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# Properties and Regulation of Adenosine 5′-Phosphosulfate Sulfotransferase from Suspension Cultures of *Nicotiana sylvestris* *

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**Abstract.** The properties and the regulation of adenosine 5′-phosphosulfate sulfotransferase extracted from cell suspension cultures of *Nicotiana sylvestris* was investigated. Optimal adenosine 5′-phosphosulfate sulfotransferase activity was obtained from the cells by extraction with 0.1 M tris-HCl, pH 8.0, containing 2 M $MgSO_4$ and 10 mM dithioerythritol. The $K_m$ for adenosine 5′-phosphosulfate in the sulfotransferase reaction was about 11 μM. Adenosine 5′-phosphosulfate in concentrations above 50 μM were inhibitory. The extractable adenosine 5′-phosphosulfate sulfotransferase activity decreased during cultivation with sulfate as the sole sulfur source, but after about 3 days it reached a constant level (50 to 100 nmol activated sulfate transferred $h^{-1}$ $mg^{-1}$ protein) which was maintained for at least 24 h. Addition of 0.5 mM cysteine to the culture medium decreased the extractable adenosine 5′-phosphosulfate sulfotransferase activity and blocked growth completely. With 0.1 mM cysteine an enzyme level of about 10% of the initial value was reached within 6 to 12 h without significant inhibition of growth. The added cysteine was absorbed rapidly and after 24 h cysteine could no longer be detected in the medium. Before the cysteine was completely depleted, the activity of adenosine 5′-phosphosulfate sulfotransferase started to increase, reaching ultimately a level which was comparable to the initial value.

**Key words:** Adenosine 5′-phosphosulfate sulfotransferase – Cell suspension culture – Cysteine – Enzyme regulation – *Nicotiana*.

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*Abbreviations:* APS = Adenosine 5′-phosphosulfate; APSSTase = adenosine 5′-phosphosulfate sulfotransferase; DTE = dithioerythritol; PAPS = adenosine 3′-phosphate 5′-phosphosulfate; 2,4-D = 2,4-di-chlorophenoxyacetic acid; BAP = benzyladenine

## Introduction

Adenosine 5′-phosphosulfate sulfotransferase (APSSTase) has been shown to be an enzyme of a pathway of assimilatory sulfate reduction in *Chlorella* (Schmidt et al. 1974). It catalyzes the transfer of the sulfonylgroup of adenosine 5′-phosphosulfate (APS) to a carrier (car-SH) to form car-S-$SO_3^-$. The carrier seems to be reduced glutathione in *Chlorella* (Tsang and Schiff 1978), but is unidentified in higher plants (Schmidt 1979). In the usual assay systems for APSSTase dithiothreitol dithioerythritol, (DTE) or glutathione are used as acceptors of the sulfonylgroup of APS (Tsang and Schiff 1976a). The enzyme is widespread among higher plants, and has been detected in more than 50 families (Schmidt 1975a). The extractable activity of the enzyme is lower in *Lemna minor* (Brunold and Schmidt 1976; 1978) and in primary leaves of *Phaseolus vulgaris* (Wyss and Brunold 1979), after the addition of cysteine to the culture medium or of $H_2S$ to the air. These regulatory phenomena caused by end products of assimilatory sulfate reduction provide evidence that APSSTase is an enzyme of this pathway in higher plants. In heterotrophic cell suspension cultures of *Catharanthus roseus,* however, cultivation with 0.5 mM cysteine as the sole sulfur source gave a peak activity of APSSTase which was about five times greater than that of extracts from cultures growing in the presence of 1.8 mM sulfate (Schwenn et al. 1978; 1979). On the other hand, an adenosine 3′-phosphate 5′-phosphosulfate (PAPS)-sulfotransferase had a lower level in extracts from cells cultivated with sulfur amino acids as the sulfur source as compared to controls with sulfate. Since PAPS-dependent sulfotransferases were demonstrated in bacteria (Tsang and Schiff 1976b) and yeast (Wilson and Bierer 1976), the question arises as to whether heterotrophic plant cells differ from autotrophic ones with respect to the sulfonyl donor of the transferase.

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In this paper we report some of the properties of APSSTase from heterotrophic cell suspension cultures of *Nicotiana sylvestris*.

## Materials and Methods

Cell suspension cultures of *N. sylvestris* Spegazz. et Comes were established from callus derived from excised leaves of haploid plants and maintained by serial subcultering in B 5 medium (Gamborg 1970) containing 0.5 mg $l^{-1}$ 2,4-D and 0.05 mg $l^{-1}$ BAP in 500 ml Erlenmayer flasks covered with two layers of aluminium foil 0.03 mm thick. The total volume of medium and inoculum was 160 ml. New cultures containing about 160,000 cells $ml^{-1}$ were started weekly by the addition of cells from a one week old culture. The Erlenmayer flasks were maintained on a gyratory shaker operated at 130 rev $min^{-1}$. Cultivation was at 26±2° C in continous light. The sterility of the cultures was checked weekly by plating aliquots on nutrient broth agar and yeast extract agar (Difco Laboratories, Detroit, Mich., USA).

The cell density was determined from 2 ml aliquots taken from the cultures. After treatment with 8 ml 10% chromic acid at 60° C for 40 min, vigorous shaking for 10 min, and dilution with water, the separated cells were counted, using an Agasse-Lafont B counting cell.

The cell fresh weight was determined by collecting the cells contained in 5 to 10 ml culture medium on a pre-weighed circular filter of nylon fabric (20 µm pore size), washing the cells with 10 ml water, draining under vacuum, and weighing.

The packed cell volume was determined by transferring 5 to 10 ml cell suspension into a conical, graduated tube and centrifuging for 5 min in a Hettich 3100 centrifuge (Hettich, Tuttlingen, FRG) at 80% of the maximal speed. It is expressed as ml cell pellet · ml culture$^{-1}$ (ml · $ml^{-1}$).

For the preparation of the extracts 5 to 10 ml of cell suspension were used and separated from the medium either by filtration or by centrifugation. The cells were washed with water, suspended in 0.1 M tris-HCl, pH 8.0, containing 2 M $MgSO_4$ and 10 mM DTE, and homogenized for 2 min in a glass homogenizer at 0–4° C. The homogenate was centrifuged for 10 min at 10,000 *g* and the supernatant retained for the measurement of APSSTase.

APSSTase activity was determined according to Schiff and Levinthal (1968), as modified by Brunold and Schmidt (1978). Protein was determined turbidimetrically according to Brunold and Schmidt (1978), using bovine serum albumin as a standard. Solutions of cysteine were sterilized by filtration through Millipore filters. Amino acids were determined using a Durrum amino acid analyzer. APS was obtained from SIGMA; $AP^{35}S$ was prepared according to Tsang et al. (1976).

The term cysteine is used in this paper when it is clear that cystine is not involved. The term cyst(e)ine indicates an unidentified mixture of cysteine and cystine.

## Results

Table 1 gives the requirements for optimal APSSTase activity extracted from cells of suspension cultures of *N. sylvestris*. DTE functions as an artifical carrier in this assay system. If it is not present in either the extraction buffer or in the assay system no activity of APSSTase can be detected. If it is only added to the extraction buffer (Table 1), it is present at a final concentration of 0.5 mM in the assay system and the activity is about 30% of the control, (Table 1) where the final DTE concentration is 10.5 mM. The pH optimum for the reaction is between 8.0 and 9.0.

**Table 1.** Requirements for optimal activity of adenosine 5′-phosphosulfate sulfotransferase (APSSTase) extracted from cells of *N. sylvestris*. The complete assay system contained, in a total volume of 400 µl, 125 mM tris-HCl, pH 9.0; 1,000 mM $Na_2SO_4$; 10 mM DTE; 18.75 µM $AP^{35}S$ and 20 µl crude extract, containing 14 µg protein. Incubation was for 30 min at 37 °C under $N_2$

| Assay system | APSSTase (nmol $h^{-1}$ $mg^{-1}$ protein) |
|---|---|
| complete | 55 |
| complete, without DTE | 15 |
| complete, without $Na_2SO_4$ | 12 |
| complete, extract heated at 80 °C for 3 min | 0 |

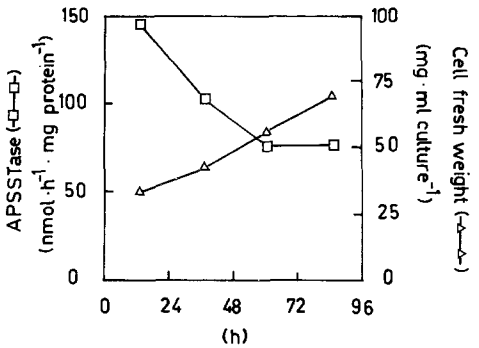


**Fig. 1.** Changes in the activity of adenosine 5′-phosphosulfate sulfotransferase (APSSTase) and in cell fresh weight · $ml^{-1}$ culture medium, with time, in a batch culture of cells of *N. sylvestris*. The culture was started at 0 h. Assay system and incubation as given in the legend to Table 1

At pH 7.5 the activity was 70 to 80% of that at the optimal pH. The $K_m$ for APS is 11.1±0.96 µM (4 determinations), and APS concentrations higher than about 50 µM are inhibitory. In the extraction buffer, 2 M $MgSO_4$ increased APSSTase activity by a factor of about 4 and stabilized the enzyme so that there was no appreciable loss of activity for 40 min after extraction. Activity was linear with time (up to 40 min) and with protein concentration (up to at least 60 µg).

Figure 1 shows that in a batch culture with sulfate as the sole sulfur source, the extractable APSSTase activity decreased during the first three days after the start of the culture, but was essentially constant during the following 24-h period. This period was routinely used for the experiments on the regulation of APSSTase activity by cysteine.

Preliminary experiments showed that addition of cysteine to the culture medium in a final concentration of 0.5 mM decreased APSSTase activity, but inhibited growth completely.

Figure 2 shows that there was a rapid decrease in extractable APSSTase activity when cysteine, in a final concentration of 0.1 mM, was added to the medium 72 h after the start of the culture. At 12 h

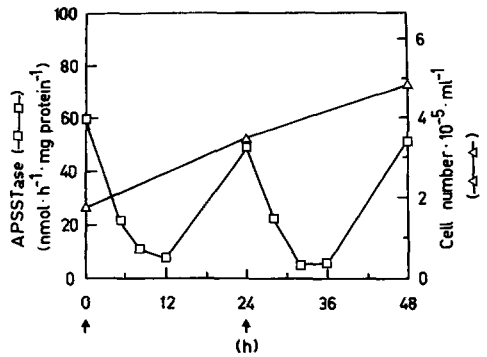


**Fig. 2.** Effect of cyst(e)ine on the activity of adenosine 5'-phosphosulfate sulfotransferase (APSSTase) and cell number · $ml^{-1}$ in batch culture of cells of *N. sylvestris*. The arrows indicate the time of addition of cysteine. After the first addition the cysteine concentrations was 0.1 mM. The second addition gave an increase in the remaining cyst(e)ine concentration of a further 0.1 mM. The culture was started 72 h before the first addition of cysteine. Assay system and incubation as given in the legend to Table 1

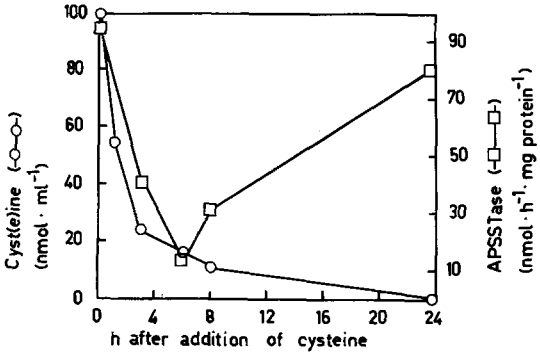


**Fig. 3.** Cyst(e)ine concentration in nutrient solution and extractable activity of adenosine 5'-phosphosulfate sulfotransferase (APSSTase) from cells of *N. sylvestris* after addition of cysteine to batch cultures to give a final concentration of 0.1 mM. Cysteine was added 72 h after the start of the culture. Assay system and incubation as given in the legend to Table 1

after the addition of cysteine APSSTase had decreased by about 90%, at 24 h it had recovered to a value comparable to the initial one. A second addition of cysteine at this time induced similar changes in APSSTase activity. No significant effect on growth was detected at this cysteine concentration.

There are several hypotheses which may explain the increase in APSSTase activity after the initial drop caused by cyst(e)ine. Since the increase was delayed in cultures with low cell densities (Jenni 1979), it seemed reasonable to assume that cyst(e)ine was depleted in a time course dependent on the cell density up to a concentration where it no longer had an effect on APSSTase.

Figure 3 shows the changes in extractable APSSTase activity and in cyst(e)ine concentration in the nutrient solution after the addition of cysteine in a final concentration of 0.1 mM to a relatively dense cell suspension culture. Initially, both APSSTase activity and cyst(e)ine concentration decreased rapidly. At a cyst(e)ine concentration of about $10^{-5}$ M APSSTase activity started to increase, reaching a value comparable to the initial one. Cyst(e)ine concentration decreased further and 24 h after the addition, it was no longer detectable. Control cultures had a comparable growth rate and an extractable APSSTase activity which was essentially constant.

Control experiments showed that addition of cysteine up to 20 mM to the APSSTase assay system had no effect on APSSTase activity, excluding the possibility that cyst(e)ine present in the extracts from cells cultivated with cyst(e)ine inhibited the enzyme in the assay.

When extracts from cells growing on nutrient solution with cysteine containing low APSSTase activity were mixed with extracts from control cultures with normal APSSTase activity, the activities were essentially additive, indicating that no inhibitor was present in the extracts with low APSSTase and no activator in the extracts with normal APSSTase.

## Discussion

The extractable activity of APSSTase from cells of suspension cultures of *N. sylvestris* is comparable to that from primary leaves of *Phaseolus vulgaris* (Wyss and Brunold 1979) and *Lemna minor* (Brunold and Schmidt 1976; 1978). The apparent $K_m$ for APS of about 11 µM is very similar to the $K_m$ of 13 µM of APSSTase in extracts from spinach (Schmidt 1976) and to the equilibrium concentration of APS of 10 µM, calculated with the assumption that ATP is present at $10^{-4}$ M, while $SO_4^{2-}$ and $P_i$ are each present at $10^{-3}$ M (Siegel 1975).

The stimulation of the APSSTase reaction with $Na_2SO_4$ observed in the present investigation has already been reported from experiments using extracts of spinach (Schmidt 1975b).

The pH optimum of APSSTase reaction in extracts from *N. sylvestris* is similar to that of the enzyme extracted from spinach (Schmidt 1975b). At pH 7.5 the rate of APSSTase is reduced by 20 to 30% in extracts from *N. sylvestris* and by about 60% in extracts from spinach. We have previously shown that in spinach, APSSTase is localized predominantly or even exclusively in the chloroplasts (Fankhauser and Brunold 1978). It is tempting to speculate that the mentioned difference at pH 7.5 reflects the fact that the spinach enzyme must have optimal activity at the relatively high pH of chloroplasts in the light, while the enzyme from heterotrophic *N. sylvestris* cells cannot take advantage of a light-induced pH increase and must therefore maintain appreciable activity at lower pH values.

The reason for the initial decrease of extractable

APSSTase activity in cultures with sulfate as the sole sulfur source is not known at present. This does not affect, however, the results from the experiments on the regulation of the enzyme, since these were done when a constant level of activity had been reached. The results from these experiments using cysteine as a regulator are similar to our previous findings using *Lemna minor* (Brunold and Schmidt 1978) and *Phaseolus vulgaris* (Wyss and Brunold 1979). The rate at which the decrease in activity occurs cannot be accounted for solely by inhibition of APSSTase synthesis and dilution of the existing molecules of enzyme during growth, and suggests that inactivation and/or degradation are involved.

Our results are in contrast to the findings of Schwenn et al. (1978; 1979). Many reasons may be responsible for this discrepancy, but at present it seems most likely that the assay system for APSSTase used by Schwenn et al. may account for the differences. This assay involves the formation of $AP^{35}S$ from $^{35}SO_4^{2-}$ and ATP catalyzed by ATP sulfurylase which is present in the crude extracts. This enzyme activity, however, shows great fluctuation in extractable activity (Schwenn et al. 1979) over the time course of the experiment, so that it is not clear whether the changes of APSSTase activity are due to varying amounts of extractable APSSTase or to varying amounts of $AP^{35}S$ available for the APSSTase reaction. We think therefore that before it can be accepted that APSSTase in *Catharanthus roseus* cells is regulated differently from *Lemna, Phaseolus*, or *N. sylvestris*, the enzyme should be measured by adding $AP^{35}S$ at an optimal concentration to the assay system.

## Conclusions

Our results show that APSSTase can be extracted from cells of suspension cultures of *N. sylvestris* in activities comparable to *Lemna minor* or *Phaseolus vulgaris* with properties similar to the enzyme extracted from spinach. The regulatory effect of cyst(e)ine on the extractable enzyme activity suggests that APSSTase takes part in the assimilatory sulfate reduction of these cells.

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