

Choline-O-Sulfate Biosynthesis in Plants¹

Identification and Partial Characterization of a Salinity-Inducible Choline Sulfotransferase from Species of *Limonium* (Plumbaginaceae)

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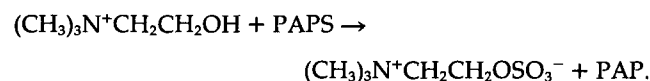
Choline-O-sulfate is a compatible osmolyte accumulated under saline conditions by members of the halophytic genus *Limonium* and other Plumbaginaceae. A choline sulfotransferase (EC 2.8.2.6) responsible for the formation of choline-O-sulfate was characterized in *Limonium* species. A simple radiometric assay was developed in which [¹⁴C]choline was used as substrate, and the h [¹⁴C]choline-O-sulfate product was isolated by ion-exchange chromatography. The choline sulfotransferase activity was soluble, required 3'-phosphoadenosine-5'-phosphosulfate as the sulfate donor, and showed a pH optimum at 9.0. Apparent *K_m* values were 25 μM for choline and 5.5 μM for 3'-phosphoadenosine-5'-phosphosulfate. Choline sulfotransferase activity was detected in various *Limonium* species but was very low or absent from species that do not accumulate choline-O-sulfate. In roots and leaves of *Limonium perezii*, the activity was increased at least 4-fold by salinization with 40% (v/v) artificial sea water. Choline sulfotransferase activity was also induced in cell cultures of *L. perezii* following salt shock with 20% (v/v) artificial sea water or osmotic shock with 19% (w/v) polyethylene glycol 6000. Labeling experiments with [¹⁴C]choline confirmed that the enzyme induced in cell cultures was active in vivo.

Many plants accumulate nontoxic, compatible osmolytes (osmoprotectants) in response to salinity or drought stress (for reviews, see Wyn Jones and Storey, 1981; Rhodes and Hanson, 1993). Glycyl betaine is a well-known example. However, it is becoming clear that plants have evolved a range of other betaines and related osmoprotectant compounds. For example, various taxa of the stress-tolerant family Plumbaginaceae accumulate CS as well as one or more betaines, and it has been suggested that this diversity is related to the specific types of abiotic stress to which these plants are adapted (Hanson et al., 1994). Understanding the physiology and biochemistry of such metabolic adaptations to stress could make it possible to genetically engineer enhanced stress tolerance (McCue and Hanson, 1990; Delauney and Verma, 1993; Tarczynski et al., 1993).

CS is particularly interesting in this respect. CS accumulates in *Limonium* species (Hanson et al., 1991) and in all other

species of Plumbaginaceae tested (Hanson et al., 1994). Because members of this family have salt glands that can secrete Na⁺ and Cl⁻, but not SO₄²⁻ (Popp, 1984; Hanson and Gage, 1991), it has been hypothesized that CS accumulation may serve to detoxify part of the SO₄²⁻ that builds up in these species (Hanson and Gage, 1991; Hanson et al., 1994). If CS synthesis can indeed function to detoxify SO₄²⁻, it could be a valuable trait to transfer to crops because SO₄²⁻ salinity occurs in many agricultural soils (Epstein and Rains, 1987).

In vivo radiotracer experiments with *Limonium* species (Hanson et al., 1991) showed that [¹⁴C]Cho was readily converted to [¹⁴C]CS. This suggests the presence of CST activity (EC 2.8.2.6), catalyzing the reaction:



There appears to be no in vitro evidence for the occurrence of such an activity in higher plants, although CST activity has been reported in bacteria (Fitzgerald and Luschinski, 1977) and in fungi (Orsi and Spencer, 1964), where CS may serve as a sulfur storage compound (Spencer and Harada, 1960). These few reports gave some information on the characteristics of the enzyme but did not consider a possible role in adaptation to osmotic stress.

In the work reported here, we developed extraction and assay procedures for a novel CST from higher plants. We characterized certain kinetic properties of this enzyme and investigated the relationship between CST level and salinity. We found that CST activity is constitutively present in roots, leaves, and cultured cells of species that accumulate CS and is further induced by salt stress or osmotic shock.

MATERIALS AND METHODS

Chemicals

All chemicals were from Sigma or BDH (Ville St. Laurent, Québec, Canada). Cell culture reagents were from Gibco

Abbreviations: APS, adenosine-5'-phosphosulfate; ASW, artificial sea water; Cho, choline; CS, choline-O-sulfate; CST, choline sulfotransferase; FABMS, fast atom bombardment mass spectrometry; MS, Murashige and Skoog medium; PAP, 3'-phosphoadenosine-5'-phosphate; PAPS, 3'-phosphoadenosine-5'-phosphosulfate.

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(Burlington, Ontario, Canada), and ion-exchange resins were from Bio-Rad (Mississauga, Ontario, Canada). [Methyl- ^{14}C]-Cho (2.1 GBq mmol $^{-1}$, from NEN-DuPont, Mississauga, Ontario, Canada) was mixed with unlabeled Cho chloride to give the desired specific activity. [^{14}C]CS was synthesized from [methyl- ^{14}C]Cho by the method of Stevens and Vohra (1955).

Plant Material and Growth Conditions

Seed sources for *Limonium* species were as follows: *Limonium sinuatum* (L.) Mill, *Limonium latifolium* (Sm.) O. Kuntze, and *Limonium perezii* (Stapf) F.T. Hubb., Park Seed (Greenwood, SC); *Limonium nashii* Small, plants collected in the St. Laurent River estuary (Rimouski, Québec, Canada); *Limonium ramosissimum* G. Donelly, Michigan State University (East Lansing, MI). Seeds of *Brassica napus* L. cv. Westar, *Brassica oleracea* L. cv. April Red, *Zea mays* L. cv. COOP 6309, and *Helianthus annuus* L. cv. Sundak were supplied by the Montréal Botanical Garden; seeds of *Hordeum vulgare* L. cv. Proctor were from stocks maintained in our laboratory. Plants were germinated in vermiculite and then grown hydroponically as described (Rivoal and Hanson, 1993). Salinization was with ASW (Flowers et al., 1990), increasing the concentration in 10% (v/v) steps every 3 d until the desired level was reached. This level was maintained for at least 7 d before harvest. Harvested roots or leaves were rinsed in distilled water and blotted dry, then frozen in liquid N $_2$ and kept at -80°C until analyzed.

Cell Cultures

A callus culture was initiated from *L. perezii*. Leaf explants from a single plant were cultured on MS medium (Murashige and Skoog, 1962) containing 1 mg L $^{-1}$ 2,4-D, 3% (w/v) Suc, and 0.6% (w/v) phytagar. The friable calli were then transferred to liquid MS medium containing 1 mg L $^{-1}$ 2,4-D to give a cell-suspension culture. Cell-suspension cultures were grown in 250-mL flasks containing 80 mL of medium on a gyratory shaker at 125 rpm, $22 \pm 2^\circ\text{C}$, 16-h day (10 μmol PAR quanta m $^{-2}$ s $^{-1}$), 8-h night. Cells were routinely subcultured every 14 d into fresh medium using an inoculum of about 3.5 g of cells per flask. Experiments were carried out with cells that had been subcultured 30 to 40 times. For salinity or osmotic shock experiments, filter-sterilized ASW (200% [v/v] in MS medium) or autoclaved PEG 6000 solution (57% [w/v] in MS medium) was added to flasks after 7 d of culture to give final concentrations of 20% (v/v) or 19% (w/v), respectively. Control flasks received appropriate additions of MS medium alone. Cells (three flasks per time point) were harvested by filtration, rinsed with fresh medium, frozen in liquid N $_2$, and kept at -80°C until analyzed.

Enzyme Extraction and Assay

Extractions were carried out at 0 to 4°C . The extraction buffer (2 mL g $^{-1}$ tissue) contained 50 mM bicine, 200 mM Na borate, 5 mM DTT, 1 mM PMSF, 20 mM Na metabisulfite, 5 mM Na ascorbate, 10% (v/v) glycerol, and 4% (w/v) insoluble PVP; the final pH was 7.6 at 4°C . Leaves and roots were

ground in extraction buffer with sand in a mortar and pestle; cells were homogenized using a Polytron (Brinkmann, Mississauga, Ontario, Canada). The brei was centrifuged for 15 min at 12,000g. The supernatant was then desalted by passage through a PD-10 column (Pharmacia) equilibrated with 10 mM bicine-KOH (pH 9.0) containing 1 mM DTT. The desalted extract was used for CST activity measurements. For the determination of K_m values, the desalted extract was dialyzed twice, for 1.5 h each time, against 300 volumes of the same buffer. In the ultracentrifugation experiments, the 12,000g supernatant was centrifuged at 113,000g for 1 h before the desalting step.

Unless otherwise stated, CST was assayed in the following medium: 0.1 M bicine-KOH (pH 9.0 at 22°C), 0.5 mM MgCl $_2$, 2.5 mg mL $^{-1}$ BSA, 150 μM PAPS (Li $^+$ salt), 300 μM [^{14}C]Cho (Cl $^-$ salt, adjusted to a specific activity of 125 kBq μmol^{-1}). Each assay tube contained 50 μL of enzyme extract (usually 20–50 μg of protein) in a final volume of 100 μL . In each experiment, the blank was an identical mixture lacking PAPS. The tubes were incubated for 1 h in a water bath at 37°C . The reaction was stopped on ice and the [^{14}C]CS formed was separated from the substrate by ion-exchange chromatography of the reaction mix on a 1-mL mixed bed column [Dowex-1 (OH $^-$):BioRex-70 (H $^+$), 2:1, v/v]. The column was washed with 3.9 mL of water. The effluent (containing [^{14}C]CS) was mixed with 2 mL of Ready Gel liquid scintillation cocktail (Beckman), and ^{14}C was assayed by scintillation counting. In experiments where Cho sulfatase activity was investigated, the assay contained 0.1 M bicine-KOH (pH 9.0 at 22°C), 0.5 mM MgCl $_2$, 2.5 mg mL $^{-1}$ BSA, 5 mM [^{14}C]CS (207 Bq μmol^{-1}), and enzyme extract, in a final volume of 100 μL . The blank was an identical reaction mix lacking the enzyme. Incubation was for 1 h at 37°C . The reaction mix was subsequently loaded onto a 1-mL Dowex-50 (H $^+$) column. The column was washed with 7.9 mL of water. This effluent contained the [^{14}C]CS. [^{14}C]Cho was then eluted with 4 mL of 2.5 N HCl. Proteins were determined by the method of Bradford (1976), with BSA as standard.

Identification of the Sulfotransferase Reaction Product

The effluent from the mixed-bed column was freeze-dried, redissolved in a small volume of water, and analyzed in two separatory systems before and after an acid hydrolysis treatment (2 h at 121°C in 2 N HCl). The first system was TLC on cellulose plates (0.1-mm layer thickness, Merck) developed with *n*-butanol:acetic acid:water (60:20:20, v/v/v). The second system was thin-layer electrophoresis on 0.1-mm cellulose plates in 1.5 M formic acid for 10 min at 1.8 kV and 6°C . The mobilities of the radioactive products were compared to those of authentic CS and Cho standards, which were visualized using the Dragendorff reagent.

FABMS Analysis

Deuterated ($^2\text{H}_6$) CS and Gly betaine standards were prepared as described (Hanson et al., 1991). Samples (50 or 100 mg) of freeze-dried, milled material were spiked with 1 μmol of [$^2\text{H}_6$]CS and 0.5 μmol of [$^2\text{H}_6$]Gly betaine and were extracted, fractionated, and analyzed by FABMS as described

(Rhodes et al., 1987; Hanson and Gage, 1991) except that a mixed-bed column [Dowex-1 (OH⁻):BioRex-70 (H⁺), 2:1, v/v] replaced Dowex-1 for CS isolation.

In Vivo Radiolabeling Experiments

Cell cultures of *L. perezii* that had been salinized with 20% (v/v) ASW for 3 d were supplied with a tracer amount (37 nmol, 74 kBq) of [¹⁴C]Cho and incubated for 6 h in the usual culture conditions. Labeled metabolites were extracted and fractionated as described by Hanson et al. (1991).

RESULTS

Optimization of Extraction Conditions for CST

These experiments were carried out with roots of *L. sinuatum*. Because difficulties were encountered in preliminary trials due to high levels of phenolics, anti-phenolic agents (4% [w/v] insoluble PVP, 0.2 M borate) were included in the extraction medium (Gegenheimer, 1990). A reducing environment provided by the addition of DTT, Na ascorbate, and Na metabisulfite increased the levels of extractable CST by a further 10 to 20%; the inclusion of PMSF in the medium resulted in an additional improvement of 10%. Bicine was used in the extraction buffer; CST activity was inhibited by several other buffers (see "Assay Requirements" below). When a root extract was centrifuged for 1 h at 113,000g, ≥98% of the activity was found in the supernatant, indicating that CST is a soluble enzyme. CST in desalted extracts was resistant to flash freezing in liquid N₂; 70 to 80% of the activity was recovered upon subsequent thawing. However,

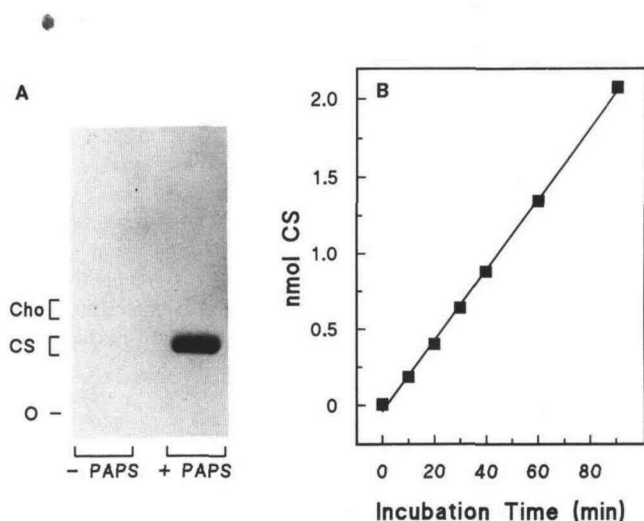


Figure 1. Identification of the reaction product of CST (A) and time course of its formation (B). CST was extracted from roots of *L. sinuatum* and assayed under standard conditions. For the identification of the reaction product, the assay was stopped at 1 h and applied to a mixed-bed column as described in "Materials and Methods." The effluent from this column was freeze-dried and analyzed by TLC followed by autoradiography. The location of the origin (O) and the running positions of CS and Cho standards are indicated. For the time course, the reaction was stopped at the indicated times by flash freezing in liquid N₂.

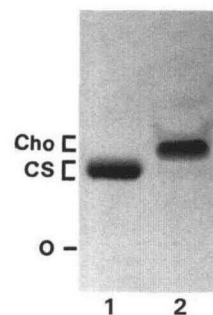


Figure 2. The ¹⁴C-labeled reaction product of CST before (track 1) and after (track 2) hydrolysis for 2 h at 121°C in 2 N HCl. Analysis was by TLC followed by autoradiography. The location of the origin and position of the standards are indicated as in Figure 1A.

the enzyme was unstable in the presence of (NH₄)₂SO₄; >90% losses in activity occurred during precipitation trials.

Design and Validation of the CST Assay

Sulfotransferase assays typically make use of [³⁵S]PAPS (Ramaswamy and Jakoby, 1987), which has the drawbacks of being unstable and costly and having a short half-life. To avoid these problems, a novel assay procedure was developed by exploiting the zwitterionic nature of CS, which allows it to pass through a column of mixed cation- and anion-exchange resins. Cho carries a positive charge and so is retained by such a mixed-bed column. Extracts were incubated in a reaction mix containing [¹⁴C]Cho. At the end of the incubation, the assay mixture was applied to a small mixed-bed column and the reaction product, [¹⁴C]CS, was washed off the column with water. The method is subject to a slight background due to trace contamination (<0.05%) of commercial [¹⁴C]Cho by [¹⁴C]Glyc betaine. This background can be lowered by purifying the [¹⁴C]Cho (Hanson et al., 1985), but this was unnecessary in our experiments since >2% of the substrate [¹⁴C]Cho was usually converted to CS.

The product of the reaction was shown to be CS by its behavior in TLC and thin-layer electrophoresis. Figure 1A shows representative data from TLC. In the absence of the sulfate donor PAPS, no labeled reaction product was detected; with PAPS present, a single band co-migrating with authentic CS was evident. Similar results were obtained when the reaction product was analyzed by thin-layer electrophoresis (not shown). The identity of CS was further confirmed by demonstrating that upon acid hydrolysis it gave a labeled compound that co-migrated with Cho in TLC (Fig. 2) and thin-layer electrophoresis (not shown).

The formation of CS was linear with time over a period of 90 min (Fig. 1B). This result indicates that although PAPS is unstable, its concentration did not become limiting under the standard assay conditions (1 h at 37°C). In these conditions, enzymatic formation of CS was also a linear function of protein concentration over a range of 1 to 50 μg per assay (not shown).

Enzymes that hydrolyze CS (Cho sulfatases) have been reported in several microorganisms (e.g. Harada, 1964; Lucas et al., 1972). Since such an activity could interfere with CST

Table I. Effects of the composition of the assay medium on CST activity

CST from *L. sinuatum* roots was assayed under standard conditions (control) or with various modifications to the composition of the assay medium. The pH was 9.0 in all cases. The data shown come from several experiments and are expressed relative to the control for each experiment. AMP, 2-Amino-2-methyl-1-propanol; TAPS, *N*-tris[hydroxymethyl]methyl-3-aminopropanesulfonic acid.

Treatment	Modification	Relative Activity % of control ^a
Control		100
Omissions	BSA	54
	MgCl ₂	79
Substitutions ^b	0.3 mM APS (PAPS)	2
	0.1 M Tricine (bicine)	54
	0.1 M TAPS (bicine)	33
	0.1 M Gly (bicine)	20
	0.1 M Tris (bicine)	15
	0.1 M AMP (bicine)	4
Additions	1 mM CS	100
	70 mM KCl	120
	70 mM NaCl	120

^a CST activity in the controls was 0.17 to 0.26 pkat. ^b The substituted component of the assay is given in parentheses.

measurement, we checked for CS hydrolysis under our assay conditions in two ways. We first assayed for a Cho sulfatase activity. In this case, 98.5% of the added ¹⁴C was recovered as CS at the end of the assay and no [¹⁴C]Cho formation could be detected. The detection limit for this assay was 16 pkat (<0.5 nkat mg⁻¹ protein). Second, unlabeled CS (1 or 10 mM) was added to the usual reaction mix at the start of the incubation. This addition did not increase the amount of [¹⁴C]CS recovered from the assay (Table I). These two control experiments show that Cho sulfatase activity, if present in *Limonium* species, does not interfere with the CST assay.

Assay Requirements

Optimal CST activity required all the assay components. Table I summarizes the effect on the level of activity of some omissions, additions, or substitutions to the standard assay. Omission of BSA lowered the activity, probably through the effect of proteases. In desalted extracts, the omission of MgCl₂

was found to lower the activity slightly. When Mg²⁺ was replaced by other divalent cations (added as Cl⁻ salts at the concentration of 0.5 mM), CST activity was inhibited, with inhibition increasing in the order Fe²⁺ < Ca²⁺ < Mn²⁺ < Zn²⁺. Similar inhibition has been observed with a plant flavonol sulfotransferase (Varin et al., 1987), although this enzyme seems not to require MgCl₂ (Varin and Ibrahim, 1992). The replacement of PAPS by APS lowered the activity by 98%, demonstrating that CST depends strictly on PAPS as sulfate donor. The replacement of bicine by several other biological buffers resulted in reductions of activity of about 50 to 95%. This effect may be due to common structural features between these buffers and Cho. As noted earlier, addition of CS had no effect on CST activity. The activity was moderately stimulated by addition of NaCl or KCl at concentrations up to 70 mM. These salts, however, were not included in the standard assay mixture.

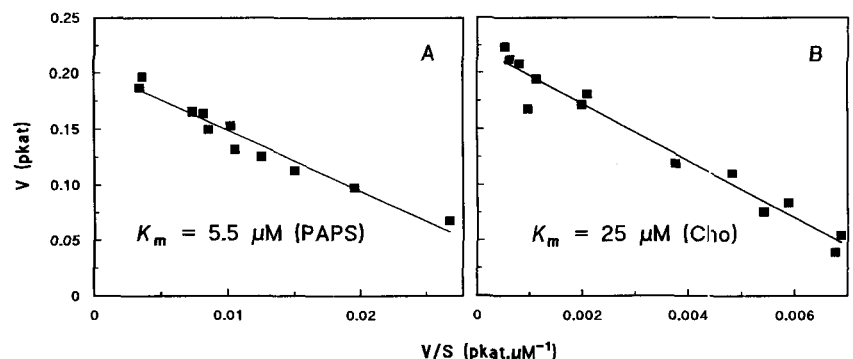
Kinetic Analysis

Kinetic parameters of CST were determined using CST from *L. sinuatum* roots. The apparent *K_m* values derived from Eadie-Hofstee plots (Fig. 3) were 5.5 μM for PAPS and 25 μM for Cho. No high-substrate inhibition could be detected for either of these compounds. The pH optimum of the reaction was determined using a combination of three buffers with overlapping buffering ranges. The buffers chosen did not inhibit CST activity. The pH optimum was around 9 (Fig. 4). Although this value is high, the decline of activity on the acidic side of the optimum was quite gradual so that the enzyme retained about 50% of its activity at pH 7.6.

CST Activity in Different Sources

The occurrence of CST was investigated in roots and leaves of various species that do or do not accumulate CS (Table II). CST activity was found in all members of the genus *Limonium* investigated, all of which accumulate CS (Hanson et al., 1994). The CST activity levels found in these species (5–12 pkat g⁻¹ fresh weight, or about 0.4–1 μmol g⁻¹ fresh weight d⁻¹) are adequate to account for their CS contents (about 5–15 μmol g⁻¹ fresh weight; Hanson et al., 1991), assuming (a) that their relative growth rates are ≤0.05 d⁻¹, and (b) that there is little turnover of CS. Among the nonaccumulators (barley, maize, sunflower, and *Brassica* spp.), none had significant CST activity.

Figure 3. Eadie-Hofstee plots showing the effect of the concentration of PAPS (A) and Cho (B) on the rate of the reaction catalyzed by CST from *L. sinuatum* roots. In each experiment, the concentration of one substrate was varied while the other was saturating. Assays contained 30 μg of protein. Apparent *K_m* values were derived from the slopes of the plots. These experiments were repeated three times, with very similar results; the data shown are representative. *V*, Initial reaction velocity; *S*, concentration of Cho or PAPS.



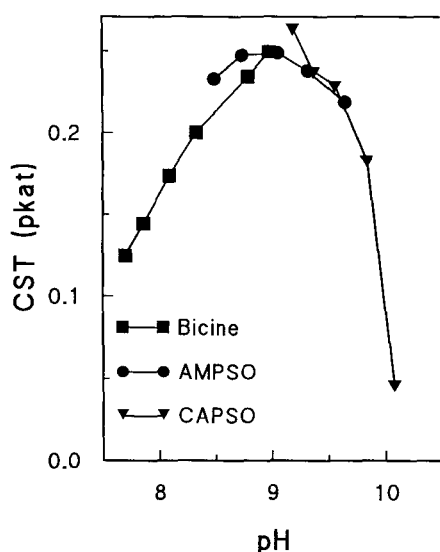


Figure 4. Effect of pH on the rate of the reaction catalyzed by CST. CST from *L. sinuatum* roots was assayed under standard conditions except that the reaction mix contained the indicated buffer at 0.1 M. Assays contained 36 μg of protein. AMPSO, 3-[(1,1-Dimethyl-2-hydroxyethyl)amino]-2-hydroxypropane-sulfonic acid; CAPSO, 3-[cyclohexylamino]-2-hydroxy-1-propanesulfonic acid.

Effect of Salt or Osmotic Stress on CST Activity in *L. perezii*

Because salt stress increases CS levels by at least 2-fold in species of *Limonium* (Hanson et al., 1991), we investigated the effect of salinization on the level of CST activity. These experiments were carried out using whole plants and cell cultures of *L. perezii*.

Table II. Occurrence of CST activity in CS-accumulating and nonaccumulating plants

CST was assayed in desalted extracts under standard conditions. Data are means \pm SE for at least three independent experiments. CS levels in nonaccumulating species were shown to be below 2 $\mu\text{mol g}^{-1}$ dry weight by FABMS analysis (Hanson and Gage, 1991; this study).

Species	Organ	CST Activity
		pkat g^{-1} fresh weight
CS accumulators		
<i>Limonium sinuatum</i>	Leaves	11.6 \pm 1.6
	Roots	5.7 \pm 0.6
<i>Limonium perezii</i>	Leaves	7.6 \pm 2.4
	Roots	8.5 \pm 2.2
<i>Limonium latifolium</i>	Roots	7.3 \pm 0.4
<i>Limonium ramosissimum</i>	Roots	5.0 \pm 0.4
<i>Limonium nashii</i>	Roots	5.3 \pm 0.6
Nonaccumulators		
<i>Hordeum vulgare</i>	Roots	<0.08
<i>Zea mays</i>	Roots	<0.06
<i>Helianthus annuus</i>	Roots	<0.04
<i>Brassica oleracea</i>	Roots	<0.02
<i>Brassica napus</i>	Roots	<0.02

Table III. Effect of salinization on CST activity in roots and leaves of *L. perezii*

Plants growing under hydroponic conditions were gradually salinized to 20 and 40% ASW. CST activity was extracted and assayed on desalted extracts. Data are means \pm SE for five independent experiments.

Organ	ASW Level	CST Activity	
		pkat mg^{-1} protein	pkat g^{-1} fresh weight
Roots	0	7.3 \pm 1.9	8.5 \pm 2.2
Roots	20	20.4 \pm 3.2	22.9 \pm 3.6
Roots	40	41.9 \pm 3.7	46.7 \pm 4.1
Leaves	0	0.7 \pm 0.2	7.6 \pm 2.4
Leaves	20	1.3 \pm 0.2	13.9 \pm 1.6
Leaves	40	2.9 \pm 0.4	33.4 \pm 4.8

Plants were grown in hydroponic conditions and salinized gradually to 20 and 40% ASW; CST activity was then assayed in roots and leaves (Table III). CST was induced by salinity, with specific activities increasing by up to 6-fold in roots and 4-fold in leaves. Expressed on a protein basis, CST activity was higher in roots. On a fresh weight basis, however, the activities in leaves and roots were comparable.

Cell cultures of *L. perezii* were subcultured every 14 d; Figure 5A shows a typical growth curve. A salt or osmotic shock was given at 7 d with 20% (v/v) ASW or 19% (w/v) PEG 6000; these treatments raised the culture medium osmotic pressure by 0.53 or 0.55 MPa, respectively. In both cases the fresh weight of the culture fell at first, but growth resumed within 1 d, indicating that the cells acclimated rapidly after the shock. CST activity per unit fresh weight in control cells rose by about 15-fold within 3 d after subculturing, and thereafter declined steadily (Fig. 5B). This pattern was equally evident when activity was expressed on a protein basis (not shown). Such variation of enzyme expression as a function of the age of the cell culture is not unusual (e.g. Frenzel and Zenk, 1990; Perez-Prat et al., 1994). The ASW or PEG shock treatments resulted in a 2- to 4-fold increase in CST activity, with maximal induction occurring 1 or 2 d after the shock (Fig. 5B). There was some variation in the relative effects of ASW and PEG from experiment to experiment, but CST induction always occurred.

FABMS was used to quantify CS in control and stressed cells (Fig. 5 legend). CS levels were in all cases very low (0.4–1.3 $\mu\text{mol g}^{-1}$ dry weight). This failure to accumulate CS could be due in part to the inability of cultured cells to produce enough Cho for both phosphatidylcholine synthesis and CS accumulation, with the former taking precedent. Consistent with this possibility, cells readily converted tracer amounts of [^{14}C]Cho to [^{14}C]CS, but also synthesized much phosphatidyl[^{14}C]choline. Thus, when 0.5 g fresh weight of salt-stressed cells were given 74 kBq (37 nmol) of [^{14}C]Cho for 6 h, they formed 5.6 kBq of [^{14}C]CS and 34.2 kBq of phosphatidyl[^{14}C]choline. Although CS was not the major fate of [^{14}C]Cho, this experiment nonetheless confirms that the CST in cell cultures is active in vivo.

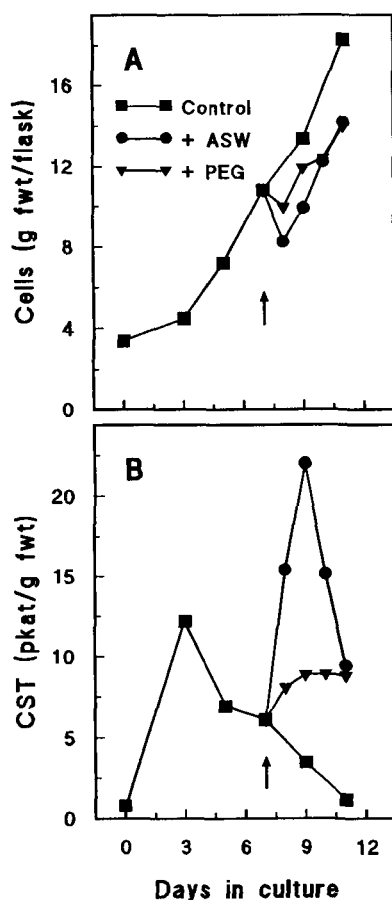


Figure 5. Effect of a salt or osmotic shock on growth (A) and extractable CST activity (B) of *L. peresii* cell cultures. Stress was imposed at 7 d (arrow) by adding ASW or PEG 6000 to the culture medium. Cells from three flasks per time point were pooled for analysis. CS levels ($\mu\text{mol g}^{-1}$ dry weight) were as follows: before stress imposition at 7 d, 0.5; at 11 d, control, 0.4; +ASW, 1.3; +PEG, 0.7. Gly betaine levels in the same samples were $<0.4 \mu\text{mol g}^{-1}$ dry weight. The experiment was repeated twice with similar results.

DISCUSSION

Previous *in vivo* studies of the incorporation of label from [^{14}C]Cho into [^{14}C]CS suggested the occurrence of CST activity in plants that accumulate CS (Hanson et al., 1991). This report presents direct *in vitro* evidence for the existence of this enzyme. The enzyme activity was detected only in plants that accumulate CS; their levels of CST activity were sufficient to account for the amounts of CS they synthesize *in vivo*.

CST occurs at a branch point of Cho metabolism and so may compete for Cho with Cho kinase (catalyzing synthesis of phosphorylcholine for phospholipid production) and also, in species that accumulate Gly betaine, with Cho monooxygenase (Hanson et al., 1994). There are no published values for the K_m for Cho of the two latter enzymes from *Limonium* species, but values from other plants for Cho kinase (about 25–260 μM , Gawer et al., 1991, and refs. therein) and for Cho monooxygenase (about 100 μM , Brouquisse et al., 1989) sug-

gest that CST, with a K_m for Cho of 25 μM , could compete effectively with both. If so, it would simplify the metabolic engineering of CS accumulation (Hanson et al., 1994). However, although the ability to compete for Cho is necessary for CST activity *in vivo*, it is not sufficient; PAPS is also required, and competition for this substrate may be intense (Schmidt and Jäger, 1992).

Salinization with ASW induced CST in roots, leaves, and cell cultures by up to 6-fold. This induction is consistent with the 2- to 3-fold increase in CS content in salinized plants (Hanson et al., 1991) and is of similar magnitude to that found for the enzymes of Gly betaine synthesis in salinized spinach (Weigel et al., 1986; Brouquisse et al., 1989; Summers and Weretilnyk, 1993). Because ASW contains both Cl^- and SO_4^{2-} (in a 19:1 ratio), our salinization data do not show whether CST induction is a specific response to SO_4^{2-} . However, this seems unlikely since CST was also induced in cultured cells by osmotic shock with PEG.

Enzymatic sulfation in animal tissues is generally considered to be a detoxification mechanism for endogenous metabolites and xenobiotics, which may then be excreted as their sulfate esters via the kidney. In these cases it is not SO_4^{2-} but the other moiety of the ester that is being detoxified, opposite to the situation of CS formation in plants. Moreover, CS is not excreted but retained by the cell, where it can have a beneficial osmoprotectant role (Hanson et al., 1994). These physiological distinctions suggest that CST may have little in common with other sulfotransferases, and perhaps evolved independently of them.

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