Existence of a plant tyrosylprotein sulfotransferase: novel plant enzyme catalyzing tyrosine *O*-sulfation of preprophytosulfokine variants in vitro

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Abstract An in vitro assay system to detect tyrosylprotein sulfotransferase (TPST) activity of higher plant cells was established, using synthetic oligopeptides based on the deduced amino acid sequence of a phytosulfokine-α (PSK-α) precursor. TPST activity was found in microsomal membrane fractions of rice, asparagus and carrot cells and it was confirmed that acidic amino acid residues adjacent to the tyrosine residues of acceptor peptides were essential to the sulfation reaction. The asparagus TPST exhibited a broad pH optimum of 7.0–8.5, required manganese ions for maximal activity and appeared to be a membrane-bound protein localized in the Golgi apparatus. These enzymes should be defined as a new class of plant sulfotransferases that catalyze tyrosine *O*-sulfation of a PSK-α precursor and other unknown proteins.

Key words: Phytosulfokine-α; Tyrosine *O*-sulfation; Tyrosylprotein sulfotransferase

1. Introduction

Protein tyrosine *O*-sulfation is the most frequent posttranslational modification which occurs in many secretory and membrane proteins of various eukaryotes [1]. In mammalian cells, the sulfation reaction is catalyzed by tyrosylprotein sulfotransferase (TPST) [2], a membrane-bound enzyme of the *trans*-Golgi network [3], that has been well characterized with respect to substrate specificity and properties [1]. Furthermore, human and mouse TPST cDNAs have been cloned, predicting type II transmembrane proteins of 370 amino acid residues with an apparent molecular mass of 54 kDa [4]. In spite of a remarkable advance in the study of mammalian TPST, nothing is known about TPST in higher plants. This might be due to the small number of sulfated proteins or peptides discovered in the plant kingdom, the only example until the early 1990s being the tyrosine sulfated glycoprotein SG 70 in volvox (*Volvox carteri*) [5].

In 1996, a novel plant growth factor, phytosulfokine-α (PSK-α), which is a disulfated pentapeptide (Tyr(SO_3H)-Ile-Tyr(SO_3H)-Thr-Gln), was isolated from a conditioned medium (CM) derived from asparagus (*Asparagus officinalis* L.) mesophyll culture [6]. Subsequent studies demonstrated the existence of PSK-α in CM derived from both monocot [7] and dicot [8,9] cell cultures. Furthermore, isolation of a rice (*Oryza sativa* L.) cDNA, *OsPSK*, encoding a putative PSK-α precursor has been achieved [10] and it was also shown that rice suspension cells contain both specific high- and low-affinity saturable PSK-α binding sites on their plasma membranes [11]. It is noteworthy that desulfated PSK-α loses both mitogenic activity and competitive ability for ligand binding [11]. The results thus strongly suggest not only the presence of TPST in the plant kingdom but also the importance of the sulfation reaction for the physiological function of PSK-α.

In the present paper, we describe the establishment of an in vitro assay system to detect TPST activity of plant cells. We adopted a synthetic tetradecapeptide (EEFHTDYIYTQDVK) modeled after the putative sulfation site of preprophytosulfokine (PP-PSK), based on the *OsPSK* cDNA sequence [10]. The reasons for choosing this PP-PSK-(74–87) as substrate are as follows: (i) it contains a mature PSK-α sequence (YIYTQ); (ii) it features two glutamic acid residues and one aspartic acid residue adjacent to the N-terminal side of the tyrosine residue. It thus appears to fulfill the criteria for an acceptor substrate because these preceding acidic residues are implicated in substrate recognition by mammalian TPST [2,12]. Using this assay, we investigated the presence of TPST in several plant cells. We could also show substrate specificity, properties and subcellular localization of the TPST.

2. Materials and methods

2.1. Chemicals

[^{35}S]PAPS (3′-phosphoadenosine 5′-phosphosulfate) (79.2 GBq/mmol) was purchased from New England Nuclear (Boston, MA, USA) and a reverse phase HPLC column, Develosil ODS-HG-5 from Nomura Chemicals (Seto, Japan). Protected amino acids for peptide synthesis were from Peptide Institute (Osaka, Japan) or PE Biosystems Japan (Chiba, Japan) and the ACS II scintillation cocktail was from Amersham Pharmacia Biotech (Tokyo, Japan). All other chemicals were obtained from Wako Pure Chemicals (Osaka, Japan).

2.2. Peptide synthesis

Preparation of the tetradecapeptide PP-PSK-(74–87) and other variants was performed with a peptide synthesizer (Applied Biosystems Model 433A) according to the manufacturer's instructions. Synthesis of chemically sulfated peptides as standards for HPLC was carried out as previously described [13].

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Abbreviations: PAPS, 3′-phosphoadenosine 5′-phosphosulfate; PP-PSK, preprophytosulfokine; PSK-α, phytosulfokine-α; TPST, tyrosylprotein sulfotransferase

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2.3. Cell culture and preparation of microsomal membrane fractions

Cell lines of rice (*Oryza sativa* L.) Oc, carrot (*Daucus carota* L.) NC, tobacco (*Nicotiana tabacum* L.) BY-2, asparagus (*Asparagus officinalis* L.) Asp-86 and tomato (*Lycopersicon esculentum* Mill.) Sly-1 were maintained with subculturing and microsomal membrane fractions of the cells were prepared by the protocol as detailed earlier [11].

2.4. Subcellular fractionation

Subcellular fractionation by linear sucrose density gradient centrifugation was performed essentially as described by Kawasaki [14]. Seven-day-old asparagus cells were harvested by filtration, mixed with 100 ml of buffer that contained 50 mM Tris/MES, pH 7.3, 2 mM dithiothreitol and 250 mM sucrose and homogenized in a blender. The homogenate was filtered through two layers of Miracloth (Calbiochem, La Jolla, CA, USA) and the filtrate was centrifuged at $10\,000\times g$ for 15 min at 4°C. The supernatant was overlaid on a cushion of 1.5 M sucrose in 20 mM Tris/MES, pH 7.3, 2 mM dithiothreitol. The resultant discontinuous gradient was centrifuged at $100\,000\times g$ for 1 h at 4°C and the membranous organelles on the cushion were collected. Aliquots were diluted with 20 mM Tris/MES, pH 7.3, 2 mM dithiothreitol to ≅18% sucrose, overlaid on 28 ml of a 20–45% sucrose gradient and centrifuged at $100\,000\times g$ for 12 h at 4°C. After centrifugation, the gradient was fractionated and each fraction was analyzed for TPST and marker enzyme activities.

2.5. Assays of the marker enzymes

The activity of Triton-stimulated IDPase as a marker of the Golgi apparatus was measured as described by Mitsui et al. [15]. The activity of cytochrome *c* oxidase as a marker of the mitochondria and antimycin A-insensitive NADH-cytochrome *c* reductase as a marker of the endoplasmic reticulum were measured as described by Yoshida [16]. Vanadate-sensitive ATPase as a marker of the plasma membrane was measured as described by O'Neill and Spanswick [17] except that the reaction mixture contained 0.02% Triton X-100.

2.6. In vitro TPST assay

TPST activity was assayed by determining the transfer of [^{35}S]sulfate groups from [^{35}S]PAPS to synthetic acceptor peptides, separated with reverse phase HPLC. The standard reaction mixture consisted of, in final concentrations, 50 mM HEPES, pH 7.0, 50 mM NaF, 5 mM $MnCl_2$, 1 mM 5′-AMP, 1% (w/v) Triton X-100, TPST preparation (50 μg of protein), 2 μM [^{35}S]PAPS and 0.1 mM synthetic peptides. The final reaction volume was 50 μl. Reactions were started by addition of [^{35}S]PAPS at 30°C. After 30 min, reactions were terminated by heating to 95°C for 3 min. After chilling on ice, the samples were centrifuged to remove the precipitated proteins and aliquots of the supernatant were subjected to HPLC on a Develosil ODS-HG-5 column (4.6×250 mm) by a linear gradient elution of 10–30% acetonitrile containing 0.1% ammonium acetate at a flow rate of 1.0 ml/min. Fractions were collected at 1 min intervals and the each aliquot was mixed with 2.0 ml of ACS II cocktail, followed by determination of the radioactivity by liquid scintillation counting.

3. Results and discussion

Tyrosine *O*-sulfation is a common posttranslational modification in mammals mediated by a Golgi enzyme called TPST [1,2]. Recently isolation and characterization of human and mouse cDNAs encoding TPST-1 and -2 were accomplished [4,18,19]. The aim of this study was to investigate the presence of TPST in higher plant cells and to determine substrate specificity and properties using a variety of synthetic peptides. For this purpose, we developed a suitable in vitro TPST assay system referring to mammalian studies [12,20]. We used PAPS as a donor substrate for plant TPST, since it is known to exist in plant cells and to act as a common sulfate donor for other plant sulfotransferases [21–23]. For the acceptor substrate of plant TPST, we employed a synthetic tetradecapeptide (PP-PSK-(74–87)), corresponding to the putative sulfation site of the PSK-α precursor of rice Oc cells [10]. Incubation of the acceptor peptide with the sulfate donor [^{35}S]PAPS and microsomal membrane fractions prepared from a suspension of cultured rice cells led to the formation of sulfated peptides (Table 1). In this in vitro assay system, the detection limit was approximately 20 fmol transfer of sulfate, allowing its use for further characterization of plant TPST.

Table 1
Distribution of plant TPSTs

Plant species	Cell line	TPST activity (pmol/min/mg protein)
Asparagus officinalis L.	Asp-86	8.21 ± 0.05
Oryza sativa L.	Oc	0.74 ± 0.05
Daucus carota L.	NC	7.91 ± 0.42
Lycopersicon esculentum Mill.	Sly-1	0.03 ± 0.001
Nicotiana tabacum L.	BY-2	0.15 ± 0.02

Tyrosylprotein sulfotransferase activity was measured in various microsomal membrane fractions prepared from five plant cultured cells. Each assay was performed in triplicate. The data shown are mean values.

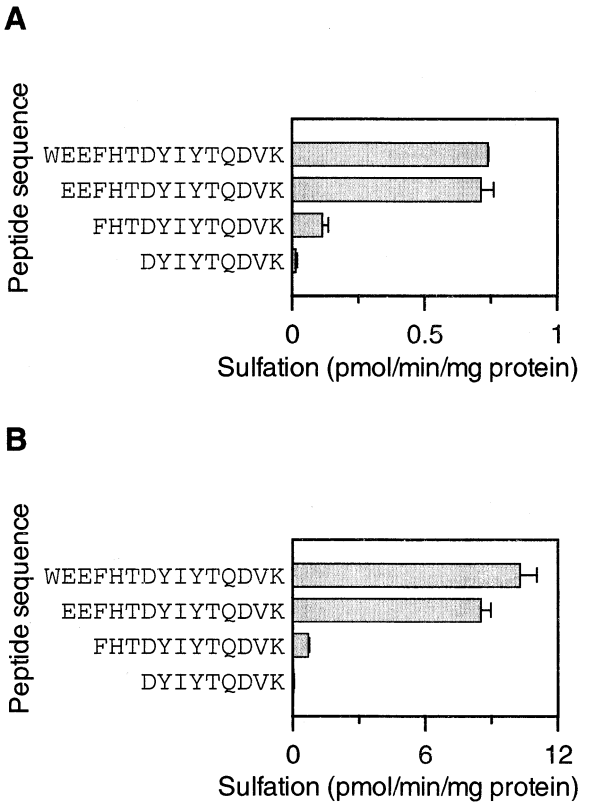

Fig. 1. Sulfation of PP-PSK variants with various lengths. The sequences of the synthetic peptides are given in single-letter code. A: PP-PSK-(73–87) and its N-truncated variants were assayed with a rice microsomal membrane fraction. B: PP-PSK-(73–87) and its N-truncated variants were assayed with an asparagus microsomal membrane fraction.

Both monocot (rice and asparagus) and dicot (carrot, tomato and tobacco) plant cells were employed for preparation of microsomal membrane fractions. As shown in Table 1, the microsomal membrane fraction of asparagus cells demonstrated the highest TPST activity in the five cell lines, with carrot cells attaining the same order of magnitude. In contrast, the TPST activity of rice cells, from which *OsPSK* cDNA had been cloned, was about 11-fold less and little

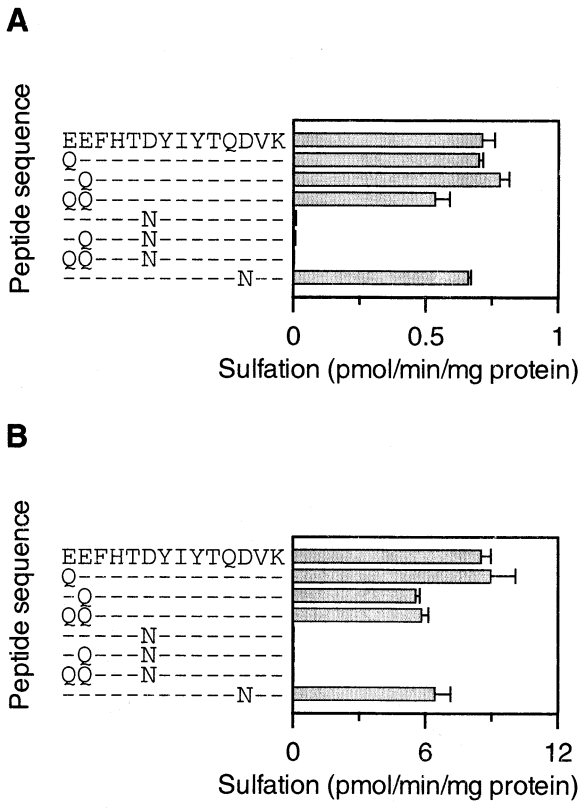

Fig. 2. Plant TPSTs may be capable of recognizing peptides containing acidic amino acids. The sequences of the synthetic peptides are given in single-letter code. Dashes indicate amino acid residues that are identical to the top sequence. A: PP-PSK-(74–87) and seven variants of this peptide were assayed with a rice microsomal membrane fraction. B: PP-PSK-(74–87) and seven variants of this peptide were assayed with an asparagus microsomal membrane fraction.

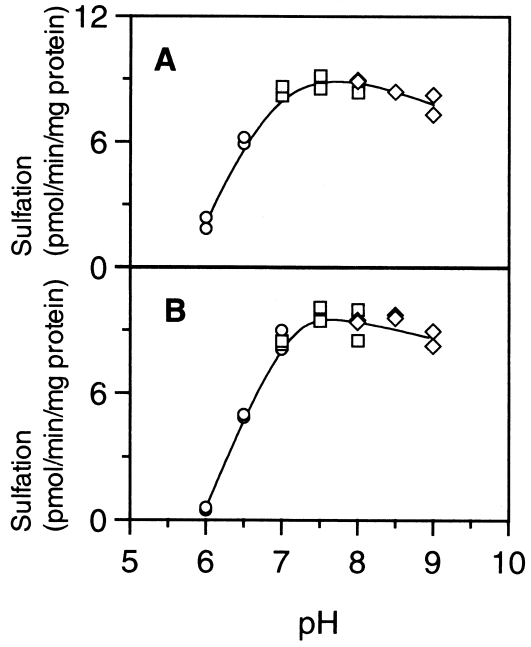

Fig. 3. Effects of pH on the sulfation of PP-PSK-(74–87) by asparagus TPST. A: Asparagus TPST activity was measured as described in Section 2 except that 50 mM MES (open circles), HEPES (open squares) or Tris (open diamonds) buffer reagents were used at the various pH values indicated. B: Stability of asparagus TPST at several pH values. The asparagus microsomal membrane fraction was incubated at differing pH as mentioned above, without addition of substrates. After incubation, 0.1% γ-globulin was added as a carrier protein. Subsequently, each aliquot was mixed 1:1 with 50% (w/v) polyethylene glycol (average molecular weight, 3350) and kept 30 min on ice. Precipitated proteins were collected by centrifugation for 15 min at 18 000×*g*, solubilized in the reaction buffer at pH 7.0 and then assayed for TPST activity.

TPST activity was detectable in the membrane fractions of tomato and tobacco cells. The results suggested some relation to the potential of PSK production, since all of the cells demonstrating TPST activity were also found to produce PSK (data not shown). Furthermore, the widespread distribution of TPST in higher plants suggests that the tyrosine *O*-sulfation may be a ubiquitous posttranslational process resulting in the biologically functional modification of proteins and peptides.

The apparent K_m values for rice and asparagus TPSTs for PP-PSK-(74–87) were 71 and 457 μM and the apparent V_{max} were 1.0 and 28 pmol/min/mg protein, respectively (Table 2). Niehrs et al. [12] showed the apparent K_m and V_{max} of bovine TPST for the synthetic peptide CCK-1, corresponding to the C-terminal nonapeptide of rat preprocholecystokinin, to be 35 μM and 3.1 pmol/min/mg protein. The apparent K_m of rice TPST and bovine TPST for CCK-1 were thus in the same range. While the apparent K_m of asparagus TPST was 6-fold higher, this may be a consequence of use of the heterologous acceptor peptide, PP-PSK-(74–87). Such a difference in kinetic parameters has also been observed for mammalian TPSTs, the bovine form having a 21-fold higher apparent K_m for the recombinant full length hirudin variant (tyrosine sulfated protein of 65 amino acid residues) than that of the homologous leech (*Hirudo medicinalis*) [20].

It remains to be estimated whether the affinity of rice TPST detected in vitro for PP-PSK-(74–87) peptide reflects the affinity of this enzyme for the full length PP-PSK protein, the larger physiological substrate. It was found, however, that the kinetic constants of leech TPST toward the recombinant full length hirudin were similar to those toward a synthetic nonapeptide corresponding to the tyrosine sulfation site of hirudin [20]. While the PP-PSK protein of 89 amino acids is slightly larger than hirudin [10], the kinetic constants of rice

Table 2
Kinetic parameters of rice and asparagus TPSTs

Substrate peptide	Rice TPST		Asparagus TPST	
	K_m (μM)	V_{max} (pmol/min/mg protein)	K_m (μM)	V_{max} (pmol/min/mg protein)
PP-PSK-(74–87)	71	1.0	457	28.0

Duplicate assays with six or nine different concentrations (100–4 μM) of substrate peptide were performed, and the kinetic parameters of TPST were determined from a linear fit of the Lineweaver–Burk plot.

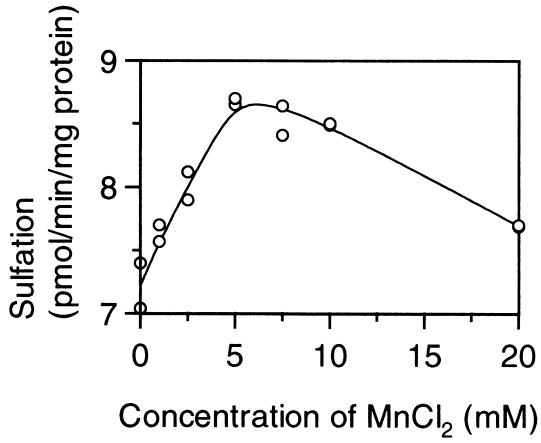

Fig. 4. Effects of Mn^{2+} concentration on the sulfation of PP-PSK-(74–87) by asparagus TPST. Tyrosylprotein sulfotransferase activity was measured using a TPST preparation (50 μg of protein) in 50 μl of 50 mM HEPES, pH 7.0, containing 0.1 mM PP-PSK-(74–87), 50 mM NaF, 1 mM 5′-AMP, 1% (w/v) Triton X-100 and 2 μM [^{35}S]PAPS and varying concentrations of $MnCl_2$ as indicated.

TPST toward the tetradecapeptide PP-PSK-(74–87) may also apply to those toward the full length PP-PSK protein.

The substrate specificity of rice and asparagus TPSTs was analyzed using synthetic peptides with varying sequences. In mammalian TPST research, sequences surrounding sulfated tyrosine residues have been shown to be important [1,12]. The hallmark of common consensus features for tyrosine *O*-sulfation is the presence of acidic amino acids adjacent to the N-terminal side of the tyrosine [12]. First, several N-terminal truncated peptides were constructed and the specificity of rice and asparagus TPSTs for these peptides was assessed (Fig. 1). Sulfate transfer to pentadecapeptide PP-PSK-(73–87) occurred at a similar level as that to Trp^{73} truncated tetradecapeptide, PP-PSK-(74–87). Elimination of the two glutamate residues of PP-PSK-(74–87) resulted in 8-fold (rice) or 12-fold (asparagus) reduction in the rate of sulfation. The nonapeptide PP-PSK-(79–87) proved to be a very poor substrate for both rice and asparagus TPSTs. Its sulfation resulted in 200-fold less activity than with PP-PSK-(74–87), despite the presence of one acidic amino acid residue (Asp^{79}).

We also investigated the influence of flanking acidic residues on tyrosine *O*-sulfation by replacing them with the respective amide residues. As shown in Fig. 2, the most important determinant for the sulfation of PP-PSK-(74–87) was found to be the aspartic acid residue at 79. More than 100-fold decrease of sulfation was observed when Asp^{79} was replaced with an asparagine residue. Contributions of Glu^{74}, Glu^{75} and the C-terminal acidic residue, Asp^{85} to the substrate specificity were also confirmed, but appeared to be less than that of Asp^{79} (Fig. 2). These results demonstrate that both adjacent and distant acidic amino acid residues impact on sulfation of the peptide, adjacent acidic amino acids being more important, in line with the prediction that plant TPSTs may also conform to consensus features of substrate recognition delineated for mammalian TPSTs.

The asparagus microsomal membrane fraction was used as a source of TPST for further characterization of the enzyme. TPST specific activity in the microsomal membrane fraction of asparagus cells was 7-fold that of the homogenate, whereas no TPST activity was observed in the cytosolic fraction (data not shown). Moreover, sulfation with the microsomal membrane fraction of asparagus cells was stimulated by the presence of Triton X-100, indicating existence as a membrane-bound form.

The pH optimum for the sulfation was found to be approximately 7.5 with a broad peak from pH 7.0 to 8.5 (Fig. 3A). The enzyme activity markedly decreased below pH 7.0, which may be accounted for by the fact that this enzyme proved unstable below pH 7.0 (Fig. 3B). In contrast, rat liver TPST displays a sharp, acidic pH optimum (pH 6.0–6.7) [1,24], presumably reflecting differences in the subcellular milieu between mammalian and plant cells.

Previous studies suggested that Mn^{2+} and/or Mg^{2+} are essential for sulfation reactions of both rat liver and bovine adrenal medulla TPSTs [1,24,25]. Accordingly, several divalent cations were examined for their ability to stimulate plant TPST activity. As shown in Table 3, both Mn^{2+} and Mg^{2+} were capable of stimulating asparagus TPST, whereas Ca^{2+} and Zn^{2+} were inhibitory. The rate of sulfate transfer was

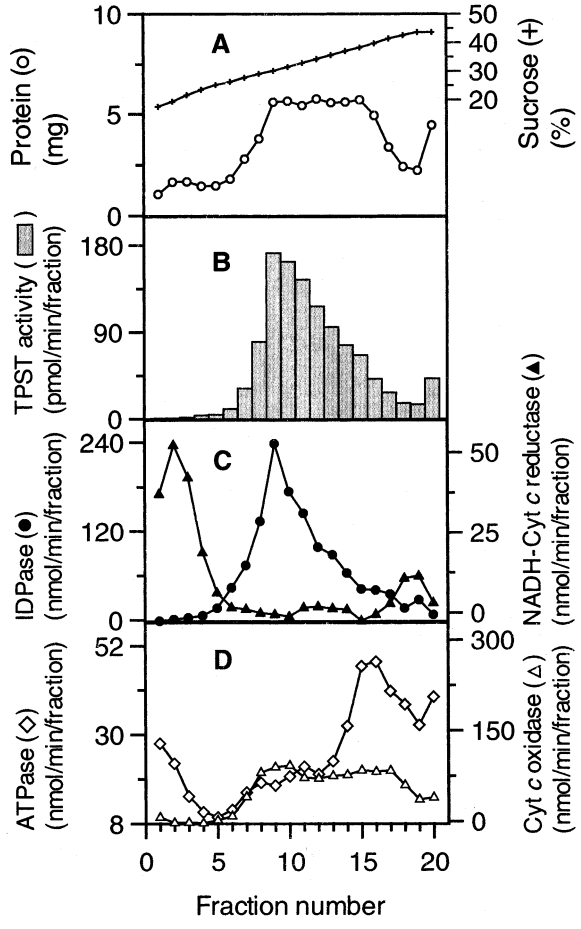

Fig. 5. Subcellular fractionation of the asparagus membrane preparation. Aliquots of asparagus membranous organelles were overlaid on 28 ml of a 20–45% sucrose gradient and centrifuged at 100 000×*g* for 12 h at 4°C. Twenty recovered fractions of approximately 1.5 ml each were collected and assayed for (A) sucrose concentration (pluses) and protein (open circles), (B) TPST activity (shaded bars), (C) activities of antimycin A-insensitive NADH-cytochrome *c* reductase (endoplasmic reticulum marker, closed triangles) and Triton-stimulated IDPase (Golgi apparatus marker, closed circles), (D) vanadate-sensitive ATPase (plasma membrane marker, open diamonds) and cytochrome *c* oxidase (mitochondria marker, open triangles).

Table 3
Effect of divalent cations on asparagus TPST activity

Cation (5 mM)	Sulfation (pmol/min/mg protein)	Relative activity
Control	8.64 ± 0.89	1.00
$MnCl_2$	10.66 ± 0.16	1.23
$MgCl_2$	9.33 ± 0.30	1.08
$CaCl_2$	3.12 ± 0.04	0.36
$ZnCl_2$	0.16 ± 0.03	0.02

Tyrosylprotein sulfotransferase activity was measured over 30 min at 30°C using 0.1 mM PP-PSK-(74–87), 50 mM HEPES, pH 7.0, 50 mM NaF, 1 mM 5′-AMP, 1% (w/v) Triton X-100, a TPST preparation (50 μg of protein) and 2 μM [^{35}S]PAPS in the presence or absence of 5 mM of the cation. Each assay was performed in triplicate and the mean values are shown.

maximally stimulated 1.2-fold when incubations were performed in the presence of 5 mM $MnCl_2$ (Fig. 4). The finding of high control activity may be caused by binding of the cations to the enzyme protein so tightly as not to dissociate during the membrane preparation. Similar results were obtained in the mammalian study of Vargas et al. [26], tyrosine sulfotransferase activity of the rat brain microsomal fraction being detected even when $MnCl_2$ was not added to the reaction mixture. Thus, both enzymes may share catalytic properties.

Lee and Huttner [3] demonstrated the mammalian TPST to be localized specifically in the *trans*-Golgi-network. Accordingly, we analyzed the subcellular localization of asparagus TPST using linear sucrose density gradient centrifugation. As shown in Fig. 5, the activities of TPST and the Golgi marker enzyme, Triton-stimulated IDPase, demonstrated closely similar distribution patterns. The specific activities of both TPST and Triton-stimulated IDPase were enriched approximately 3.5-fold in the 30% sucrose fraction (fraction 9) as compared to the crude membrane fraction. Furthermore, 74% of the original TPST activity in the crude membrane fraction could be recovered in the three fractions from 9 to 11. The activity of other marker enzymes, Vanadate-sensitive ATPase or NADH-cytochrome *c* reductase, was distributed in the different fractions. These observations indicated that asparagus TPST is predominantly localized in the Golgi apparatus.

In summary, we have described the existence of a Golgi-localized, posttranslational enzyme, tyrosylprotein sulfotransferase, in a series of plant cells and characterized its substrate specificity and kinetic and catalytic properties. Purification of the enzyme protein and molecular cloning of the cDNA are now in progress.

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