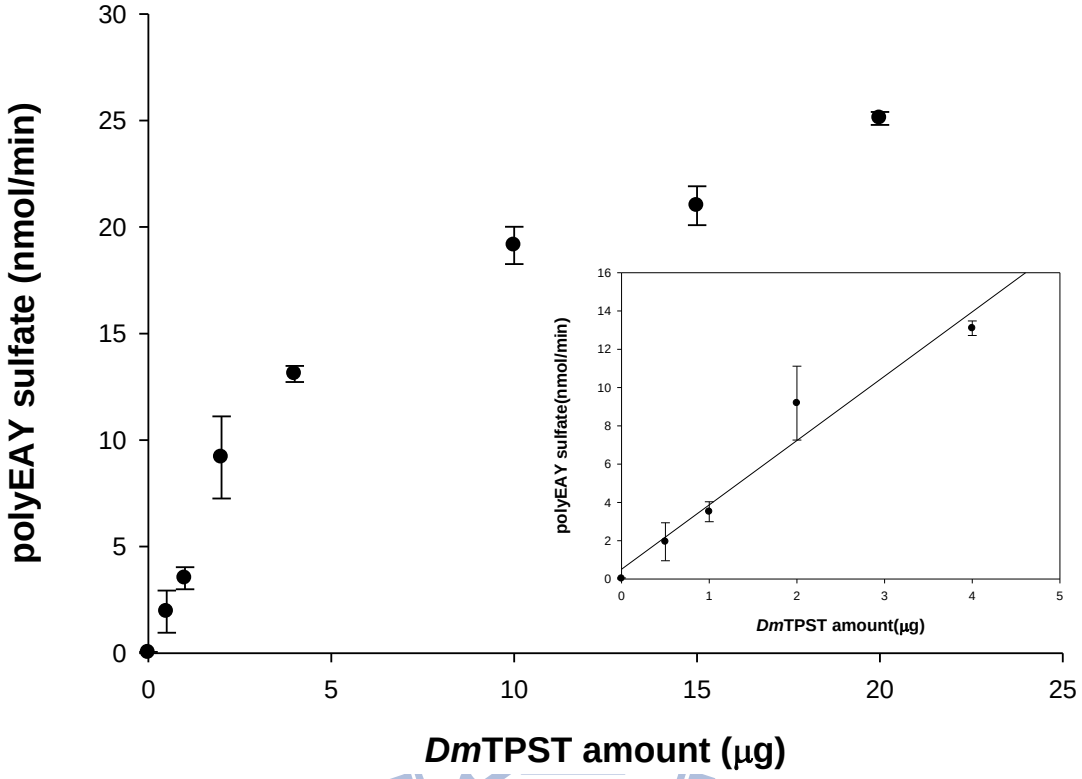


Figure 6. Effective range of *Dm*TPST assay.
polyEAY sulfation catalyzed by the variable amount of recombinant *Dm*TPST (from 0.5 to 20 μg) was determined under the standard condition. Each point and bar represented the mean and SD, respectively, obtained from three experiments.

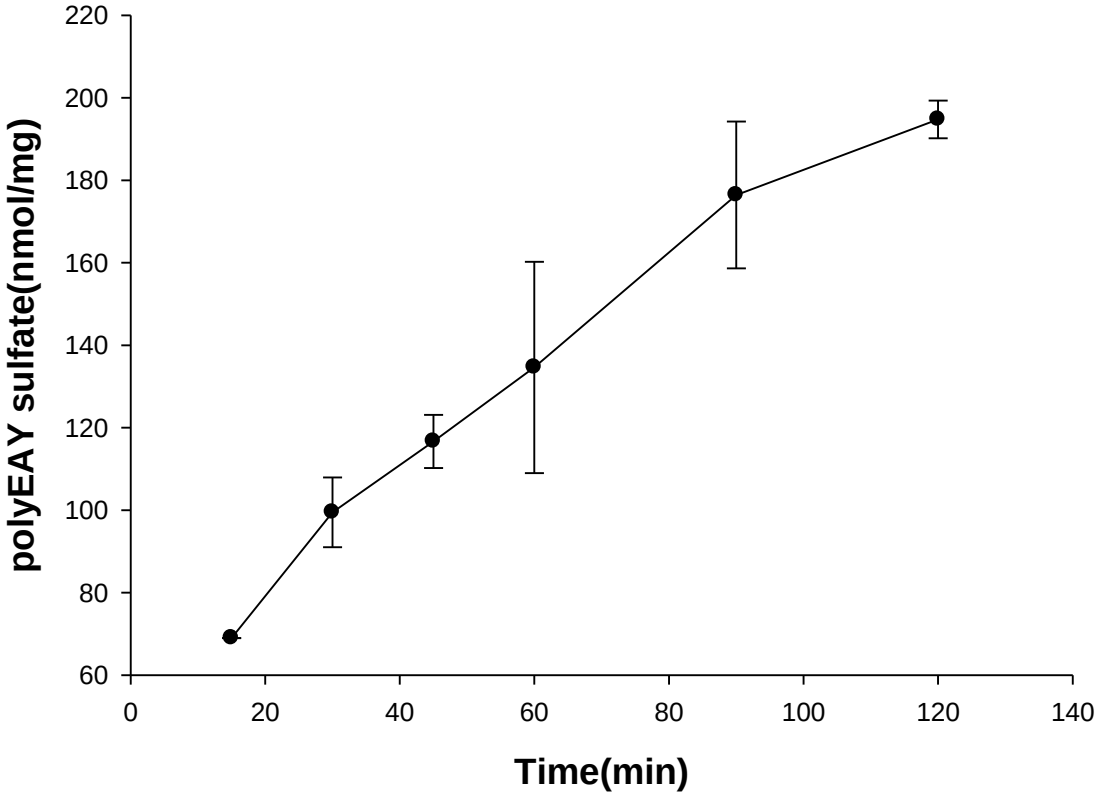


Figure 7. Time course of the activity of recombinant *Dm*TPST.

Time course effected on the activity of *Dm*TPST. Activities of *Dm*TPST were measured in different time (15, 30, 45, 60, 90, 120 min) under the standard condition. Each point and bar represented of three experiments.

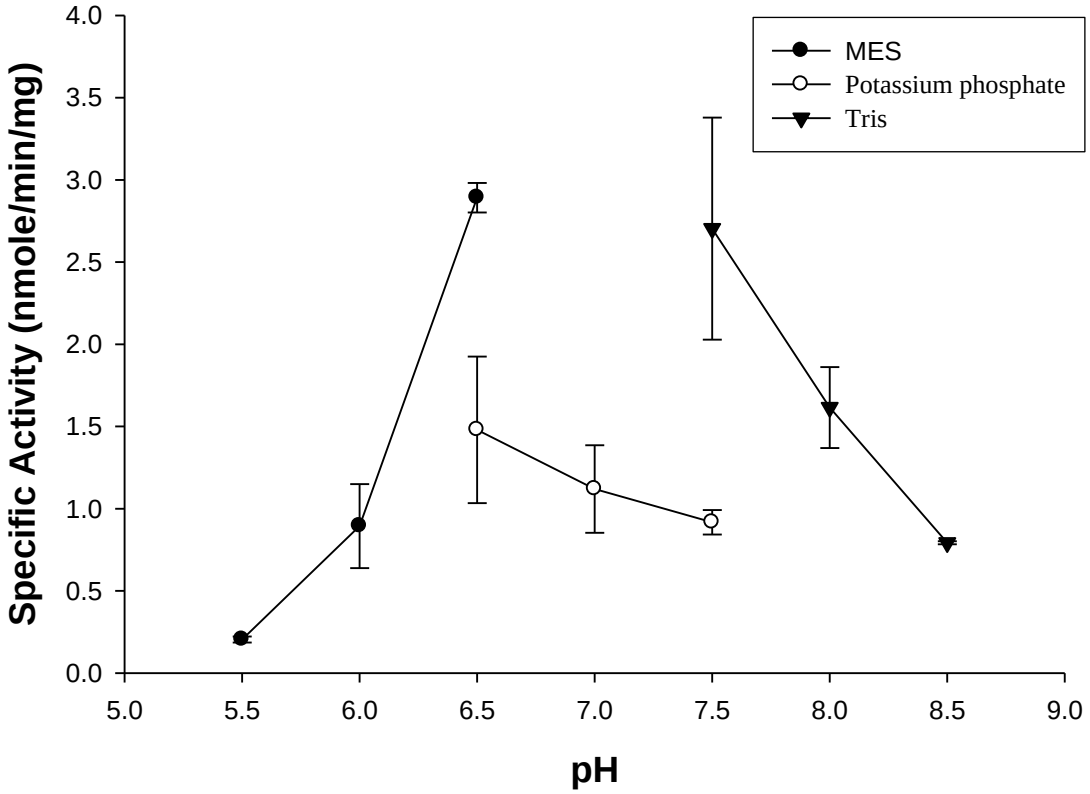


Figure 8. pH profile effected on the activity of recombinant *Dm*TPST.

The pH profile of *Dm*TPST activity. TPST activities were measured in 50 mM buffer at selected pHs (MES for pH 5.5, 6.0, 6.5, potassium phosphate for pH 6.5, 7.0, 7.5, and Tris for pH 7.5, 8.0, and 8.5) under the standard condition. Each point and bar represented the mean ± SD of three experiments.

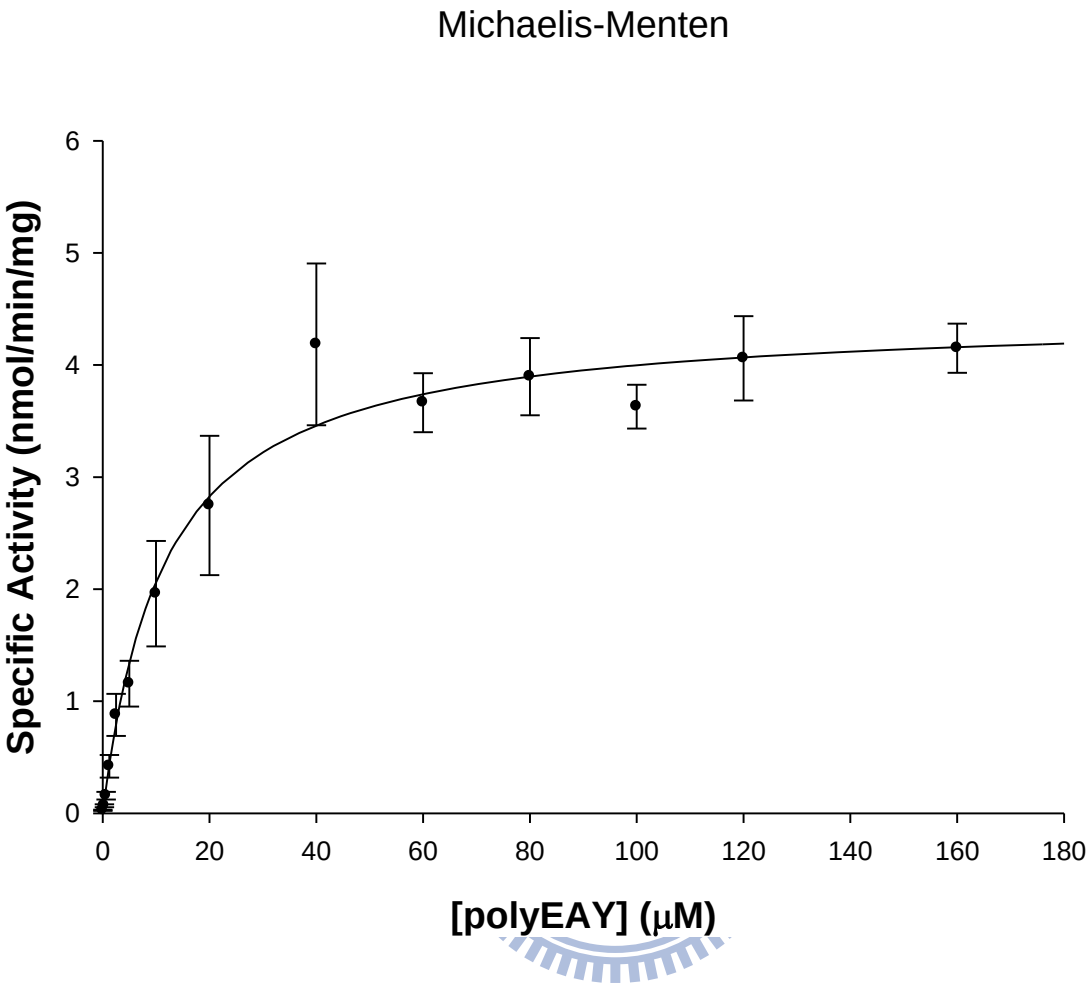


Figure 9. Kinetics of *Dm*TPST using polyEAY as substrate.

The *Dm*TPST kinetics activities were determined under the standard condition with various polyEAY concentration from 0.3125 to 160 μM. Each point and bar represented the mean ± SD of three experiments. The data indicated the K_m and V_{max} was 11.5 ± 2.5 mM and 4.5 ± 0.2 nmole/min/mg, respectively.

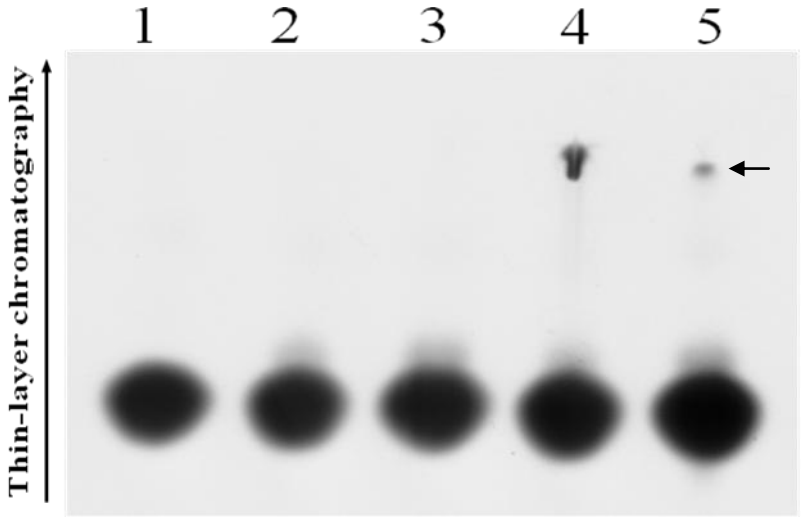
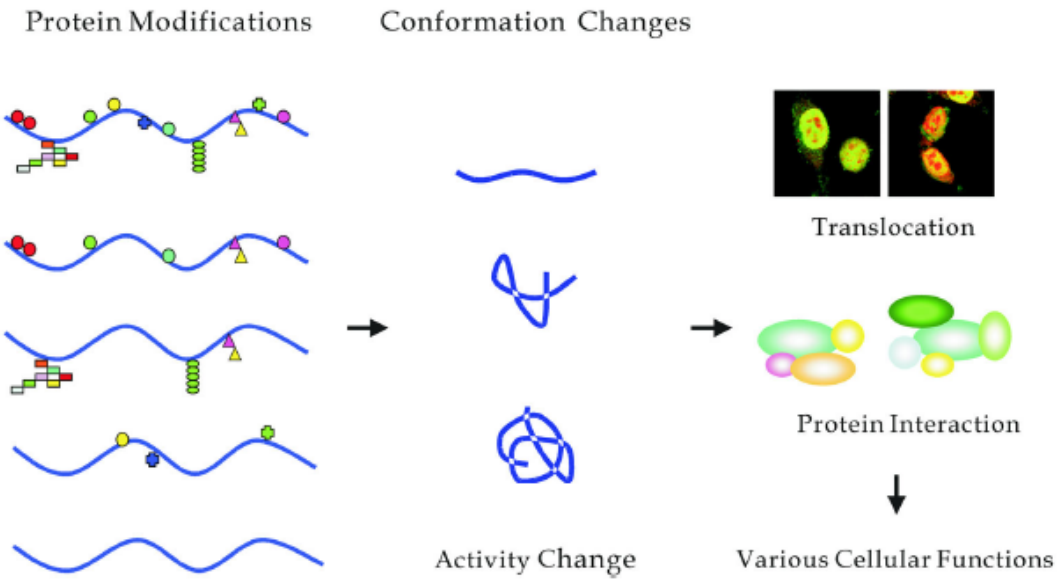


Figure 10. Autoradiography of [^{35}S]-labeled Drosulfakinin catalyzed by *Dm*TPST.

Lanes 1 to 4 were controlled reactions in the absence of PAPSS (lane 1), substrate (lane 2), and *Dm*TPST (lane 3), respectively. Lane 4 using polyEAY as substrate proceed sulfation reaction was positive control, and lane 5 was full reaction that using drosulfakinin as substrate. The arrowhead indicated the [^{35}S]sulfated drosulfakinin peptides and [^{35}S]sulfate.

Appendix



Appendix 1. Schematic representation of protein modifications related to the regulation of biological processes. (Adapted from Seo *et al*. 2004)

Table 1. Some common and important post-translational modifications

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.

Appendix 2. Some common and important post-translation modifications (Mann *et al*., 2003).

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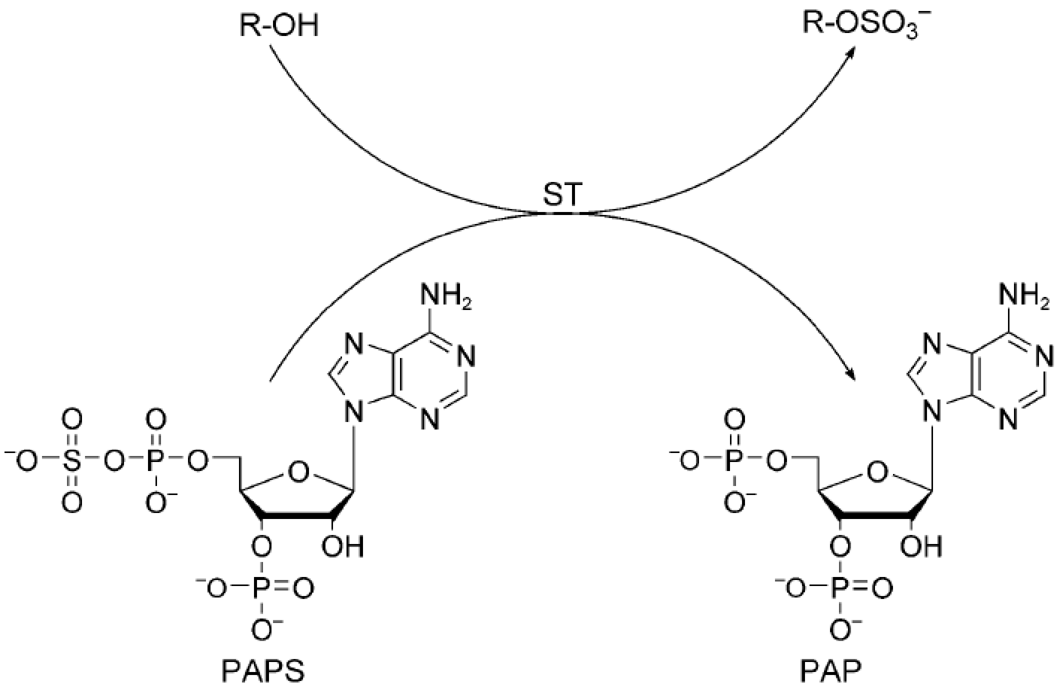
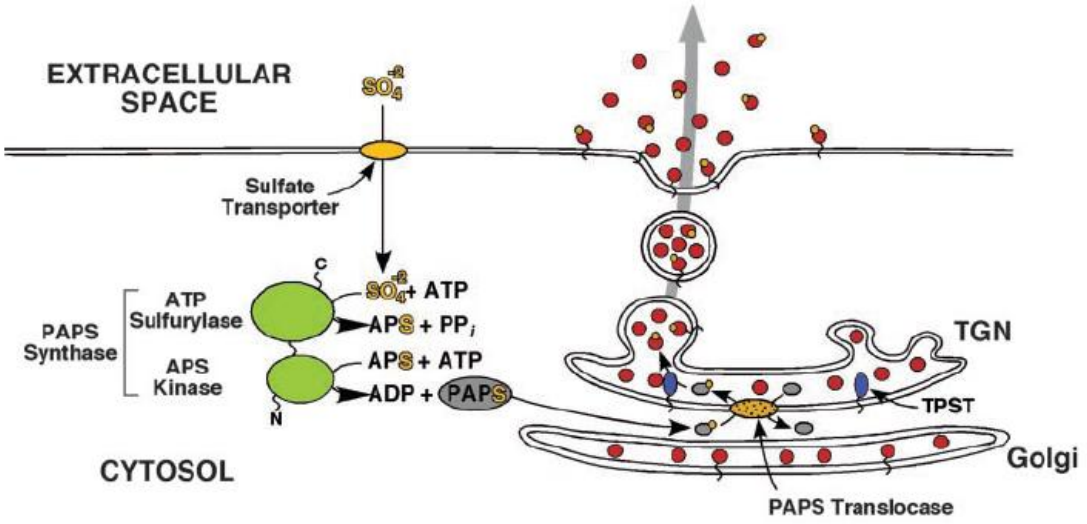
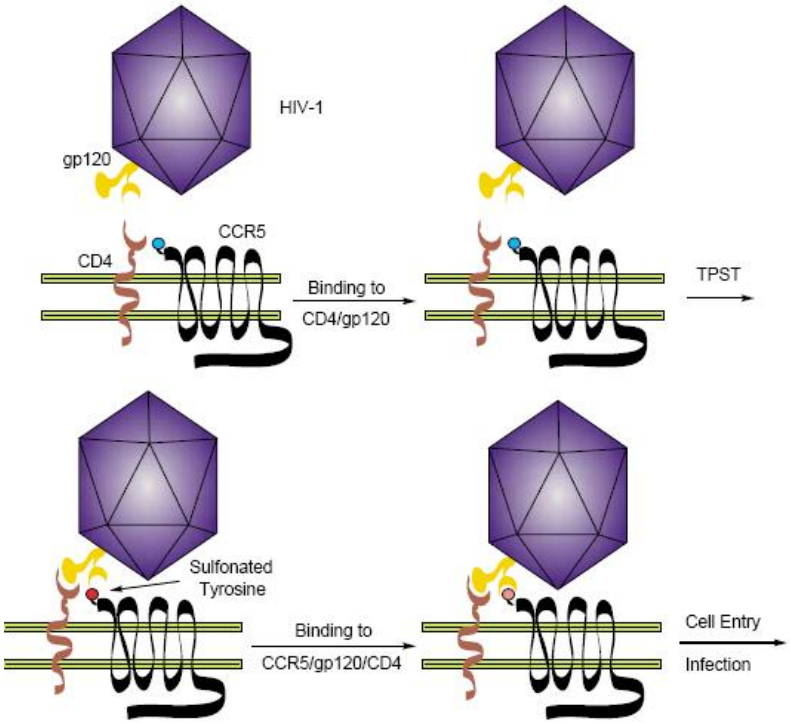


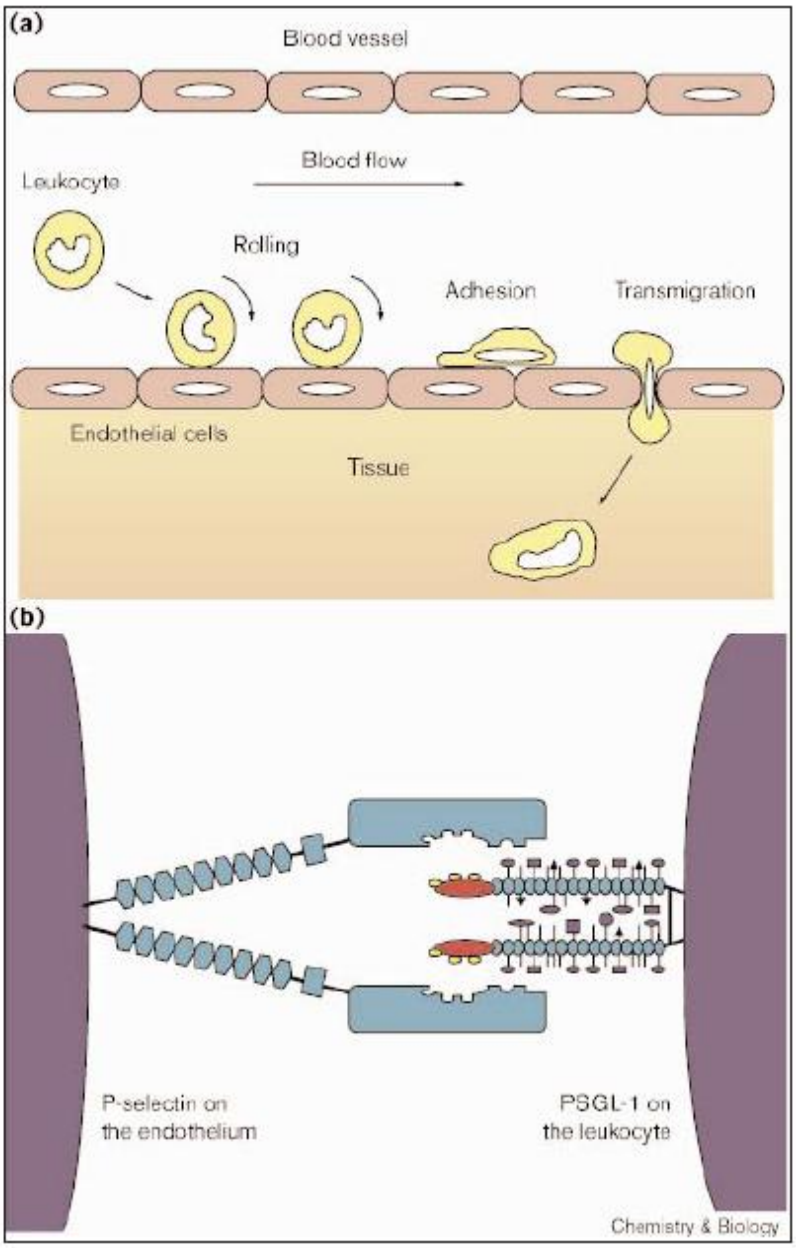
Appendix 3. General ST-catalyzed reaction with PAPS as the cosubstrate (Chapman *et al*., 2004).



Appendix 4. Sulfate activation and tyrosine *O*-sulfation. Inorganic sulfate enters the cell by the action of one of several sulfate transporters. Once in the cytosol, sulfate is then activated by the action of one of two PAPS synthases (*PAPSS1* or *PAPSS2*). These bifunctional enzymes contain a C-terminal ATP sulfurylase domain and an N-terminal adenosine phosphosulfate (APS) kinase domain. In the first step of sulfate activation, ATP and inorganic sulfate are converted to APS and pyrophosphate by ATP sulfurylase. APS is then channeled directly between the ATP sulfurylase and APS kinase active sites. In the second step catalyzed by the APS kinase domain, a second ATP is consumed to phosphorylate the 3'-hydroxyl of the ribose ring of APS to yield PAPS and ADP. PAPS is then transported into the Golgi lumen by a PAPS translocase that has been purified but not yet cloned. This transporter functions via an antiporter mechanism with PAP as the returning ligand. Once inside the Golgi lumen PAPS acts as the sulfate donor for TPSTs and all other carbohydrate sulfotransferases, and the sulfated products are either secreted or retained in the membrane of lysosomes, secretory vesicles, and/or the plasma membrane. *TGN*, *trans*-Golgi network. (Moore, 2003).



Appendix 5. Schematic representation of cell entry by HIV-1 following sulfonation of CCR5 by a tyrosylprotein sulfotransferase (Chapman *et al*., 2004).



Appendix 6. Tyrosine sulfation plays an important role in the immune response. (a) Leukocytes roll upon, adhere to and transmigrate between endothelial cells at sites of inflammation. P-selectin and its ligand, PSGL-1, are often required for this process. (b) PSGL-1 is a mucinlike glycoprotein that appears to be an extended rod shape *in vivo*. The extreme amino terminus of PSGL-1 carries three tyrosine sulfation sites, shown in yellow. These sulfate esters, and specific glycans on PSGL-1, are key binding determinants for P-selectin (Kehoe and Bertozzi, 2000).

pET-43.1a-c(+) Vector

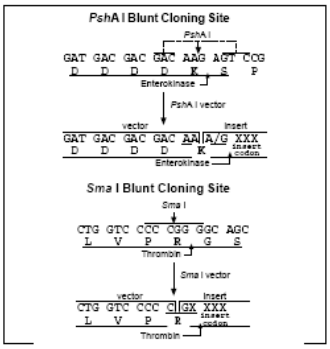
TB288 09/00

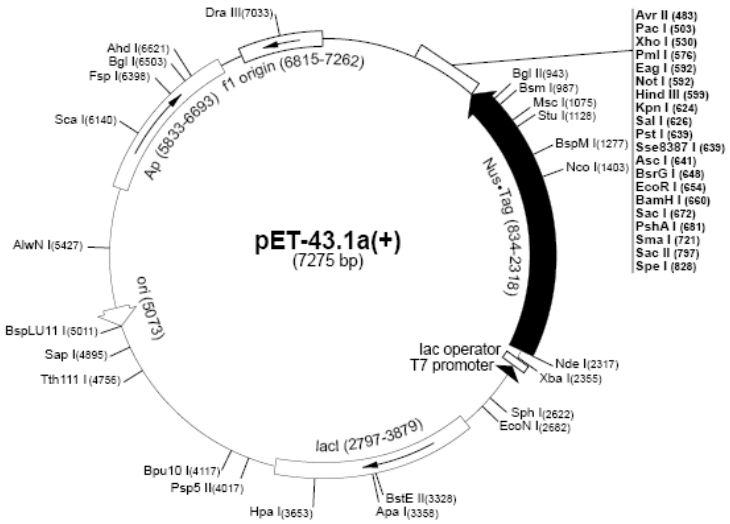
	Cat. No.
pET-43.1a(+) DNA	70939-3
pET-43.1b(+) DNA	70940-3
pET-43.1c(+) DNA	70941-3

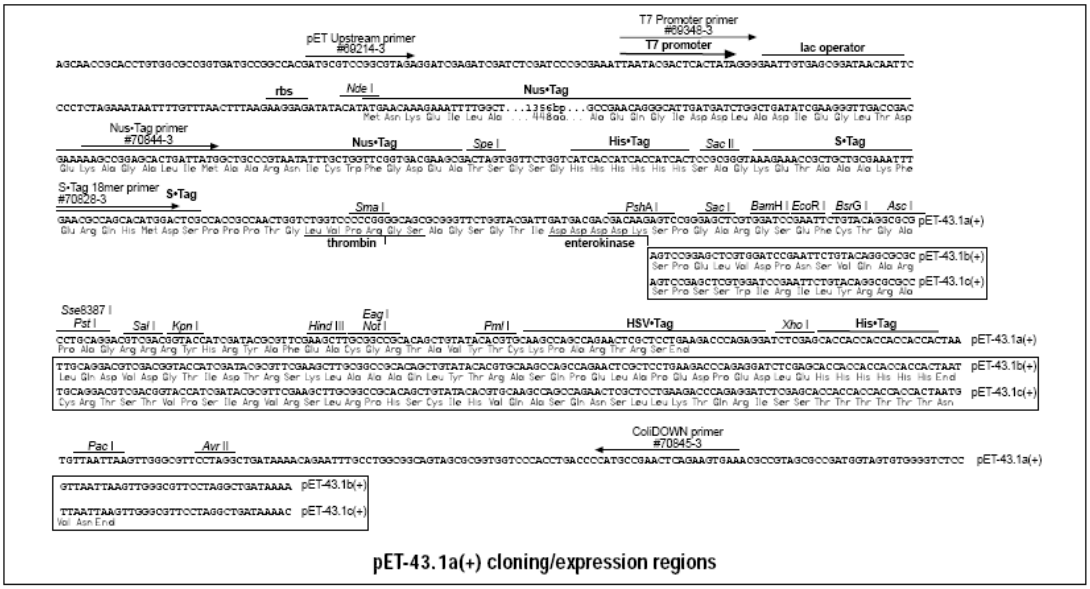
pET-43.1a(+) sequence landmarks

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

Appendix 7. pET-43.1a-c(+) Vector exposition

Mascot Search Results

Protein View

../data/20100630/Ft	TPST_DROME	1	false	44
0	0.05	0	1	1025
2	-1	0		

Match to: **TPST_DROME** Score: **112**
Protein-tyrosine sulfotransferase OS=Drosophila melanogaster GN=Tango13 PE=2 SV=2
Found in search of MI806075.pkl

Nominal mass (M_r): **58013**; Calculated pI value: **7.66**
NCBI BLAST search of TPST_DROME against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Drosophila melanogaster

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **4%**

Matched peptides shown in **Bold Red**

```
    1 MRLPYRNKKV TLWVLFGIIV ITMFLFKFTE LRPTCLFKVD AANELSSQMV
   51 RVEKYLTDDN QRVYSYNREM PLIFIGGVPR SGTTLMRAML DAHPDVRCGQ
  101 ETRVIPRILQ LRSHWLKSEK ESLRLQEAGI TKEVMNSAIA QFCLEIIAKH
  151 GEPAPRLCNK DPLTLKMGSY VIELFPNAKF LFMVRDGRAT VHSIISRKVT
  201 ITGFDLSSYR QCMQKWNHAI EVMHEQCRDI GKDRCMMVYY EQLVLHPEEW
  251 MRKILKFLDV PWNDAVLHHE EFINKPNGVP LSKVERSSDQ VIKPVNLEAM
  301 SKWVGQIPGD VVRDMADIAP MLSVLGYDPY ANPPDYGKPD AWVQDNTSKL
  351 KANRMLWESK AKQVLQMSSS EDDNTNTIIN NSNNKDNNNN QYTINKIIPE
  401 QHSRQRQHVQ QQHLQQQQQQ HLQQQQHQRQ QQQQQREEES ESEREAEPDR
  451 EQQLLHQKPK DVITIKQLPL AGSNNNNINN NINNNNNNNN IMEDPMADT
```

Appendix 8. MS analysis of *Dm*TPST

MATRIX SCIENCE Mascot Search Results

Protein View

../data/20100630/Ft	NUSA_ECOLI	1	false	44
0	0.05	0	1	1025
2	-1	0		

Match to: NUSA_ECOLI Score: 321
Transcription elongation protein nusA OS=Escherichia coli (strain K12) GN=nusA PE=1 SV=1
Found in search of MI806075.pkl

Nominal mass (M_r): 54837; Calculated pI value: 4.53
NCBI BLAST search of NUSA_ECOLI against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Escherichia coli K-12

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: 10%

Matched peptides shown in Bold Red

```
    1 MNKEILAVVE AVSNEKALPR EKIFEALESA LATATKKKYE QEIDVRVQID
   51 RKSGDFDTFR RWLVVDEVTQ PTKEITLEAA RYEDESLNLG DYVEDQIESV
  101 TFDRITTQTA KQVIVQKVRE AERAMVVDQF REHEGEIITG VVKKVNRDNI
  151 SLDLGNNAEA VILREDMLPR ENFRPGDRVR GVLYSVRPEA RGAQLFVTRS
  201 KPEMLIELFR IEVPEIGEEV IEIKAAARDP GSRAKIAVKT NDKRIDPVGA
  251 CVGMRGARVQ AVSTELGGER IDIVLWDDNP AQFVINAMAP ADVASIVVDE
  301 DKHTMDIAVE AGNLAQAIGR NGQNVRLASQ LSGWELNVMT VDDLQAKHQA
  351 EAHAAIDTFT KYLDIDEDFA TVLVEEGFST LEELAYVPMK ELLEIEGLDE
  401 PTVEALRERA KNALATIAQA QEESLGDNKP ADDLLNLEGV DRDLAFKLAA
  451 RGVCTLEDLA EQGIDDLADI EGLTDEKAGA LIMAARNICW FGDEA
```

Appendix 9. MS analysis of NusA