

C500 REPORT

SPRING 2006

1.EXPRESSION OF TYROSYLPROTEIN SULFOTRANSFERASE 1 AND 2..

2.THE REFOLDING AND PURIFICATION OF TPST1.

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INTRODUCTION

The chemokine receptors are membrane bound GPCRs (G Protein Coupled Receptors) found in Eucaryotes ..As with other GPCRs', their activation leads to production of second messengers downstream (intracellular) which, in this case are; phosphatidylinositol triphosphate (IP₃) ,diacylglycerol (DAG) and calcium from the endoplasmic reticulum. These second messengers lead to the ultimate effects of chemokines.(1).This ultimate effects of chemokines affect inflammation, tumor metastasis and blood cell infection by various strains of HIV(Human Immunodeficiency Virus). The Stone lab has developed a soluble system that contains many of the features of natural chemokine receptors.

As with other secreted or membrane proteins in Eucaryotes, these receptors can undergo a specific kind of post translational modification ;Tyrosine- O-⁴sulfation.Tyrosine-O⁴- sulfation is catalysed by two enzymes, Tyrosylprotein Sulfotransferase's. Tyrosylprotein Sulfotranferase 1 (TPST1) and Tyrosylprotein Sulfotransferase 2 (TPST 2); both EC2.8.2.20, named in the order of their discovery, are enzymes that catalyze Tyrosine –O⁴-sulfation in cognate proteins. The sulfate group is transferred from Adenosine 3'-phosphate 5'-phosphosulfate (PAPS) to the hydroxyl group of a peptide-tyrosine residue with concomitant formation of 3',5' ADP and Tyrosine O⁴ sulfate ester.

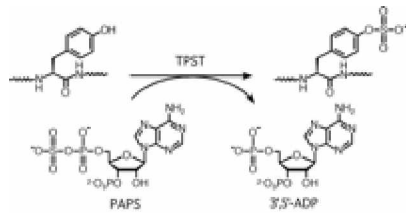


Fig1: The tyrosylprotein reaction(Ref :Kevin Moore *J.Biol Chem.*278,27,pp24244 ,2003)

A lot of interest in the field has since focused on the role of tyrosine *O*-sulfation in G-protein-coupled receptor (GPCR) function after CCR5, a major HIV co-receptor, was shown to be tyrosine-sulfated.(2)It has also been shown that the effects of altering the action of TPST1 and /or TPST2 can be significant to mice.(3). This action may therefore lead to a change in downstream activities of chemokines affecting inflammation, tumor metastasis and blood cell infection by various strains of HIV(Human Immunodeficiency Virus)

Recent data suggest that TPST-1 and TPST-2 are membrane bound Golgi enzymes, and the catalytic domain is soluble and active even in the absence of the transmembrane domain and also the N –terminal.(4,5)

By understanding the factors that optimize the activities of TPST1 and 2, we can be able to understand their regulation and the factors that determine their activities in the human body. In the long run, it is hoped that ways of preventing premature or excessive activities of these proteins in the human body can be obtained thus ameliorating their deleterious effects.

EXPERIMENTAL TECHNIQUES AND PROCEDURES

***E coli* Competent Cells Preparation**

This was done in the regular way as per the manufacturer's protocol. A plate of BL21(DE3)*E coli* (Novagen, Madison, WI) was streaked on Luria Broth(LB) without any antibiotics. It was incubated overnight at 37⁰ C. A colony was then picked and placed in a flask containing 50 mL of LB without antibiotics. This was grown at 37⁰C while shaking until the OD₆₀₀ was about 0.4 The cells were then spun at 5000 rpm for ten minutes into a pellet. The supernatant was then discarded .The pellet was then resuspended in 10mL of sterile and chilled (4⁰C) CaCl₂. Then left the suspension on ice for about 10 minutes.

Transformation of *E.coli*

About 1 microlitre (μL) of plasmid for each separate TPST was added to the bottom of an individual Falcon tube and placed in ice for about 10 minutes. Then added 200 microliters of competent *E coli* cells .

Then mixed gently and allowed to sit on ice for 1 hr. The separate tubes (with TPST1 and TPST2) were then immersed for 45 seconds in a waterbath of 42°C . to heat shock the cells. Then was added 800 μL of fresh sterile SOC into the Falcon Tubes. This was then placed in a shaker incubator for 45 minutes. About 200 μL of cell suspension was then plated per plate with Ampicillin.

Pre-culture

To each of two 50 mL LB solutions with Ampicillin, added, by streaking the respective plates, the transformed *E coli* cells containing TPST1 and TPST2 . The solutions were then grown in a shaker incubator while monitoring the OD_{600} until the respective ODs were 0.47 and 0.43. Induction was done by adding IPTG to a final concentration of 1 Mm.

Harvest

Bacterial cells were then harvested by centrifugation at 8000 rpm for 15 minutes. The supernatant and the pellet fraction from each kind of TPST was then kept in separate tubes at various time intervals as shown ; immediately after induction, after 2hr, 4 hr and finally, after 8 hours.

Inclusion body extraction

This was done by modifying the standard protocol. To the culture resuspended 35 mL PBS /1 Litre culture(i.e140ml added). Added PMSF to make a final concentration of 0.7mM.The suspension was then kept cool on ice. The cells were then disrupted with the Cell Cracker, using the Cell Cracker protocol. Two runs through the cracker circuit were performed .Due to some loss by leakage during the process, the final volume obtained was about 250mL. To this volume was then added 500µL of more PMSF to ensure proteolysis does not take place .The solution was then centrifuged at 15,000 rpm for 45 minutes. After discarding the supernatant, resuspended the pellet with wash detergent(consisting of 0.5M Guanidium HCl, 1% triton,20mM Tris at pH of 7.2).The supernatant was stored separately while the pellet was again resuspended on loading buffer containing 10mMTris,100mM NaH₂PO₄,6M Guanidine, 10Mm β ME,10% glycerol at pH of 8.0 This suspension was centrifuged again at 15,000 rpm for 45 minutes. After this the supernatant was collected as the inclusion body extracts.

Purification by the Ni-NTA Column

This was done according to the standard protocol. After filtering the protein solution ,applied onto Ni-NTA agarose column. Then batch adsorbed at room temperature on rocker for 1hour.This was followed by standing the column and allowing the agarose to settle down .The supernatant was then collected using gravity flow. Then washed the column with five times the bed volume of the loading buffer(10Mm Tris HCl,100Mm NaH₂PO₄,6 M Guanidine HCl,10mM β Mecarptoethanol,10% Glycerol adjused pH to8.0) followed by a second wash with ten times the bed volume of wash buffer one(10mM Tris HCl,100mM NaH₂.PO₄, 6M Guanidine HCl 10 mM β

ME, 10% Glycerol at pH 6.3.) The third wash consisted of five times the bed volume of wash buffer two (similar to the wash buffer one but with pH of 5.9). Finally, the proteins, TPSP 1 solution was eluted (this elution buffer was similar to the above buffers but with pH of 4.5) with six times the bed volume while collecting each fraction to be equivalent to the bed volume. Therefore collected each 4 mL for each fraction..Collected six fractions.

Determined the Optical Density(O.D.) of these fractions.

The fraction that had the highest OD was then subjected to the solubility experiment as shown in the following protocol.

TPST-1 Solubility Experiment

EXP #	Vol. of B μ L) Loading Buffer (μ L)	Vol. of Vol. of Sol B + Loading Buffer (μ L)	Vol of Sol A (μ L)	Guanidine in Combined Sol Loading Buffer (M)	Final conc.of Guanidine HCL b+L After mixing (M)	Optical Density Observed
1	540	0	540	60	0	0.068
2	510	30	540	60	0.33	0.084
3	480	60	540	60	0.67	0.104
4	450	90	540	60	1	0.12
5	420	120	540	60	1.333	0.153
6	390	150	540	60	1.667	0.174
7	350	190	540	60	2.111	0.165
8	300	240	540	60	2.667	0.173

Solution A consisted of the elution Fraction #2 from the Ni-NTA column.

Solution B consisted of 10mM Tris,HCl, 100 mM sodium phosphate at pH 8.0

After adding the indicated solutions the tubes were inverted several times to mix and then left at room temperature for one hour. The tubes were then spun at 13,000 rpm for 15 minutes. The O.D. was measured for each sample.

Preparation Prior to Running SDS- PAGE Electrophoresis

Since the above buffer solutions contained Guanidine, it was imperative that the protocol be modified before running the SDS-PAGE since the Gels will be distorted in the presence of this compound. The protocol proceeded as follows. To 50 μ L of each sample added 500 μ L of cold 10%TCA in acetone. After mixing well, put tubes in -20 ° C freezer for one and a half hours. Then the the tubes were spun at 13,000rounds per minute for fifteen minutes. Each of the samples was then subjected to careful aspiration of the supernatant leaving the pellet. These were then allowed to air dry for forty minutes. To this was then added 80 μ L of 1x loading buffer. After boiling this for five minutes, loaded to each well in the SDS –PAGE 20 μ L. After completion of electrophoresis and staining Then observe the gel for the presence of a thick band at about 52k Da

TPST Refolding Protocol.

The Ni-NTA agarose fractions 1 to 3 were combined and the O.D. was then taken at 280nm. After adjusting the pH of these fractions to 8.0 using NaOH , D.T.T. (Dithiothreitol) was added to give a final concentration in the solution of 100Mm.The solution was then stirred at room temperature for 30 minutes. The solution was then dialyzed against 250 mL of Dialysis Buffer #2(4M Guanidine, 10Mm HCl) for about 29 hours while stirring gently. The solution was then diluted with nine parts of Refolding

Buffer (0.6M Arginine HCL,,0.15M NaCl,50Mm TAPS,1mM EDTA, 4.88Mm GSSG, 1.63mM GSH, 20 μ M PAP at pH of 9.0) to give a final volume of ~90mL. Then stirred at room temperature for four days. The solution was then dialysed against dialysis buffer #3 (consisting of 1M NaCl ,20mM MOPS,10% Glycerol,0.02% Na N₃ at pH of 7.5) for twelve hours then against dialysis buffer # 4 (similar to buffer 3 but with 50mM NaCl) twice each time for twelve hours. After this centrifuged the samples at 15,000 r.p.m. for 30 minutes. Decanted and combined the supernatants which gave a volume of ~130ml. Then checked the O.D. of the combined supernatants. Afterwards resuspended the pellets in an equal volume of dialysis buffer# 4. Then compared the combined supernatants and the combined resuspended pellets on an SDS-PAGE gel.

RESULTS AND DISCUSSION

After harvesting the cells, electrophoresis was done on the fractions collected at the various time intervals and compared to the molecular weight marker Mark 12. The results for TPST 1 and TPST 2 showed that these proteins were expressed in large amounts four hours after induction in each case. (see Figure 3 a,b below.)

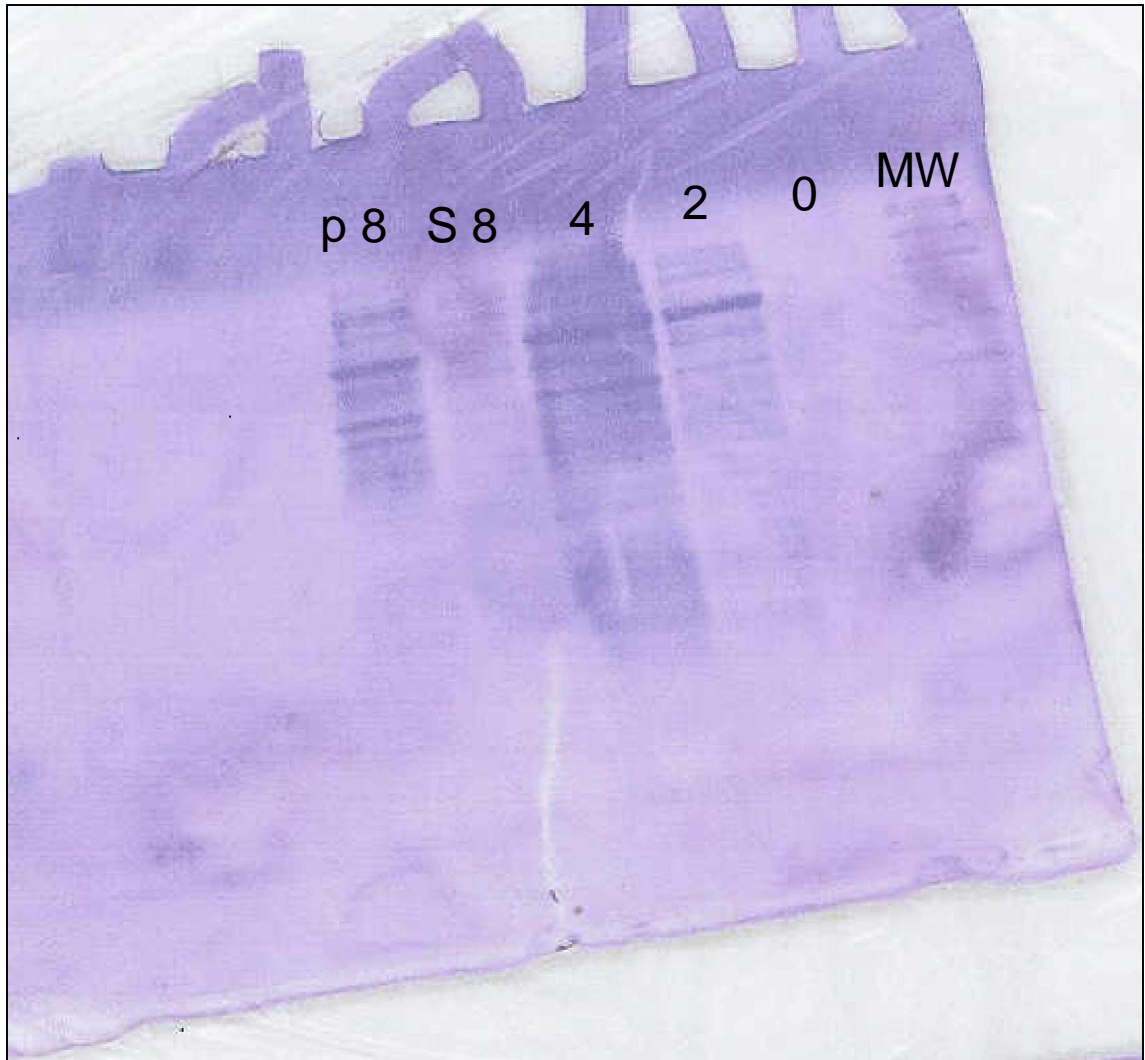


Fig3(A). The proteins observed at various time intervals (in hours) after induction with plasmid containing TPST 1 . P8 refers to pellet after 8 hours and S8 to supernatant after 8 hours from induction.. The thick band corresponds to about 52 kDa.

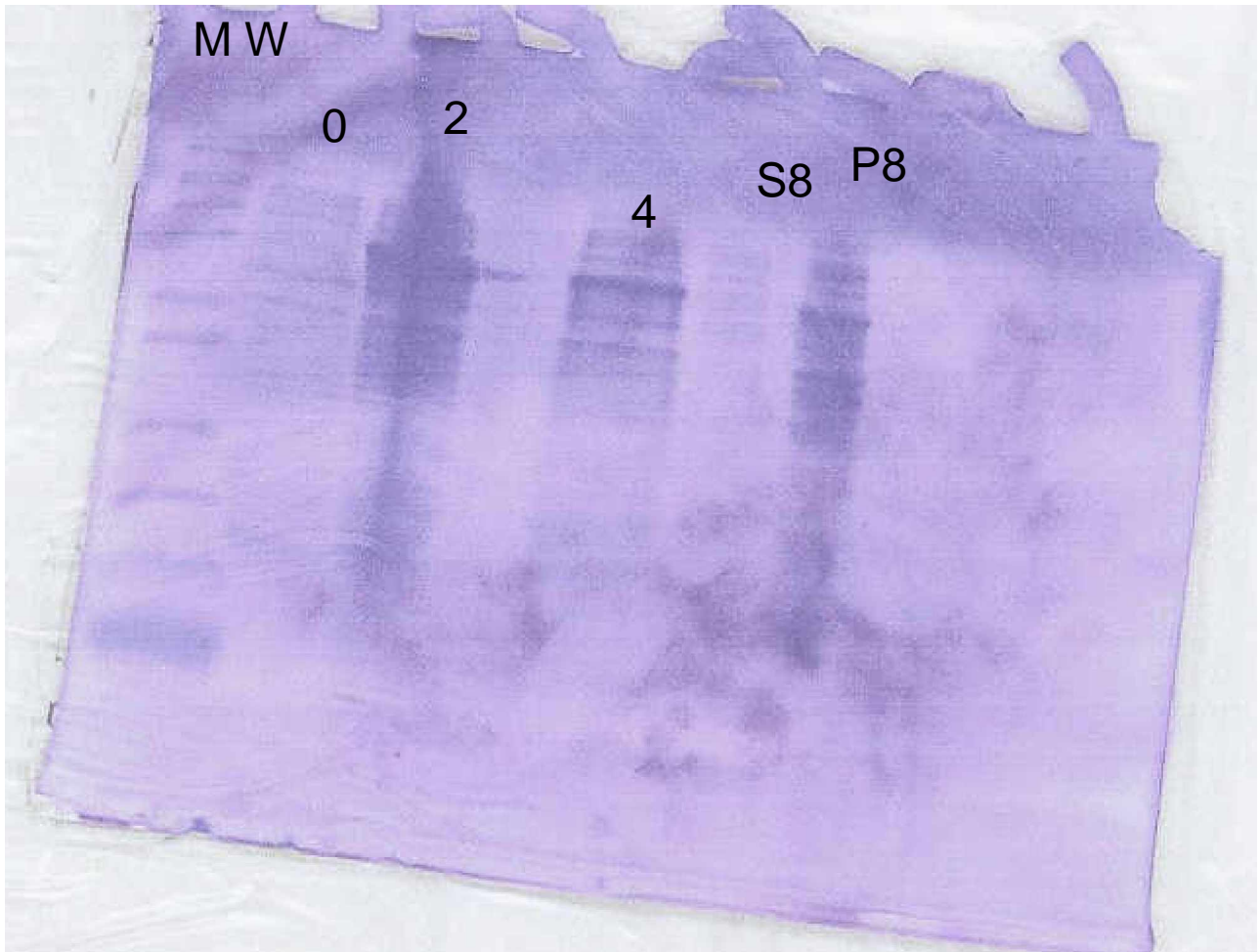


Figure 3 (b)The proteins observed at various time intervals after induction in the case of a plasmid containing TPST2.S8 refers to the supernatant after 8 hours while P8 refers to the pellet after 8 hours from induction. The thick band corresponds to about 58kDa.

In addition, it was found that the pellet fraction contained these proteins after sonication of the pellet fraction eight hours after induction.

On the basis of the above results bacterial cells were grown in a larger scale .Four litres were grown and induced to synthesize TPST 1 and harvested after four hours .The cells were then lysed using the Cell Cracker Protocol. While performing this protocol

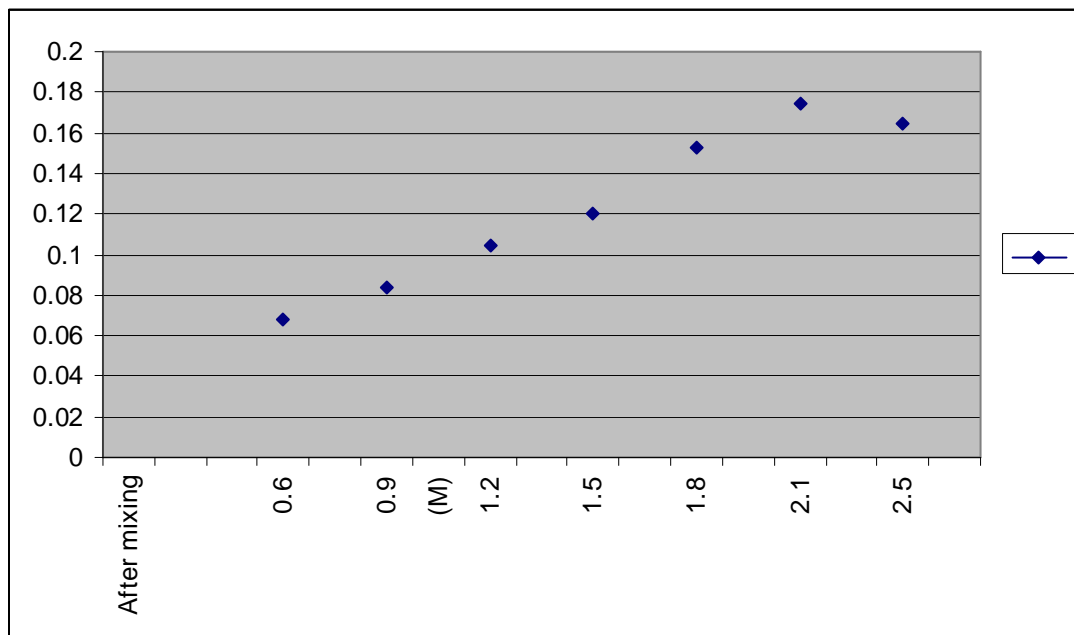
some of the sample cells were lost in the process of trying to remove a blockage in the gadget. The final volume was about 200ml. 500 μ L of PMSF was then added to this.

After performing the Ni-NTA elution the amount of protein in each of the six fractions

(each ~4ml) was then determined by observing the Optical Density(O.D) at 280 nm. The O.D. were as follows, 0.470,1.1222,0.911,0.715,0.589 and 0.515. The O.D. at 280 nm of the combined fractions was determined to be 0.867.

Solubility Experiment

It was observed that as the final concentration of guanidinium was increased the OD also increased to stabilize at about 0.173.



Refolding Experiment

After performing the refolding experiment as described earlier, the samples were subjected to SDS-PAGE Electrophoresis. However, it was difficult to observe the TPST

1. band in the gel. It was determined that the amount of protein may have been too small to be observed at about 1.453mg for about two liters growth media. This aspect of the experiment will be attempted again in order to determine whether these results are reproducible. It is also hoped that further studies will shed light on the catalytic mechanism of TPST .

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