Purification and Partial Characterization of the *Streptomyces viridochromogenes* Tü494 Phosphinothricin-*N*-acetyltransferase Mediating Resistance to the Herbicide Phosphinothricin in Transgenic Plants

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A purification scheme for the enzyme phosphinothricin-*N*-acetyltransferase (PAT) originating from *Streptomyces viridochromogenes* (*pat*-gene product from *Streptomyces viridochromogenes*) and mediating herbicide resistance to transgenic plants was developed. The enzyme was isolated from a transformed and overproducing *Escherichia coli* strain. With a combination of ammonium sulfate fractionation, chromatography on DEAE-Sephadex A50-, Phenylsepharose-, Hydroxylapatite- and FPLC-Superose 12-columns it was possible to obtain PAT which was at least 90 % homogeneous on the basis of SDS-PAGE. The properties of the isolated PAT were compared with the properties of PAT from *S. hygroscopicus* (*bar*-gene product from *S. hygroscopicus*) previously isolated and characterisized by Botterman, J., Gosselé, V., Thoen, C., Lauwereys, M. (1991), Gene **102,** 33–37. Differences were observed in the molecular masses of the two native enzymes (PAT from *S. viridochromogenes* being a dimer of 40 kD and PAT from *S. hygroscopicus* being a monomer of 21 kD), and in the temperature sensitivity of the two enzymes (the PAT from *S. viridochromogenes* being slightly more temperature stable than PAT from *S. hygroscopicus*). However, since the *pat* and the *bar*-gene are to 85 % homologous, substantial similarities exist between the two enzymes especially in the kinetic values and the substrate specificity. The isolated *S. viridochromogenes* PAT did not acetylate putative substrates present in the plant cell. Antibodies were raised against the isolated protein. This antiserum was able to detect PAT in transgenic plants and therefore is suitable to analyse the fate of the protein in such plants under various stress conditions.

Introduction

The antibiotic phosphinothricyl-alanyl-alanine (bialaphos, Ptt) is produced by the Gram$^+$ soil bacteria *Streptomyces hygroscopicus* (Kondo *et al.*, 1973) and *S. viridochromogenes* (Bayer *et al.*, 1972). Ptt is transported into bacterial cells by an oligopeptide transport system (Diddens *et al.*, 1976) and cleaved intracellulary by peptidases. The released phosphinothricin (Pt) inhibits not only procaryotic (Bayer *et al.*, 1972) but also eucaryotic glutamine synthetases (Lea *et al.*, 1984). Hence, it can be used in agriculture as a non-selective herbicide. The synthesis of Ptt in *S. hygroscopicus* has been studied intensively (Murakami *et al.*, 1986; Hara *et al.*, 1991), and a corresponding pathway was also identified in *S. viridochromogenes* (Alijah *et al.*, 1991). Interestingly, the enzyme promoting step 10 in the biosynthetic pathway has a double function, since it also mediates resistance to Pt. This enzyme catalyzes the *N*-acetylation of demethylphosphinothricin (DMPT) and of the herbicide Pt as well giving rise to *N*-acetyl-DMPT and *N*-acetyl-Pt, respectively (Murakami *et al.*,

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Abbreviations: AEP, 2-aminoethylphosphonic acid; APB, 2-amino-4-phosphono butyric acid; *bar*, bialaphos-resistance gene from *Streptomyces hygroscopicus* coding for a phosphinothricin-*N*-acetyltransferase; DMPT, demethylphosphinothricin; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); MSO, methionine sulfoximine; *pat*, gene from *Streptomyces viridochromogenes* Tü494 coding for a phosphinothricin-*N*-acetyltransferase; PAT, phosphinothricin-*N*-acetyltransferase; PPO, 4-methylphosphino-2-oxo-butyric acid; Pt, phosphinothricin; Ptt, Pt-alanyl-alanine, Pt-tripeptide, bialaphos; TNB, 2-nitro-5-thiobenzoic acid.

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1986; Thompson *et al.,* 1987; Strauch *et al.,* 1988). The corresponding genes, *bar* from *S. hygroscopicus* and *pat* from *S. viridochromogenes* have been isolated and transferred to plants. Since *N*-acetyl-Pt is not able to interact with glutamine synthetase, the expression of the bacterial genes in plants confers strong resistance to the herbicide in all plants tested so far (e.g. De Block *et al.,* 1987; Wohlleben *et al.,* 1988). Both, the *pat*-gene and the *bar*-gene which show an overall identity of 85 % (Wohlleben *et al.,* 1988) code for proteins of 183 amino acids. Differences in the amino acid sequence are more frequent in the C- and N-terminal domains indicating that the central part of the protein may carry the functional domain. In addition to the *bar*- and the *pat*-gene a third gene coding for a Pt-*N*-acetyl-transferase has been identified in *S. coelicolor.* However, this gene shares only 30 % homology with either the *pat*- or the *bar*-gene (Bedford *et al.,* 1991). The protein encoded by the *bar*-gene has already been characterized (Thompson *et al.,* 1987; Botterman *et al.,* 1991).

At present the non-selective herbicide Pt (= glufosinate) is mainly used in vineyards and orchards, since its uptake is restricted to the green parts of the plant. Although it has been demonstrated with sterile plant cultures that Pt is also assimilated by roots (Broer *et al.,* 1989; Dröge *et al.,* 1992), the uptake of Pt by plant roots is of minor importance, since Pt is rapidly degraded in unsterile soil by microorganisms (Bartsch and Tebbe, 1989; Tebbe and Rebber, 1991). The utilisation of transgenic herbicide resistant plants would extend the possible applications of non-selective herbicides, and this could be of advantage for the natural environment, since in contrast to selective herbicides, only one compound has to be applied to the field and this one compound should lead to a minor toxicity than different selective herbicides. However, the consequences of the expression of a foreign gene in a complex organism like a plant should carefully be investigated before transformed plants can be used in agriculture. Therefore, transgenic plants are analysed in the field prior to their economic use. Out of 393 releases of transgenic plants in 1991, 159 plants were herbicide resistant. 77 of the varieties used carried either the *bar*- or the *pat*-gene (Chasseray and Duesing, 1992). Several aspects of the biosafety of Pt-resistant plants, such as the stability of transgene expression (Walter *et al.,* 1992) or the metabolism of the herbicide in transformed and untransformed plants (Dröge *et al.,* 1992; Dröge-Laser *et al.,* 1994) have already been analysed. One of the remaining questions related to PAT is the substrate specificity of the introduced enzyme. Because of the great number of acetylations occurring in plants, this question is difficult to answer *in planta.* Therefore, isolation and purification of the enzyme became necessary to allow a characterization of the enzyme *in vitro.* Since the *bar*- and the *pat*-gene products show an approximately 15 % difference in their amino acid sequence (Wohlleben *et al.,* 1988), deviations in their properties can be imagined. Therefore, we isolated the *S. viridochromogenes* PAT from an overproducing *Escherichia coli* strain, examined its characteristics, compared its properties with those of the *bar*-gene product from *S. hygroscopicus* and raised a polyclonal antiserum against the purified protein. The availability of specific antibodies raised against PAT will help to analyse the fate of the protein *in planta.* This is especially desirable, since we published that loss of herbicide resistance occurs in transgenic alfalfa suspension culture cells after a heat treatment (Walter *et al.,* 1992). In order to analyse the mechanism of this phenomenon it has to be shown whether the loss of Pt resistance occurred at the translational level or is possibly due to an inactivation of the enzyme.

Materials and Methods

Bacterial strain

Escherichia coli JM 83 pES6.1 was the same as described by Strauch *et al.* (1988). This strain carries a 0.8 kb DNA fragment containing the *pat*-coding region fused to the *lac*Z-promoter. *E. coli* JM83 pES6.1 was cultivated in PA medium consisting of 17.5 g Penassay Broth (Difco) in 1 l. Transgenic plants containing *pat* were derived from *Nicotiana tabacum* SR1 (line C29.3) and are described in Broer *et al.* (in prep.).

Growth of E. coli JM83 pES6.1 and preparation of cell extract

E. coli JM83 pES6.1 was cultivated overnight at 37 °C in 10 l bottles in PA medium containing ampicillin (100 mg/l) for selection in a stream of air.

Fifty liters cell suspension resulting in 131 g packed cells were used for isolation of PAT. The cells were harvested by centrifugation, washed once with 10 mM Na_2HPO_4 adjusted to pH 7 with HCl and containing 10 mM NaCl, then resuspended in the same buffer to give a cell concentration of 1 mg fresh cells/ml, and passed through a chilled French pressure cell at 137.8 MPa. The extract was centrifuged 10 min at 14000 x *g* to remove unbroken cells and the supernatant was frozen at -20 °C for further use.

Preparation of tobacco extracts

Untransformed and transgenic tobacco plants were grown as previously described (Dröge *et al.*, 1992). Extracts of leaves from these plants were prepared as described in Dröge *et al.* (1992).

PAT activity test

PAT activity was determined spectroscopically at 22 °C by following the formation of 2-nitro-5-thiobenzoate (TNB) from 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) and S-CoA which is released in the PAT catalyzed reaction from *N*-acetyl-CoA. The assay conditions were according to Thompson *et al.* (1987) with slight modifications. The reaction mixture (total volume 0.94 ml) contained 0.096 M Tris-HCl, pH 7.8, 0.96 mM DTNB, 0.096 mM acetyl-CoA, 0.18 mM L-Pt, and the enzyme as indicated. The molar extinction coefficient of TNB at 412 nm is 14150 M^{-1} cm^{-1} according to Riddles (1983) and 13600 M^{-1} cm^{-1} according to Shaw (1975). The latter one was used for our calculation.

Determination of the molecular mass of native PAT

PAT was chromatographed on a FPLC-Superose 12 column (Pharmacia). As reference proteins catalase from bovine liver (240 kD), aldolase from rabbit muscle (158 kD), bovine serum albumin (68 kD), ovalbumin (45 kD), DNaseI from bovine pancreas (31 kD), and cytochrome c from bovine heart (12.4 kD) were used.

Protein was determined according to Bradford (1976). SDS page and immunoblot experiments were performed with standard procedures according to Laemmli (1970) and Stott (1989), respectively. A polyclonal antiserum against PAT was raised from a rabbit as described by Schmid *et al.* (1993).

Results

Purification of PAT

For the isolation of PAT we used the *Escherichia coli* strain JM83 pES6.1 (Strauch *et al.*, 1988). Due to the fusion of the *pat*-coding region to the *lac*Z-promoter this strain overproduces the enzyme. During the cloning procedure the original signal for translation termination has been eliminated, therefore 10 amino acids are added to the C-terminal domain compared to the protein originating from *S. viridochromogenes*.

Fifty liters of *E. coli* cell suspension overproducing PAT resulted in 131 g packed cells that were used for the isolation of the enzyme (see Materials and Methods). All subsequent steps were performed at 4 °C or on ice. For purification of PAT the extract was fractionated with ammonium sulfate. The protein fraction precipitating between 30–65 % ammonium sulfate saturation was resuspended in 25 mM Tris-HCl, pH 7.5, containing 1 mM EDTA, and dialyzed overnight against 10 mM Tricine-HCl, pH 8.0. After centrifugation and dialysis, the protein solution was loaded on a DEAE-Sephadex A50 column (size 3x20 cm) which was equilibrated with 10 mM Tricine-HCl, pH 8.0. The protein fraction which did not bind to the column and which contained PAT, was dialyzed against 20 mM potassium phosphate buffer, pH 7.5, containing 1 M ammonium sulfate, and was then chromatographed on a phenylsepharose column (size 3x10 cm) which was equilibrated with 20 mM potassium phosphate, pH 7.5, containing 1 M ammonium sulfate. After applying the protein and washing the column with the above buffer, PAT was eluted from the column with a linear gradient consisting of 250 ml each of 20 mM potassium phosphate buffer, pH 7.5, containing 1 M ammonium sulfate and buffer without ammonium sulfate (total volume of gradient was 500 ml). The most active fractions were combined and dialyzed against 30 mM potassium phosphate buffer, pH 7.5. After dialysis the protein sample was chromatographed on a hydroxylapatite column (size 2x2.5 cm) which was equilibrated with 30 mM potassium phosphate buffer, pH 7.5. Elution was with a lin-

Table I. Purification of the *Streptomyces viridochromogenes* PAT isolated from a transformed and overproducing *E. coli* strain. Details of the purification procedure are given in the text. 1 unit of enzyme corresponded to 1 μmol TNB formed/min at 22 °C.

Purification step	Protein total [mg]	PAT activity Total (Units)	Spec. activity (Units/mg Protein)
French press extract	3000	1003	0.33
Ammonium sulfate precipitation (30–60%)	2400	953	0.40
DEAE Sephadex A50-column	1815	823	0.45
Phenylsepharose-column	70	285	4.10
Hydroxylapatite-column	5.9	75.5	12.70
FPLC-Superose 12-column	0.78	10.6	13.60

ear gradient which ranged from 30 to 100 mM potassium phosphate buffer, pH 7.5 (total volume of gradient was 300 ml). The most active fractions were combined and dialyzed against 20 mM potassium phosphate buffer, pH 7.5, containing 5 mM NaCl and 30 % polyethylene glycol for concentration. The concentrated protein (2.7 ml) was applied in 200 μl fractions to a Superose 12 column (Pharmacia) coupled to a FPLC system and eqilibrated with 20 mM potassium phosphate buffer, pH 7.5, containing 50 mM NaCl. The results of a typical purification are given in Table I. The isolated PAT was at least 90 % homogenous on the basis of SDS-PAGE (Fig. 1).

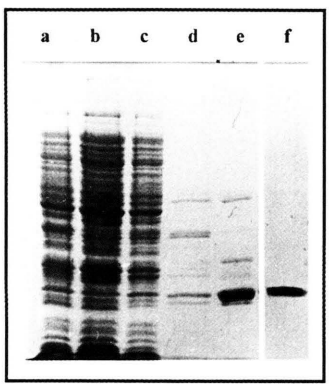


Fig. 1. Coomassie brilliant blue stained SDS-polyacrylamide gel (12 % acrylamide) of protein samples from the various PAT purification steps. Lane a: crude extract (55 μg protein applied to the gel); lane b: 30–65 % ammonium sulfate fraction (95 μg protein); lanes c-f: pooled PAT containing fractions. Lane c: after DEAE-Sephadex A50-column (60 μg protein), lane d: after Phenylsepharose-column (6 μg protein); lane e: after Hydroxylapatite-column (5 μg protein); lane f: after FPLC-Superose 12 column (5 μg protein).

Molecular mass determinations

The apparent molecular mass of the native PAT was determined by gel filtration on a Superose 12 column (Pharmacia) coupled to a FPLC system and shown to be 40 (+/-3) kD. SDS-PAGE of the purified enzyme gave one band which corresponded to 20.9 kD. This indicates that the enzyme consists of 2 subunits of equal molecular mass.

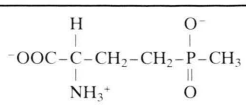
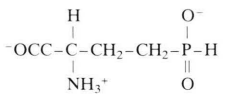
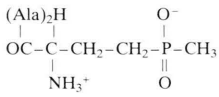
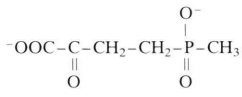
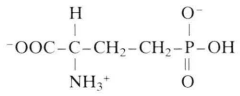
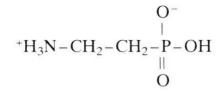
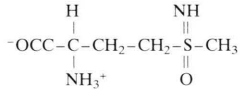
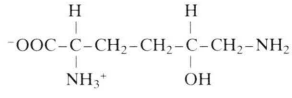
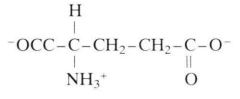
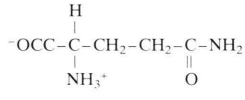
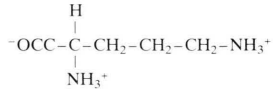
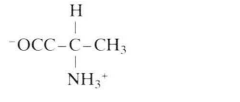
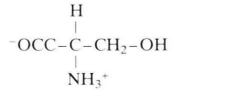
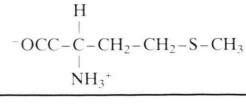
Substrate specificity of PAT and its inhibitors

As previously shown, PAT converts phosphinothricin in the presence of acetyl-CoA to *N*-acetyl phosphinothricin. The turn-over number for the highly purified PAT corresponded to 9.2 s^{-1} at 22 °C and 27 s^{-1} at 35 °C (Pt and acetyl-CoA as substrates and at pH 7.8). A number of phosphinothricin derivatives or precursors (such as PPO, Ptt, DMPT, APB and AEP) and two other inhibitors of glutamine synthetase (MSO and δ-Hydroxylysine, Lea and Ridley, 1989) were tested as possible substrates for PAT. Besides Pt only DMPT and MSO were acetylated by PAT with rates corresponding to 10 and 16 % of the rate for Pt, respectively (Table II). We also tested a number of L-amino acids (for details see Table II), but none of these proved to be a substrate for PAT under our test conditions. PAT activity was inhibited by PPO (23 and 67 % inhibition with 0.3 and 1 mM PPO, respectively). L-Glu, L-Gln or δ-hydroxylysine (up to concentrations of 10 mM) did not inhibit PAT.

Reaction characteristics

The K_M values for the two substrates corresponded to 0.056 mM for Pt (in presence of 0.096

Table II. Substrate specificity of the purified PAT. The concentrations of the substances are given as final concentration of the L-form in the spectroscopic assay mixture as described under Materials and Methods. The acetylation rates are given as relative reaction rates – the rate with PAT beeing 100%.

Substrate		Structure	Concentration [mM] tested	Relative reaction rate (%)
Pt-derivatives or precursors	Pt		0.18	100
	DMPT		0.18	10
	Ptt		1	0
	PPO		10	0
	APB		10	0
	AEP		10	0
Inhibitors of glutamine synthetase	MSO		0.18	16
	δ-Hydroxylysine		10	0
Selected amino acids	Glutamate		10	0
	Glutamine		10	0
	Ornithine		10	0
	Alanine		10	0
	Serine		10	0
	Methionine		10	0

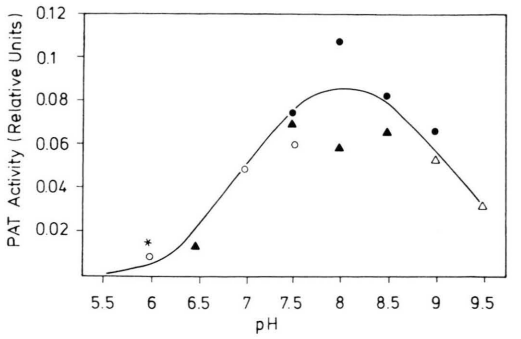


Fig. 2. PAT-activity as a function of pH. The buffers used were Mes (∗), Hepes (○), Tricine (▲), Tris (●) and Ches (△).

mM acetyl-CoA) and to 0.12 mM for acetyl-CoA (in the presence of 0.18 mM Pt). The pH optimum was at pH 8.0. The enzyme was inactive at pH 5.5 and had approximately 10 % and 30 % of its maximal activity at pH 6.5 or 9.5, respectively (Fig. 2). With Tris-HCl, pH 8.0, the activity was about 40% higher than with Tricine-HCl, pH 8.0. The temperature optimum for the PAT reaction was in the range of 40 °C to 50 °C – being variable in this range depending on the preincubation conditions used (Fig. 3A). The conditions chosen to determine the temperature optimum for the PAT reaction were the same as described in Botterman *et al.* (1991).

Temperature stability

In Fig. 3B the temperature stability of PAT is given. When preincubated at 38 °C the purified enzyme had lost 50 % of its activity after 80 min. After 90 min of incubation at 35, 38 or 45 °C the remaining PAT activity corresponded to 45, 42 or 38 %, respectively.

Properties of the antiserum raised against purified PAT

The antiserum raised against purified PAT detected PAT in crude extracts of *E. coli* JM83 pES6.1 at a dilution of 1 to 50000 in immunoblot experiments (Fig. 4A). In Fig. 4B it is shown that at dilution of 1 to 1000 the antiserum could detect amounts of purified PAT as low as 31 ng. Thus, the antiserum is very sensitive and is able to detect even small amounts of PAT. The antiserum inhibited PAT activity to 50 % at a dilution of 1 to 16 and to 86 % at a dilution of 1 to 1 (data not shown). Moreover, it was tested whether the antiserum could be used for detection of PAT in tobacco plants which were transformed as described in Broer *et al.* (in prep.). Although PAT could easily be detected in plants grown at 25 °C, in heat treated tobacco plants (37 °C) PAT was not detectable by the antiserum (Fig. 5) indicating that the level of the enzyme is greatly reduced. Thus, the

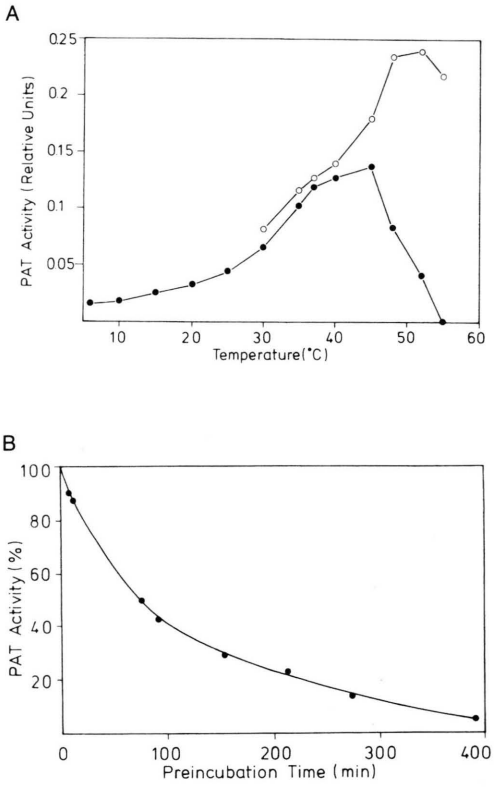


Fig. 3. PAT-activity as a function of temperature. A: Determination of temperature optimum of the PAT reaction: The assay mixture was the same as described under Materials and methods. The dependence of PAT activity on temperature was determined under two conditions. a: The assay mixture was preincubated with the substrate at the temperatures given in the figure, and the reaction was started by adding the enzyme (○---○). b: The assay mixture without Pt was preincubated with the enzyme for 15 min at the temperatures indicated in the figure, and then the reaction was started with the substrate Pt (●---●). B: Determination of temperature stability of PAT at 38 °C: The assay mixture was the same as described under materials and Methods. PAT was preincubated in the assay mixture (without Pt) at 38 °C for the times indicated in the figure. After the preincubation the reaction was started by adding Pt.

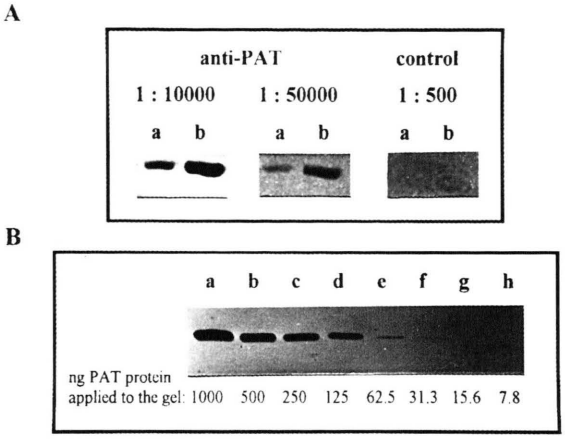


Fig. 4. Sensitivity of the antibody raised against the isolated PAT. A: Immunoblots with the antibody raised against the here purified PAT. The antiserum was used at 1 to 10000 and 1 : 50000 dilution and the control serum at 1 : 500 dilution. The samples applied to the SDS-polyacrylamide gel used for blotting were in lane a: crude extract from the PAT-producing *E. coli* JM83 pES6.1 strain (40 µg protein) and in lane b: purified PAT (4 µg protein). B: In lane 1–8 descending amounts of purified PAT were applied to the SDS polyacrylamide gel. The amount of PAT on the gel in lanes a to h are indicated in the figure. The SDS gel was immunostained with the antibody raised against the purified PAT (antibody dilution 1 to 1000).

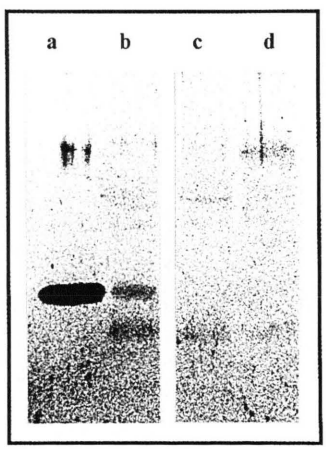


Fig. 5. Detection of PAT in transgenic tobacco plants. Lane a: Purified PAT as a control (90 ng protein on the gel). Lane b: Crude extract from *Nicotiana tabacum* SR1 C29.3 (100 µg protein on the gel) grown under regular conditions; Lane c: Crude extract from heat treated *Nicotiana tabacum* SR1 C29.3 (100 µg protein on the gel) grown at 37 °C as described in Broer *et al.* (in prep.) Lane d: Crude extract from *N. tabacum* SR1 untransformed (100 µg protein on the gel).

antiserum can be used to study the fate of the PAT protein in plants grown under different conditions.

Discussion

Purification of PAT originating from *S. viridochromogenes* but isolated from an overproducing *E. coli* strain enabled us to investigate its characteristics *in vitro* and additionally to analyse its fate in transgenic plants using the raised antibody. With the purification scheme developed in this paper it was possible to obtain PAT which was at least 90 % homogenous on the basis of SDS-PAGE. When we compared the results obtained here for PAT with those previously published by Thompson *et al.* (1987) and Botterman *et al.* (1991) for the *bar*-gene product from *S. hygroscopicus*, small but unexpected differences were observed. A summary of this comparison is given in Table III. Although both proteins should, according to their DNA-derived amino acid sequence, have the same subunit molecular weight (21 kD) (Wohlleben *et al.*, 1988), they differ in the molecular mass of the native enzyme. The PAT isolated here is a dimer with an apparent molecular mass of 40 kD, while from the results of Fig. 6A in Bottermann *et al.* (1991) it can be deduced that the native *bar*-encoded enzyme is about 21 kD in size, thus implying that the protein is a monomer. This is possibly due to the difference in the amino acid sequence (85% identity). Nevertheless it cannot be excluded that the dimerisation might also be caused by the 10 amino acids added to the C-terminal domain of the modified protein produced in *E. coli*. Another difference between the two PAT proteins is that the PAT from *S. viridochromogenes* is slightly less temperature sensitive than the PAT from *S. hygroscopicus*. This may be explained by the fact, that *S. viridochromogenes* was isolated from soil in Cameroun while *S. hygroscopicus* was isolated in Japan (Wohlleben *et al.*, 1988). However, the temperature stability tests have been performed *in vitro* using the modified protein derived from *E. coli* and have not been proven with the original protein isolated from *S. viridochromogenes*. Supposed, the stability of PAT also occurs *in planta* this property could be of advantage in the field under high temperature conditions and might qualify *pat* as a good marker protein for transformation of plants. Besides the above presented

Table III. Comparison of properties of the isolated *pat*-gene product from *Streptomyces viridochromogenes* (data from this work and from Wohlleben *et al.* [1988]) and the *bar*-gene product from *S. hygroscopicus* isolated by Thompson *et. al.* (1987) and Bottermann *et al.* (1991).

	pat-gene product	*bar*-gene product
Origin	*Streptomyces viridochromogenes*	*Streptomyces hygroscopicus*
Isolated from	*E. coli* JM83 pES6.1 (Strauch *et. al.*, 1988)	*E. coli* NF1 pGSFRTC1 (Bottermann *et al.*, 1991)
Number of amino acids calculated on basis of nucleotide sequence of gene	183[a]	183
Molecular mass calculated on basis of nucleotide sequence	20.6	20.6
Apparent molecular mass of:		
Native enzyme	40 kD	21 kD[b]
Subunit(s)	20.9 kD	21 kD
Substrates acetylated	Pt ≫ MSO > DMPT[c]	Pt ≫ DMPT > MSO > δ-Hydroxylsine > Glutamate[d]
Turnover number:		
at 22 °C	9.2 s^{-1}	–
at 35 °C	27.0 s^{-1}	24.4 s^{-1} [e]
K_M values		
for Pt	0.056 mM	0.06 mM
for Acetyl CoA	0.116 mM	n.d.
pH optimum (Tris buffer)	pH 8	pH 7.5
Temperature optimum:		
Preincubation with PAT	45 °C	35 °C
Preincubation with Pt	52 °C	45 °C

[a] The here isolated PAT was 10 amino acids longer than the original enzyme from *S. viridochromogenes* due to the cloning procedure used in Strauch *et al.* (1988); [b] not explicitly said by Bottermann *et al.* (1991) but deduced by us from the data given in Fig. 6 A; [c] based on relative reaction rates, see also Table II and text; [d] based on K_M-values given in Thompson *et al.* (1987; Pt: 0.06 mM, DMPT: 2.0 mM, MSO: 36 mM, δ-Hydroxylsine: 56 mM, Glutamate: 240 mM), see also text; [e] not explicitly said by Thompson *et al.* (1987) and Bottermann *et al.* (1991) but calculated by us from the data given in their papers.

deviations, the *pat*- and the *bar*-gene product show, as expected, great similarities in their respective K_M-values, turnover number and pH-optimum.

In addition to the characteristics described above, we analysed the substrate specificity of PAT. This can be of importance, since both, the *pat*-gene and the *bar*- gene are used to construct transgenic herbicide resistant crop plants. The specificity of the transgene encoded protein in the plant is of great importance for biosafety analysis concerning the usage of transgenic plants in agriculture. Unspecific reactions can in principle disturb metabolic pathways in the plant and lead to undesired and unknown reaction products. One of the most obvious putative substrates for PAT would be the structural analogue of Pt glutamate. We tested glutamate concentrations up to 10 mM, which is already high. The concentration of glutamate in tobacco is about 1.8 μmol/g fresh weight (T. Becker, personal communication), but no acetylation of glutamate could be observed. This seems to contradict the findings of Thompson *et al.* (1987), who found acetylation of glutamate. In their work they presented the K_M of PAT for glutamate which was as high as 240 mM. A comparison between their and our results is difficult, because we did not determine K_M-values for glutamate, therefore the comparison of the substrate specificity of PAT based on relative reaction rates and BAR based on K_M must be considered with some reservations. Since the K_M-values for Pt are identical for both enzymes, it can not be excluded that acetylation of glutamate at very high concentrations may also occur with PAT originating from *S. viridochromogenes*. Nevertheless, from the fact that no reaction occurs with glutamate at physiological concentrations *in vitro*, we assume, that neither the *pat*- nor the *bar*-gene product will lead to higher *N*-acetyl-glutamate concentrations in transgenic plants. The same is true for the acetylation of δ-hydroxylysine that has been observed by Thompson *et al.* (1987). They estimated the

K_M-value for this substrate to be 56 mM, while we limited the substrate concentration to 10 mM. We found no acetylation of other substrates tested with the exception of MSO (another glutamine synthetase inhibitor with a similar structure to Pt; Abell and Villafranca, 1991) and DMPT, the Pt precursor and substrate of PAT in the biosynthesis of Ptt. These substrates were also acetylated by PAT from *S. hygroscopicus* with K_M-values of 36 mM (MSO) and 2.0 mM (DMPT) (Thompson *et al.,* 1987). Neither DMPT nor MSO occur in plant metabolic pathways and therefore can not be a substrate for PAT *in planta*. It seems that the phosphino group is important for binding the substrate to the enzyme. Among the amino acids tested in this work besides Pt and DMPT only the sulfoximine group of MSO is similar to the phosphino group (see also Table II), while the other amino acids show no similar structure. The important role of the phosphino group is supported by the fact that PPO is able to inhibit the PAT reaction. PPO is the 2-oxo-derivative of Pt (see also Table II) and can therefore bind to the functional domain of PAT with its phosphino group but cannot be acetylated because of the missing amino group. It has to be stated that, although we found a high specifictity for PAT *in vitro*, we cannot entirely exclude that under *in vivo* conditions PAT might catalyze other acetylations.

As the results of the corresponding immunoblot experiments show, we were able to raise an antibody against the isolated PAT with very high sensitivity. This antibody was able to detect PAT in transgenic tobacco plants. Moreover, the results indicate that in heat treated plants PAT must be greatly reduced, since the protein is no longer detectable by the antibody – however small amounts of PAT must still be present, since the plant retains a resistance against low concentrations of Pt but this small amount is not detectable by the antiserum.

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