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TECHNICAL NOTE

Fluorescence assay for protein post-translational tyrosine sulfation

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Abstract We developed a fluorescent assay to conveniently determine the kinetics of protein sulfation, which is essential for understanding interface between protein sulfation and protein–protein interactions. Tyrosylprotein sulfotransferase (TPST) catalyzes protein sulfation using 3′-phosphate 5′-phosphosulfate (PAPS) as sulfuryl group donor. In this report, PAPS was regenerated following sulfuryl group transfer between adenosine 3′,5′-diphosphate and 4-methylumbelliferyl sulfate catalyzed by phenol sulfotransferase (PST). The TPST and PST coupled enzyme platform continuously generated fluorescent 4-methylumbelliferone (MU) that was used to real-time monitor protein sulfation. Using a recombinant N utilization substance protein A fused *Drosophila melanogaster* tyrosylprotein sulfotransferase, we demonstrated that the activity of TPST determined through MU fluorescence directly correlated with protein sulfation. Kinetic constants obtained with small P-selectin glycoprotein ligand-1 peptide (PSGL-1 peptide, MW 1541) or its large glutathione *S*-transferase fusion protein (GST-PSGL-1, MW 27833) exhibited significant variation. This assay can be further developed to a high-throughput method for the characterization of TPSTs and for the identification and screening of their protein substrates.

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Introduction

Protein sulfation is one of the common post-translational modifications. It is catalyzed by tyrosylprotein sulfotransferase (TPST) through the transfer of sulfuryl group (SO_3^{-1}) from 3′-phosphoadenosine 5′-phosphosulfate (PAPS) onto a tyrosyl residue within a target protein. Protein sulfation plays a critical role in protein–protein interactions that modulates virus infection, inflammation, immune responses, and other biological events [1–3]. Protein sulfation is known to increase the probability of viral infection by the surface protein interactions between cells and virus. Protein sulfation changes chemical properties of the protein surface and in turn affects protein folding, protein conformation, protein stability, and protein–protein interactions. Despite that the importance of post-translational protein sulfation is well appreciated, the biological functions of protein sulfation are still largely unknown. To determine the activity of protein sulfation is one of the important issues to understand its catalytic mechanism and biological functions, and an efficient assay for TPST activity is critical for the study of post-translational protein sulfation.

Mass spectra and isotope labeling are current methods used for monitoring the tyrosine sulfation and analyzing TPST properties [4, 5]. These methods are end point assays that usually require tedious procedures and expensive instruments. In particular, due to the need for the separation of sulfated products, these two methods are limited to use a small peptide as a substrate of TPST [6]. In vivo protein sulfation usually appears on large proteins and it is

important to determine kinetic constants and substrate specificities of TPST using a protein substrate.

This report describes a continuous assay for protein sulfation, which is not limited by the size of substrates and can be used conveniently for screening protein substrates or peptides. We developed a phenol sulfotransferase–tyrosylprotein sulfotransferase (PST-TPST) coupled enzyme assay to continuously monitor TPST activity by fluorescence. PST coupled enzyme assay has been successfully developed for the assay of several sulfotransferases including sulfotransferase 1A3 (SULT1A3) and sulfotransferase 2A1 (SULT2A1) [7, 8]. Using a peptide and its GST fusion protein as substrates, we demonstrated that the reported fluorescent method can efficiently monitor protein sulfation in real time regardless of the size of substrates. As an optical method, only basic biochemical tools are needed and this method can be to easily develop to a high-throughput analytical technique.

Experimental

Materials

P-selectin glycoprotein ligand-1 peptide (PSGL-1, ATEYEYLDYDFL) was synthesized by Genemed Synthesis, Inc. (Texas, USA). Adenosine PAPS, adenosine 3′,5′-diphosphate (PAP), 2-mercaptoethanol, MES hydrate, 4-methylumbelliferone (MU), and 4-methylumbelliferyl sulfate (MUS) were purchased from Sigma (St. Louis, MO, USA). All other reagents were of the highest grades commercially available.

Preparation of recombinant proteins

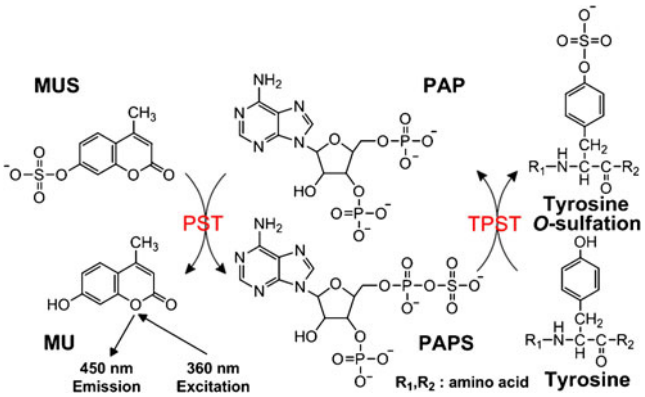
Recombinant PST (K65ER68G of a rat PST) [9], glutathione *S*-transferase fused P-selectin glycoprotein ligand-1 (GST-PSGL-1) peptide [10], and N utilization substance protein A fused tyrosylprotein sulfotransferase (NusA-*Dm*TPST) [11] were expressed in *Escherichia coli* according to published procedures using expression vectors of pET-3c, pGEX-4T1, and pET-43a, respectively. All the proteins were purified to homogeneity following published procedures (Electronic Supplementary Material Fig. S1).

PST assay

In this study, the function of PST was to catalyze the generation of PAPS from PAP and its assay has been developed previously [7]. PST activity was determined according to variation of MU fluorescence at 450 nm with excitation at 360 nm. The reaction mixture (200 μl) comprised 50 mM MES buffer (pH 6.5), 5 mM 2-mercaptoethanol, 2 mM MUS, 20 μM PAP, and 0.3–2 μg K65ER68G PST. The rate of PAPS production is equivalent to that of MU. One unit of PST was defined as 1 μmol of PAP converted to PAPS per minute under the PST assay condition. The emission coefficient was determined with commercial MU at specific temperature and pH.

TPST assay

A PST-TPST coupled enzyme assay (Scheme I) developed in this report was used to determine TPST activity. The reaction mixture included 50 mM MES buffer (pH 6.5), 5 mM 2-mercaptoethanol, 30 μM PAPS, 2 mM MUS, 120 μM PSGL-1 peptide, and 17 mU (28 μg) K65ER68G PST and NusA-*Dm*TPST (5–25 μg) in a final volume of 200 μl. The mixture was preincubated in 37 °C for 5~10 min in the absence of TPST. Preincubation was needed to assure that all contaminated PAP converted to PAPS before the beginning of protein sulfation catalyzed by TPST. In the coupled enzyme system, it is essential that PST activity is significantly higher than that of TPST to completely convert the variation of PAP to signal and maintain the concentration of PAPS in TPST assay. Following the completion of the preincubation period, NusA-*Dm*TPST was added last into the reaction mixture to start the protein sulfation. A spectrofluorometer (Hitachi F-7000, Japan) was used to detect the increase of fluorescent molecule (MU, monitored as described for PST assay) and to determine the activity of TPST.



Scheme I Fluorescence assay of protein tyrosine sulfation. Protein sulfation was continuously monitored through a two-enzyme platform. PST catalyzed the transfer of sulfuryl group from MUS to PAP and produced MU (fluorophore) and PAPS (sulfuryl group donor). The PST catalyzed reaction could not proceed continuously unless PAP was regenerated by TPST-catalyzed sulfuryl group transfer from PAPS to a tyrosine in a protein substrate. In this study, a rat PST mutant K65ER68G [9] and a recombinant NusA-DmTPST were used. PST is insensitive to peptide or protein substrates and TPST does not use phenol substrates

Analysis of kinetic data

Initial rate was determined from TPST assay, as described above. The apparent Michaelis–Menten constant (K_m), inhibition constant (K_i), and turnover number (k_{cat}) were obtained using nonlinear regression by Sigmaplot V12 and its Enzyme Kinetics Module (SPSS Inc., Chicago, IL). The following equations for non-inhibitory reactions (1), substrate inhibition reactions (2), and calculation of k_{cat} (3) were used:

$$v = V_{max}[S]/(K_m + [S]) \quad (1)$$

$$v = V_{max}[S]/\{K_m + [S](1 + [S]/K_i)\} \quad (2)$$

$$V_{max} = k_{cat}[E] \quad (3)$$

These equations described the relationship of enzymatic reactions among the measured reaction rate v, a maximum reaction rate V_{max}, and the concentration of a substrate [S].

Result and discussion

Scheme of TPST assay

A fluorescent assay for protein sulfation can be highly sensitive for rapid kinetic determination of TPST and high-throughput screening of its potential protein substrates and inhibitors. Scheme I illustrates that fluorescence produced through MU reflects the protein sulfation catalyzed by PST and TPST coupled enzyme assay. The fluorescence can be continuously observed only when sulfuryl group of PAPS is transferred to a protein substrate catalyzed by TPST. Such scheme has been tested successfully for the determination of the activities of SULT1A3 and SULT2A1 [7, 8]. PAPS is efficiently produced by PST coupled enzyme assay and the sensitivity is comparable to the end point assay by isotope labeling with added advantage to continuously monitors the catalytic reaction [6]. In this study, we demonstrated that this platform was useful not only to monitor the activity of TPST but also to screen for its potential protein substrates without size limitation either for small peptide or the whole protein.

Measurement of TPST activity

The PST-TPST coupled enzyme assay proposed in Scheme I was validated as shown in Fig. 1. Under the assay conditions, the change of fluorescence could be observed only when complete reactants (PAPS, PSGL-1 and MUS) and enzymes (PST and TPST) were present. Elimination of any one of the reactants or enzymes resulted in no continuous change of fluorescence. A reducing reagent 2-mercaptoethanol was used to stabilize PST. No significant effect could be observed in the absence of 2-mercaptoethanol since excess amount of PST was used. Data shown in Fig. 1 clearly demonstrated that the change of fluorescence observed in the coupled enzyme assay imitated the sulfation of PSGL-1 catalyzed by TPST. Protein sulfation was further confirmed by Western blot by anti-sulfotyrosine antibody and by [35S] autoradiography as shown in Electronic Supplementary Material Figs. S2 and S3, respectively, and comparable results were obtained.

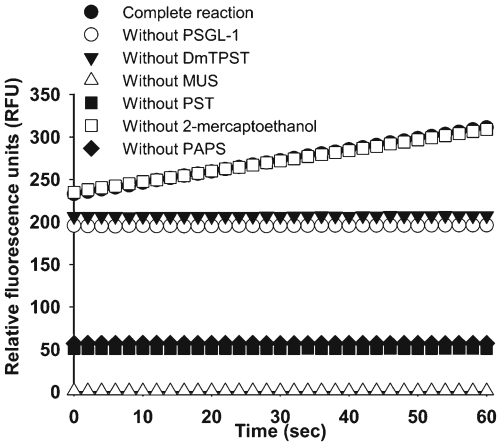


Fig. 1 Validation of fluorescence assay for protein sulfation. Complete reaction (*filled circles*) mixture comprised 50 mM MES buffer (pH 6.5), 5 mM 2-mercaptoethanol, 30 μM PAPS, 2 mM, MUS, 120 μM PSGL-1 peptide, 17 mU (28 μg) K65ER68G PST, and NusA-*Dm*TPST (10 μg) in a final volume of 200 μl. The reaction mixture was preincubated at 37 °C for 5~10 min in the absence of TPST. Preincubation was to assure sulfation of PAP that may contaminate with commercial PAPS [7, 14]. NusA-*Dm*TPST was added last to start the protein sulfation. Reactions each in the absence of one of the following reagents were also performed individually: *Dm*TPST (*filled triangles*), PST (*filled squares*), MUS (*open triangles*), PAPS (*filled diamonds*), 2-mercaptoethanol (*open squares*), and PSGL-1 (*open circles*). These reactions served as controls to ensure that the change of fluorescence was solely followed by the sulfation of protein/peptide substrate

Analysis of kinetic data

To obtain maximum TPST activity in the coupled enzyme assay, excess amount of PST was used. Our previous study described the optimal conditions (from pH 6.0–9.0) in which PST effectively produces fluorescent signal of MU [7]. In Fig. 2a, we demonstrated that the change of fluorescence was not dependent on the amount of PST added when more than 10 mU of PST was used in the coupled enzyme assay. This result suggested that PST activity over 10 mU gave maximum TPST activity. In this study, 17 mU (28 μg) of PST was established as a standard assay condition. With this amount of PST activity, we can ensure that the rate-limiting step and the observed activity of the coupled

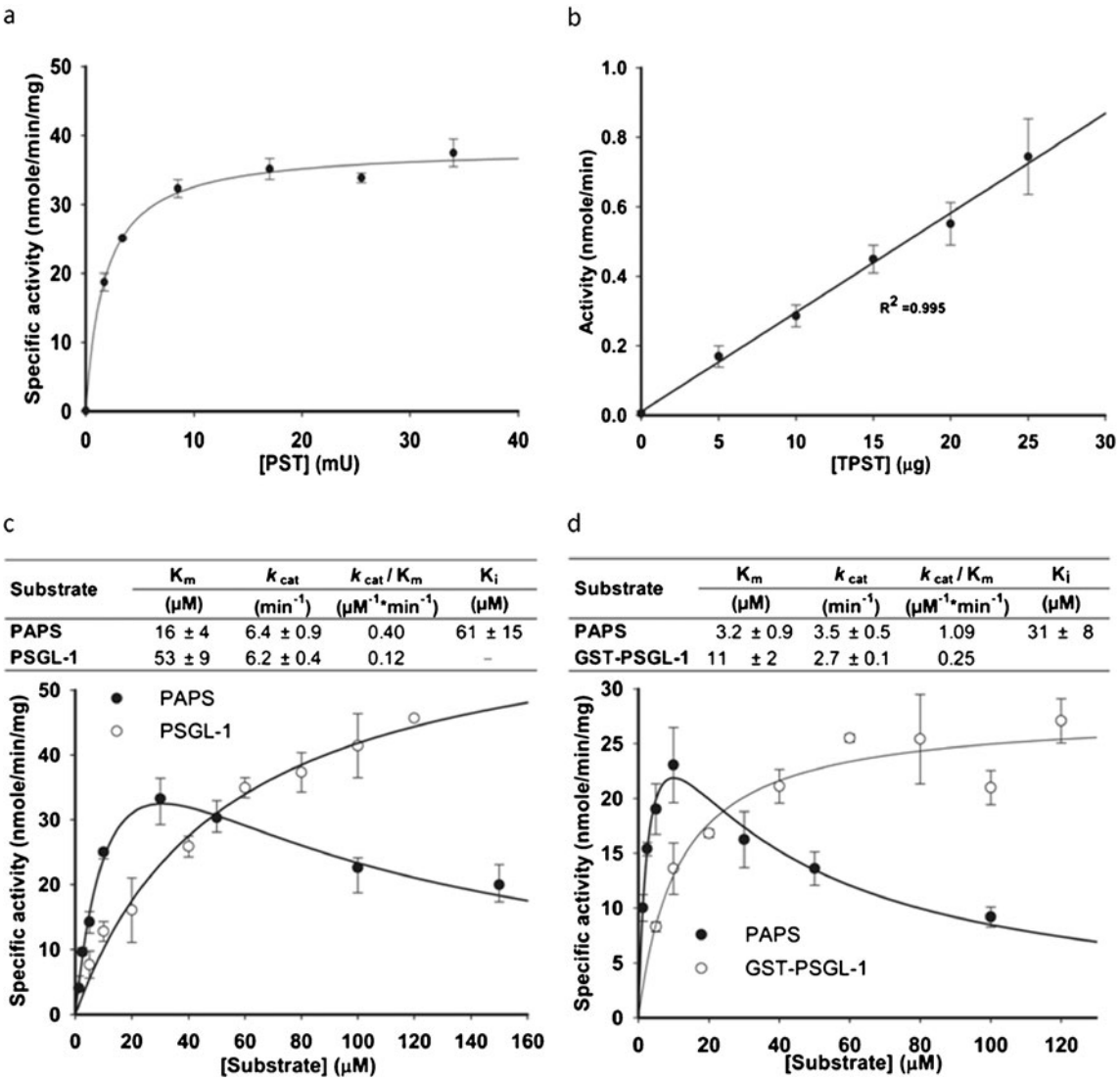


Fig. 2 Activities of *Dm*TPST determined through PST-TPST coupled enzyme assay. **a** Effect of PST on protein sulfation. Reaction condition is the same as described in "Experimental" section for TPST assay except that 10 μg NusA-*Dm*TPST and different amounts of K65ER68G *rat* PST (1.7, 3.4, 8.5, 17, 25.5, and 34 mU) were used. **b** Effective range for *Dm*TPST assay. Reaction condition is the same as described in "Experimental" section for TPST assay. **c** Determination of kinetic constants of *Dm*TPST at varied concentrations of PSGL-1 peptide(*open circles*) or PAPS (*filled circles*). **d** Determination of kinetic constants of *Dm*TPST at varied concentrations of GST-PSGL-1 (*open circles*) or PAPS (*filled circles*). All the reaction conditions are the same as described in the "Experimental" section for TPST assay except those mentioned specifically. Each data were the average of three independent measurements

enzyme reaction solely depended on the protein sulfation. As demonstrated in Fig. 2b, the activity of the PST-TPST coupled enzyme assay was linearly dependent on the amount of TPST. Figure 2a, b established the appropriate amount of PST needed as well as the linear range of the TPST coupled enzyme assay. Under the assay condition, the effective (linear) range of TPST assay (Fig. 2b) can reach to approximately 1 nmol/min activity. Kinetics of TPST was determined through initial rate data as shown in Fig. 2c, d. Kinetic data of *Dm*TPST were first reported here in Fig. 2c, d using small peptide and its fusion protein, respectively, as substrates. Although it is not unusual for other sulfotransferases, it was first reported that PAPS exhibited substrate inhibition to *Dm*TPST. It is interesting to note that the size of substrate significantly affected the kinetic constant of *Dm*TPST. It appeared that large substrate gave much lower K_m indicating a better affinity to the enzyme. These results indicated that the size of protein may also be important as substrate of TPST. Both the K_m and k_{cat} were comparable to those recently reported for human TPST using radioactive labeling assay [12]. Much lower TPST activity (k_{cat}=0.50±0.03 min^{-1}) was observed by a mass spectrometric kinetic analysis of human TPST [13].We proposed that the PAPS purity influenced by different supplemental system may cause the variation of TPST activity reported in the literatures. In this report, PAPS concentration was continuously supplemented through PST catalyzed

reaction and maintained at constant level even following the sulfation of PSGL-1 catalyzed by TPST.

Application of fluorescent TPST assay

The advantage of this assay was to continuously monitor the progress of the protein sulfation catalyzed by TPST without complicated procedures or expensive instruments. This method was not limited by the size of substrate and may become a useful tool to further examine specificities of TPST by simply replacing PSGL-1 substrate used in this study with other proteins of interest. In addition, due to the simplicity of the procedure, a high-throughput method can be further developed and applied to screening protein sulfation sites and TPST inhibitors or for the detailed studies of TPST kinetics. It can also be a useful tool for the future study of the physiological responses and development of the medicine to treat the diseases that are related to protein sulfation.

Conclusion

Fluorescence assay for TPST-catalyzed protein sulfation was demonstrated. This was a continuous and rapid method and could use peptide or protein substrates without size limitation. We expect that this method would be an easy and convenient tool for the study of post-translational protein sulfation.

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