

## Characterization of dual specificity protein kinase from maize seedlings

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A protein kinase of 57 kDa, able to phosphorylate tyrosine in synthetic substrates pol(Glu<sub>4</sub>,Tyr<sub>1</sub>) and a fragment of Src tyrosine kinase, was isolated and partly purified from maize seedlings (*Zea mays*). The protein kinase was able to phosphorylate exogenous proteins: enolase, caseins, histones and myelin basic protein. Amino acid analysis of phosphorylated casein and enolase, as well as of phosphorylated endogenous proteins, showed that both Tyr and Ser residues were phosphorylated. Phosphotyrosine was also immunodetected in the 57 kDa protein fraction. In the protein fraction there are present 57 kDa protein kinase and enolase. This co-purification suggests that enolase can be an endogenous substrate of the kinase. The two proteins could be resolved by two-dimensional electrophoresis. Specific inhibitors of typical protein-tyrosine kinases had essentially no effect on the activity of the maize enzyme. Staurosporine, a nonspecific inhibitor of protein kinases, effectively inhibited the 57 kDa protein kinase. Also, poly L-lysine and heparin inhibited tyrosine phosphorylation by 57 kDa maize protein kinase. The substrate and inhibitor specificities of the 57 kDa maize protein kinase phosphorylating tyrosine indicate that it is a novel plant dual-specificity protein kinase.

Although protein Tyr phosphorylation is a rare post-translational event (Cooper *et al.*, 1983), its importance in controlling fundamental cellular processes such as growth, pro-

liferation, and differentiation has been well established (Uchida *et al.*, 1994). In mammals, tyrosine phosphorylation is catalysed by both specific (receptor and cytoplasmic)

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**Abbreviations:** 2DE, two-dimensional electrophoresis; DSK, dual-specificity kinase; MAPK, mitogen activated protein kinase; MAPKK, MAPK kinase; MBP, myelin basic protein; PK, protein kinase; PTK, protein-tyrosine kinase; RR-Src, fragment of Src tyrosine kinase.

protein tyrosine kinases (PTKs, reviewed in Hunter, 1998) and DSKs (Dual Specificity Protein Kinases) which, in addition to Tyr, also phosphorylate Ser and/or Thr residues (reviewed in Lindberg *et al.*, 1992; Dhana-sekaran & Reddy, 1998).

To date, Tyr phosphorylation of plant proteins has not been sufficiently elucidated. Although this modification has been observed in many plant species (Elliot & Geytenbeek, 1985; Torruella *et al.*, 1986; Trojanek *et al.*, 1996), its function remains unknown. There are only few reports on this matter. It was determined that profilin (from *Phaseolus vulgaris*), a component of the actin cytoskeleton, contained phosphotyrosine (Guillén *et al.*, 1999). Recently, reversible tyrosine phosphorylation has been suggested to play an important role in the regulation of stomatal function in *Arabidopsis* (Luan, 2002). Also, Tyr phosphorylation of low molecular mass proteins of pea mitochondria seems to be involved in respiratory redox signalling (Håkansson & Allen, 1995). In pea mitochondria, coconut embryos, and transformed roots of *Catharanthus roseus*, tyrosine phosphorylation is inhibited by genistein (Håkansson & Allen, 1995; Islas-Flores *et al.*, 1998; Rodriguez-Zapata & Hernández-Sotomayor, 1998), a specific inhibitor of animal PTKs. The presence in plants of an activity of phosphorylating RR-*Src* (a synthetic peptide derived from *Src* tyrosine kinase), which can be inhibited by genistein indicates PTK activity (Islas-Flores *et al.*, 1998; Rodriguez-Zapata & Hernández-Sotomayor, 1998).

Isolated from *Arabidopsis thaliana* cDNA encoding protein kinase (APK1) comprises mosaic of sequence characteristic for Ser/Thr as well as Tyr kinases. Recombinant APK1 was able to phosphorylate several exogenous proteins on Tyr (Hirayama & Oka, 1992). Screening of an *A. thaliana* expression library with an anti-phosphotyrosine antibody allowed cloning of another protein kinase (ADK1). Recombinant ADK1 was autophosphorylated on Ser, Thr, and Tyr residues and was able to

phosphorylate Tyr in the synthetic peptide poly(Glu<sub>4</sub>,Tyr<sub>1</sub>) (Ali *et al.*, 1994).

A cDNA encoding a protein with characteristic of a receptor-like kinase (PRK1) was isolated from a *Petunia inflata* library (Mu *et al.*, 1994). Recombinant cytoplasmic domain of PRK1 autophosphorylated on Ser and Tyr, suggesting that it is a DSK. The receptor-like structure of this kinase, its localization and interaction with calcium binding protein (KIP1) suggest that it modulates microtubule dynamics during pollen development (Skirpan *et al.*, 2001).

The occurrence in plants of DSKs with essential roles in signal transduction has been well documented. Mitogen Activated Protein Kinase Kinases (MAPKKs/MEKs), the main components of MAPK cascades, phosphorylate MAPKs on both Thr and Tyr residues of the conserved activation motif (Thr-Glu-Tyr, Thr-Pro-Tyr or Thr-Gly-Tyr). In plants, as in yeast and mammals, specific MAP kinase pathways participate in transduction of a variety of stimuli, among them well recognized are ethylene signaling pathway and innate immunity cascade induced by flagellin (Morris *et al.*, 1997; Asai, 2002; Chang, 2003; Ouaked *et al.* 2003; Guo & Ecker, 2004). The best characterized is SIMPKK (Salt Stress Induced MAPK Kinase). There is evidence directly demonstrating that the salt-induced activation of SIMK is mediated by the dual-specificity SIMKK (Kiegeri *et al.*, 2000). Another plant PK phosphorylating Tyr with a defined function is the dual specificity kinase, named PK12. This is a member of the LAMMER family of PKs, related to cyclin-dependent kinases and MAPKs. Recombinant PK12 undergoes autophosphorylation *in vitro* on Ser, Thr, and Tyr residues. The postulated functions of tobacco PK12 are transduction of ethylene signal(s) (Sessa *et al.*, 1996) and alternative splicing modulation (Savaldi-Goldstein *et al.*, 2003).

A role in signaling of the mobilization of seed storage reserves was suggested for a

cloned Ser/Thr/Tyr kinase from peanut (*Arachis hypogaea*). This developmentally regulated DSK, different to MAPKs, is induced by abiotic stresses (Rudrabhatla & Rajasekharan, 2002).

There are indications suggesting an involvement of protein tyrosine phosphorylation in the control of plant development (Mu *et al.*, 1994; Barizza *et al.*, 1999; Rudrabhatla & Rajasekharan, 2002). Considerable protein tyrosine phosphorylation was detected in etiolated maize seedlings (Trojanek *et al.*, 1996) at the intensive elongation phase, therefore presently we have focused on identification of a protein kinase responsible for this phosphorylation process.

## MATERIALS AND METHODS

**Biological material.** Maize (*Zea mays*) seeds, after soaking for 24 h at room temperature in distilled water, were grown at 27°C for 72 h in the dark. The apical 1 cm of the etiolated seedlings was harvested into liquid N<sub>2</sub>, then stored at -80°C.

**Chemicals.** Mouse anti-phosphotyrosine antibody (PY-20) was from ICN Biochemicals, Immobilon P PVDF was from Millipore, TLC cellulose plastic sheets and Na<sub>2</sub>CO<sub>3</sub> were from Merck, BCIP and NBT were from Promega, P-11 phosphocellulose and 3MM paper were from Whatmann, [ $\gamma$ -<sup>32</sup>P]ATP was from Amersham, Superose 12 column was from Pharmacia LKB, trichloroacetic acid was from Reachim, LMW, HMW, 2DE standards and Bio-Lyte 3/10 and 5/7 Ampholites were from BIO RAD. All other chemicals were from Sigma.

**Buffers.** Buffer A, 20 mM Tris/HCl, pH 7.0, 250 mM sucrose, 25  $\mu$ M Na<sub>3</sub>VO<sub>4</sub>, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF); buffer B, 20 mM Tris/HCl, pH 7.0, 25  $\mu$ M Na<sub>3</sub>VO<sub>4</sub>, 1 mM EDTA, 0.1 mM PMSF, 10 mM 2-mercaptoethanol; buffer C, 50 mM Tris/HCl, pH 7.5, 0.1 mM EDTA, 10  $\mu$ M Na<sub>3</sub>VO<sub>4</sub>, 10 mM 2-mercaptoethanol, 10 mM

MnCl<sub>2</sub>; buffer D, 10 mM Tris/HCl, pH 7.5, 1 mM EDTA, 30  $\mu$ M Na<sub>3</sub>VO<sub>4</sub>, 10% (v/v) glycerol, 0.01% (v/v) Triton X-100, 0.1 mM PMSF, 10 mM 2-mercaptoethanol; buffer E, buffer B plus 10% (v/v) glycerol; buffer F, 50 mM Tris/HCl, pH 8.0, 5 mM 2-mercaptoethanol; buffer TBST, 100 mM Tris/HCl, pH 8.0, 50 mM NaCl, 0.05% (v/v) Tween 20.

**Purification of protein kinase(s).** Purification procedures up to the first chromatography step were performed essentially as described previously (Trojanek *et al.*, 1996) except that 10-fold more material was used. Fifty grams of frozen etiolated maize seedlings was ground in liquid N<sub>2</sub> in a mortar, then transferred to a second mortar and mixed with 160 ml of buffer A. The extract was centrifuged for 30 min at 100 000  $\times g$  and the supernatant was filtered through glass wool before being applied to a phosphocellulose column (100 ml), equilibrated with buffer B. The column was washed with buffer B, then eluted with a linear gradient of 0 to 0.8 M NaCl in buffer B (300 ml). Fractions of 3 ml were collected and to each fraction glycerol was added to a final concentration of 10% (v/v). The enzymatically active fractions were pooled, dialyzed overnight against buffer D, then applied to a poly L-lysine agarose column (1.5 ml) previously equilibrated with buffer E. The column was washed with buffer E, until the protein concentration was below 10  $\mu$ g/ml, then the bound proteins were eluted with a linear gradient of 0 to 0.4 M NaCl in buffer E (30 ml); 1.5 ml fractions were collected. All steps of the purification were performed at 4°C. Protein concentration was estimated by Bradford's method (Bradford, 1976) using bovine serum albumin (BSA) as a standard.

**Phosphorylation of synthetic polypeptide poly(Glu<sub>4</sub>,Tyr<sub>1</sub>).** Total reaction mixtures contained: the appropriate enzymatic fraction, 12.5  $\mu$ M [ $\gamma$ -<sup>32</sup>P]ATP (0.5  $\mu$ Ci) and 2  $\mu$ M poly(Glu<sub>4</sub>,Tyr<sub>1</sub>) in buffer C. Phosphorylation of endogenous proteins without adding poly(Glu<sub>4</sub>,Tyr<sub>1</sub>) was used as a reference.

The  $^{32}\text{P}$  incorporated in the reaction products was determined as described previously (Trojanek *et al.*, 1996).

**SDS/PAGE.** Samples containing 0.2–2.0  $\mu\text{g}$  of protein were subjected to the slab gel system of Laemmli (1970). Routinely, 10% polyacrylamide gels were used. After electrophoresis, the gels were stained with silver, dried and autoradiographed.

**Immunoblotting with anti-phosphotyrosine antibody.** Proteins were precipitated by addition of trichloroacetic acid to a final concentration of 20% (v/v) and after centrifugation washed with cold acetone and dried. The precipitates were dissolved in sample buffer, boiled, and proteins were separated on 10% polyacrylamide gel. Additionally, phosphotyrosine conjugated with BSA (as an antigen-positive control) was run on the gel. After SDS/PAGE, the proteins were transferred to Immobilon P in the buffer system containing 25 mM Tris, 192 mM glycine, 20% (v/v) methanol. Next, the membrane was blocked with 5% (v/v) non-fat milk in TBST buffer for 1.5 h at room temperature followed by incubation with a 1:1000 diluted mouse anti-phosphotyrosine antibody in TBS for 1 h at room temperature with gentle rocking. Washing, incubation with secondary antibody and staining were performed as described previously (Trojanek *et al.*, 1996).

**Separation of phosphoamino acids by thin-layer chromatography.** Enzymatic fraction (100  $\mu\text{g}$ ) was phosphorylated in a mixture containing buffer C, 10 mM  $\text{MnCl}_2$  and 12.5  $\mu\text{M}$  [ $\gamma$ - $^{32}\text{P}$ ]ATP (25  $\mu\text{Ci}$ ) alone, or with casein (100  $\mu\text{g}$ ) or enolase (100  $\mu\text{g}$ ) for 30 min at 30°C. Then, trichloroacetic acid was added to a final 20% (v/v) and the precipitated proteins were dissolved in sample buffer and separated by SDS/PAGE in 10% gels. Samples containing proteins of appropriate molecular masses were cut out of the gels and extracted (Neufeld *et al.*, 1989) in a buffer containing 50 mM  $\text{NaHCO}_3$ , 0.5% (v/v) SDS, and 32 mM dithiothreitol (DTT). After 40 h incubation at room temperature, pro-

teins were precipitated by 20% trichloroacetic acid and hydrolyzed in 6 M HCl for 2 h at 110°C in sealed glass tubes. After hydrolysis, the samples were lyophilized and the residues were dissolved in 100  $\mu\text{l}$  water, re-lyophilized (two times), and finally dissolved in 15  $\mu\text{l}$  of water. Thin-layer chromatography was performed on cellulose plates according to a described procedure (Neufeld *et al.*, 1989; Trojanek *et al.*, 1996). Standards of Ser(P), Thr(P) and Tyr(P) were run in parallel and detected by reaction with 0.1% (w/v) ninhydrin, whereas the  $^{32}\text{P}$ -labelled amino acids were detected by autoradiography.

**In-gel kinase activity assay.** Enzyme activity was measured in gel with immobilized substrates according to described procedure (Geahlen *et al.*, 1986). Ten percent SDS/polyacrylamide gels contained 0.5 mg/ml copolymerized: poly(Glu<sub>4</sub>Tyr<sub>1</sub>) or enolase. After electrophoresis, SDS was removed by washing the gels with washing buffer (50 mM Tris/HCl, pH 8.0, 20% (v/v) 2-propanol) twice for 30 min each at room temperature; the gels were then equilibrated in buffer F (twice for 30 min). Proteins were denatured with 6 M guanidine  $\cdot$  HCl in buffer F (twice for 30 min), followed by overnight renaturation at 4°C in buffer F with 0.04% (v/v) Tween 40. Subsequently, the gels were incubated for 1 h at room temperature in 10 ml of buffer C with 10 mM  $\text{MnCl}_2$  and 20  $\mu\text{M}$  ATP supplemented with 50  $\mu\text{Ci}$  [ $\gamma$ - $^{32}\text{P}$ ]ATP. Reactions were terminated and unincorporated [ $\gamma$ - $^{32}\text{P}$ ]ATP was removed by washing the gels with 5% (v/v) trichloroacetic acid and 1% (v/v) sodium phosphate. Finally the gels were stained with Coomassie Brilliant Blue R 250, dried, and exposed to Hyperfilm MP.

**Two-dimensional electrophoresis (2DE)** was carried out as described by O'Farrell (1975). Samples containing 10–20  $\mu\text{g}$  protein were applied in buffer (9.5 M urea, 2% (v/v) Triton X-100, 5% (v/v) 2-mercaptoethanol, 1.6% (v/v) Biolyte 5/7 ampholite, and 0.4% (v/v) Biolyte 3/10 ampholite) on tube gels containing 4% (w/v) acrylamide, 9.2 M urea,

2% (v/v) Triton X-100, 1.6% (v/v) Biolyte 5/7 ampholite, 0.4% (v/v) Biolyte 3/10 ampholite. The size of the tube gels was  $45 \times 1$  mm. Isoelectric focusing was developed for 6 h at 435 V with 20 mM NaOH in the upper chamber and 10 mM  $\text{H}_3\text{PO}_4$  in the lower chamber. Then, the gels were removed from the tubes and applied on second dimension 10% slab gels (size  $85 \times 55$  mm, 1 mm thin). Electro-

correlated with the peak of activity phosphorylating casein, which was about 50% higher than phosphorylation of tyrosine in poly(Glu<sub>4</sub>Tyr<sub>1</sub>) (Fig. 1A). This procedure resulted in 70-fold purification of the enzyme with more than 40% recovery (Table 1).

For further purification and estimation of the molecular mass of the protein kinase, the preparation after poly L-Lys chromatography

**Table 1. Purification of protein kinase phosphorylating tyrosine from maize seedlings.**

Step of purification	Protein (mg)	Total activity (pmol·min <sup>-1</sup> )	Specific activity (pmol·min <sup>-1</sup> ·mg <sup>-1</sup> )	Fold of purification	Recovery %
Homogenate	352	686	2	1	100
P-cellulose	17	651	38	20	95
Poly L-lysine agarose	2	281	134	70	41
Superose 12	0.16	84	526	270	12

Fifty grams of maize seedlings was used for the first three steps of purification. Three independent preparations after poly L-lysine/agarose chromatography were pooled concentrated and applied on Superose 12. For the determination of enzymatic activity poly(Glu<sub>4</sub>Tyr<sub>1</sub>) was used as a substrate (see Materials and Methods).

phoresis was carried out for 45 min at 150 V and the gels were autoradiographed. The 2DE marker proteins were: conalbumin 76 kDa, pI 6.0–6.3, BSA 66 kDa, pI 5.4–5.6, actin 43 kDa, pI 5.0–5.1, carbonic anhydrase 31 kDa, pI 5.9–6.0.

## RESULTS

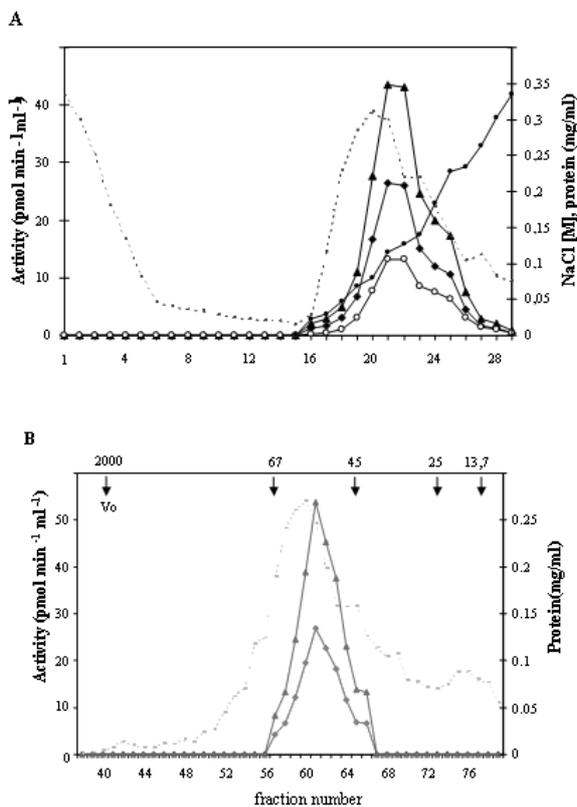
### Purification and identification of the protein kinase

A protein kinase from maize seedlings phosphorylating Tyr was initially separated by chromatography on phosphocellulose as previously described (Trojanek *et al.*, 1996). The enzymatically active fractions eluted by 0.2–0.3 M NaCl gradient were further purified by chromatography on poly L-Lys/agarose. The peak of activity phosphorylating Tyr in poly(Glu<sub>4</sub>Tyr<sub>1</sub>) eluted from immobilized poly L-lysine by 0.10–0.17 M NaCl was

was subjected to gel filtration using a Superose 12 column attached to FPLC system. The activity toward phosphorylation of poly(Glu<sub>4</sub>Tyr<sub>1</sub>) or casein, eluted in a single peak with maximal activity corresponding to 55–57 kDa (Fig. 1B). The same position of elution of both activities, as well as the symmetry of the peak eluted from the column indicate that only a single PK is present in the region of maximal activity (fr. 61–63). The small shoulder on the upper part of the elution (Fig. 1B, fr. 65–66) may suggest that another PK of about 45 kDa is separated. By Superose gel filtration a further 4-fold purification of the 57 kDa protein kinase was achieved. The specific activity of the 57 kDa kinase was about 526 pmol·min<sup>-1</sup>·mg<sup>-1</sup> (Table 1). For identification of the protein kinase responsible for Tyr phosphorylation, the activity was monitored at different steps of purification by using the in-gel kinase assay with poly(Glu<sub>4</sub>Tyr<sub>1</sub>) as a substrate (Fig. 2A). Detection of activity at the different steps of pu-

rification revealed a predominant 57 kDa band. Immunoblot analysis with anti-phosphotyrosine antibody recognized a 57 kDa protein in the fractions exhibiting maximal activity separated by poly L-lysine agarose

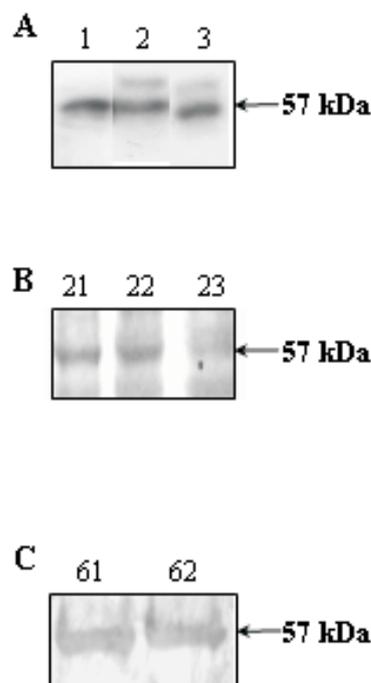
phosphotyrosine residues. Immunoblot analysis with anti-phosphotyrosine antibody confirmed the presence of the 57 kDa protein in active fractions after the Superose 12 chromatography (Fig. 2C).



**Figure 1.** Elution profiles of protein kinase activity from poly L-Lys/agarose (A) and from Superose 12 (B).

The activity was assayed in solution as described in Materials and Methods using poly(Glu<sub>4</sub>Tyr<sub>1</sub>) (◆) and casein (▲) as substrates or without any substrate (○); protein concentration is marked by the broken line whereas in A NaCl concentration (●). Vertical arrows (left to right) (B) indicate the elution positions of the following molecular mass standards: blue dextran 2000 used for void volume (V<sub>0</sub>); bovine serum albumin (67 kDa), ovalbumin (45 kDa), chymotrypsinogen (25 kDa), and ribonuclease (13.7 kDa).

chromatography (Fig. 2B). These results confirm that the 57 kDa protein(s) contains



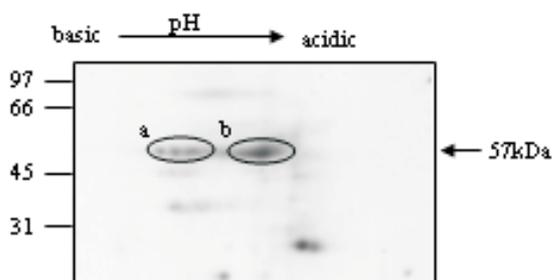
**Figure 2.** Identification of protein kinase phosphorylating tyrosine.

Protein kinases were analysed by in-gel kinase assay with immobilized poly(Glu<sub>4</sub>Tyr<sub>1</sub>) at different stages of purification (A) homogenate (lane 1), enzymes eluted from: P-cellulose column with 0.2–0.3 M NaCl (lane 2) and from poly L-Lys/agarose (lane 3) and Western blotting with anti-phosphotyrosine antibody of proteins from the enzymatically active fractions (frs. 21–23) after poly L-Lys/agarose (B) and Superose 12 chromatography (frs. 61 and 62) (C).

Two-dimensional electrophoresis (2 DE) was used to estimate the purity and isoelectric point of the isolated protein kinase. As can be seen from Fig. 3, the proteins of about 57 kDa were phosphorylated. The more acidic protein (b) of pI about 5.2 was predominantly phosphorylated, whereas the second protein (a) probably occurred in three isoforms, differentially phosphorylated (pI about 5.8, 5.7, 5.6) (Fig. 3).

### Substrate specificity and modulation of enzymatic activity

Electrophoretic separation of the proteins after gel filtration revealed the presence of other phosphorylable protein of 57 kDa apart from protein kinase. This protein, which copurified with the studied protein kinase, was analysed by time-of-flight mass spectrometry (Q-TOF MS) and identified by the database computer program Mascot, (Perkins *et al.*, 1999) as enolase (not shown). Therefore,



**Figure 3.** Separation of maize phosphoproteins by two-dimensional gel electrophoresis.

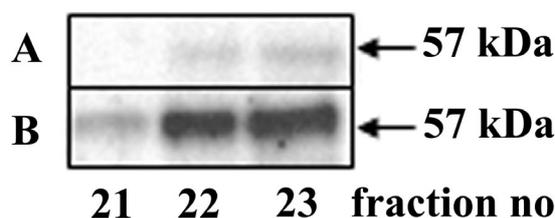
Autoradiogram of  $^{32}\text{P}$ -labelled proteins after gel filtration on Superose 12. Separated proteins are marked "a" and "b". Numbers indicate molecular mass standards in kDa.

to test whether enolase can serve as a substrate of the 57 kDa protein kinase, we used in-gel kinase assay with immobilized enolase (from rabbit muscle). High activity of the 57 kDa protein kinase toward phosphorylation of immobilized enolase was observed (Fig. 4B), while without immobilized enolase only traces of endogenous phosphorylation of the 57 kDa protein could be detected (Fig. 4A). Also, other immobilized proteins, like casein, histone H1 or myelin basic protein (MBP) were phosphorylated by 57 kDa protein kinase (not shown).

Examination of the substrate specificity of the maize protein kinase was continued with in-solution assays using different substrates of PTKs: poly(Glu<sub>4</sub>,Tyr<sub>1</sub>), poly(Glu<sub>1</sub>,Tyr<sub>1</sub>),

Src peptide (RRLIEDAEYAARG), enolase and commonly used substrates of protein kinases: casein, MBP, histones, phosvitin, casein kinase-1 substrate (RRKDLHDDEEEDAM-SITA). The relative phosphorylation of the Src peptide and enolase was comparable to that of histones and most of caseins with the exception of kappa casein which showed approximately 3-fold higher phosphorylation. The specific substrates of casein kinases, namely phosvitin and casein kinase-1 substrate, were not phosphorylated (Fig. 5).

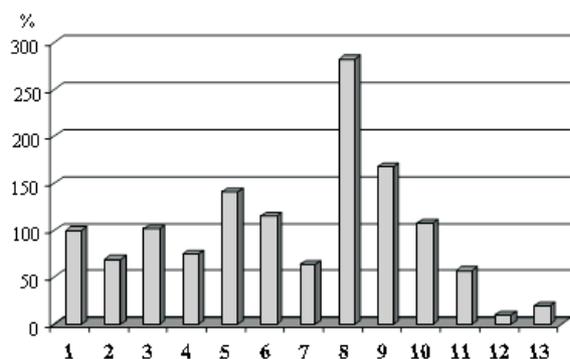
Further characterization of the maize protein kinase involved an elucidation of the effect of various modulators of mammalian PTKs on the plant enzyme. Specific inhibitors of mammalian enzymes: genistein, an erbstatin analogue, herbimycin A and tryphostine AG 126 and PP1 even at high concentrations (370, 100, 44, 232 and 178  $\mu\text{M}$ , respectively) did not affect the activity of the



**Figure 4.** Phosphorylation of enolase by 57 kDa maize protein kinase.

Activity of three fractions after poly L-Lys/agarose chromatography was tested by in-gel kinase assay without (A) and with (B) immobilized enolase.

maize protein kinase (not shown). Only lavendustine showed some effect on the phosphorylation of poly(Glu<sub>4</sub>,Tyr<sub>1</sub>) (IC<sub>50</sub> 68.8  $\mu\text{M}$ ). Poly L-lysine and heparin, activators of mammalian PTKs, inhibited the activity of the maize enzyme, but staurosporine, which is a general inhibitor of protein kinases, exhibited the strongest inhibitory effect. Inhibition of poly(Glu<sub>4</sub>,Tyr<sub>1</sub>) phosphorylation by staurosporine was more pronounced than casein (IC<sub>50</sub> 0.074 and 0.620  $\mu\text{M}$ , respectively)



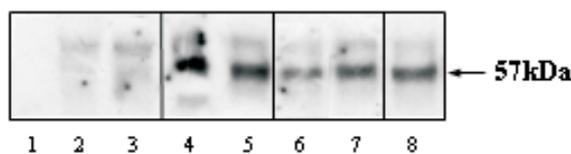
**Figure 5. Substrate specificity of maize protein kinases.**

The following peptides and proteins were tested: poly(Glu<sub>4</sub>Tyr<sub>1</sub>) (1), poly(Glu<sub>1</sub>Tyr<sub>1</sub>) (2), Src peptide (3), enolase (4), whole casein (5),  $\alpha$ -casein (6),  $\beta$ -casein (7),  $\kappa$ -casein (8), MBP (9), histone H1 (10), histone H2b (11), phosvitin (12), and casein kinase 1 substrate (13). The activity with the peptides and proteins was tested at 0.1 mg/ml except of the Src peptide – 7.6 mg/ml (1.25 mM). Phosphorylation of Tyr in poly(Glu<sub>4</sub>Tyr<sub>1</sub>) was taken as 100.

(Table 2). To confirm the inhibition results observed in solution, the gel-kinase assays were performed. Staurosporine at 0.5  $\mu$ M inhibited fully the activity of 57 kDa protein kinase (Fig. 6, lane 1), whereas at lower concentrations (0.1 and 0.05  $\mu$ M) only traces of the activity were visible (Fig 6, lane 2, 3). Genistein and lavendustin had no major effect on the activity of the plant enzyme (Fig. 6, lanes 4–7).

#### Identification of phosphorylated amino acids

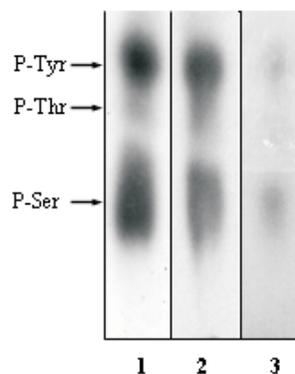
In order to establish which amino acids were phosphorylated by the maize protein kinase, casein, enolase and maize protein fraction of 57 kDa were subjected, after phosphorylation, to acid hydrolysis followed by TLC separation of <sup>32</sup>P-labelled amino acids and autoradiography (Fig. 7). The positions of phosphoamino acids were estimated based on the position of ninhydrin-stained spots of P-Tyr, P-Thr and P-Ser standards spotted in parallel



**Figure 6. Effect of selected protein kinases inhibitors on the activity of maize protein kinase.**

The enzyme activity was assayed by in-gel kinase assay with immobilized poly(Glu<sub>4</sub>Tyr<sub>1</sub>) in the presence of staurosporine: 0.5  $\mu$ M (lane 1), 0.1  $\mu$ M (lane 2), 0.05  $\mu$ M (lane 3); genistein: 185  $\mu$ M (lane 4), 92.5  $\mu$ M (lane 5); lavendustin: 131  $\mu$ M (lane 6), 65.5  $\mu$ M (lane 7); and the enzyme alone (lane 8).

with the hydrolysates. In the case of the endogenously phosphorylated 57 kDa proteins, two radioactive spots were present: P-Tyr and P-Ser (Fig 7, lane 3). Tyr and Ser were also intensively phosphorylated in casein and enolase (Fig. 7, lanes 1 and 2) but only traces of P-Thr were visible.



**Figure 7. Thin-layer chromatography of [<sup>32</sup>P] phosphoamino acids in acid hydrolysate of <sup>32</sup>P-labelled proteins (autoradiogram).**

Proteins phosphorylated by maize protein kinase: whole casein (lane 1), enolase (lane 2), and endogenous phosphorylation of 57 kDa maize proteins (lane 3).

#### DISCUSSION

A protein kinase phosphorylating Tyr residues was partially purified from etiolated maize seedlings by chromatography on P-celulose and poly L-Lys/agarose. The molecular

mass of the kinase was estimated by in-gel kinase assay as 57 kDa. Moreover, gel filtration chromatography in nondenaturing conditions showed molecular masses in the range of 55–57 kDa, indicating that the 57 kDa kinase is enzymatically active as a monomer.

Poly L-lysine, a known activator of PTKs, had an inhibitory effect on the plant enzyme. The inhibition was dependent on the substrate, which suggests that poly L-lysine could modulate the interaction of the protein kinase with particular substrates (Abdel-Ghany *et al.*, 1990). Poly L-lysine might interact with the substrates and induce conformational changes that make them either more or less available for phosphorylation. Our results indicate that in addition to the postulated role of this polycation as a substrate modulator,

Staurosporine, a non-specific inhibitor of protein kinases, efficiently inhibited the activity of the 57 kDa protein kinase from maize seedlings. Staurosporine binds with an extraordinarily high affinity (1–20 nM) to most Ser/Thr protein kinases (Prade *et al.*, 1997), and with a lower affinity (10–1000 nM) to PTKs (Secrist *et al.*, 1990). Staurosporine bound to the adenosine pocket of protein kinase A catalytic subunit causes a considerably induced fit rearrangement of the enzyme, yielding a unique open conformation. This inhibitor can induce a variety of conformational changes of neighboring enzyme residues (Prade *et al.*, 1997). However, a few protein kinases are relatively insensitive to staurosporine. Namely, Ser/Thr kinases: CK1 and CK2, MAPK, CaM-kinase III, and ty-

**Table 2. Influence of effectors of mammalian tyrosine kinases on the activity of maize protein kinase phosphorylating tyrosine**

Effector	Concentration used [ $\mu$ M]	IC <sub>50</sub> [ $\mu$ M]	
		poly(Glu <sub>4</sub> ,Tyr <sub>1</sub> )	casein
Staurosporine	0.02–20	0.074	0.620
Lavendustin A	16.4–131	68.8	–
Poly L-lysine	0–5.5	0.982	0.118
Heparin	0–24.9	2.76	–

–, lack of inhibition.

poly L-Lys efficiently binds the maize protein kinase. This affinity was exploited for efficient purification of the enzyme using poly L-Lys immobilized on agarose. The polyanion heparin also inhibited the maize enzyme in a substrate dependent manner. A strong inhibition of the enzymatic activity was observed when poly(Glu<sub>4</sub>,Tyr<sub>1</sub>) was used as a substrate, whereas casein phosphorylation was not effected by heparin (Table 2). In contrast, phosphorylation of casein by mammalian protein tyrosine kinases was inhibited by heparin (Wong & Goldberger, 1984; Borowski *et al.*, 1993).

rosine kinases: CSK (C-Src kinase) and IGF-IR which are inhibited by micromolar concentration of staurosporine (Meggio *et al.*, 1995).

Genistein is often used as a diagnostic inhibitor of PTKs (Akiyama *et al.*, 1987) and has been shown to inhibit protein Tyr phosphorylation in a plant signal system (Håkansson & Allen, 1995; Islas-Flores *et al.*, 1998; Rodriguez-Zapata & Hernández-Sotomayor, 1998). In contrast, the maize 57 kDa protein kinase, although it phosphorylates Tyr residues, was not inhibited by genistein.

Data on *A. thaliana* genome sequencing does not show sequences homologous with typical PTKs (European Union Arabidopsis Genome Sequencing Consortium, and The Cold Spring Harbor, Washington University in St. Louis, and PE Biosystems Arabidopsis Sequencing Consortium, 1999). There are, however, molecular indications that DSKs are responsible for Tyr phosphorylation of plant proteins (Hirayama & Oka, 1992; Ali *et al.*, 1994; Mu *et al.*, 1994). Presently, we provide biochemical data showing that the properties of the 57 kDa maize protein kinase are similar to those of dual specificity protein kinases. These include broad substrate specificity and the ability to phosphorylate Tyr as well as Ser in exogenous and endogenous proteins. The maize protein kinase was able to phosphorylate Tyr residues in exogenous substrates of PTK such as poly(Glu<sub>4</sub>Tyr<sub>1</sub>) and the Src peptide, but also Ser and Tyr residues in casein and enolase. Besides, the maize protein kinase was able to phosphorylate Tyr and Ser residues in endogenous proteins. The broad substrate specificity distinguishes the 57 kDa protein kinase from the known dual specificity MAP kinase kinases (MAPKKs). In contrast to the maize kinase, MAPKK from the cell line A 431 stimulated by epidermal growth factor, phosphorylated MBP and histones with low efficiency and was not able to phosphorylate poly(Glu<sub>4</sub>Tyr<sub>1</sub>) and Src (Seger *et al.*, 1992). Also casein kinases 1 and 2 (CK1 and CK2) seem to be dual specificity kinases because *in vitro* they are able to phosphorylate Tyr residues (Hoekstra *et al.*, 1994; Wilson *et al.*, 1997; Marin *et al.*, 1999). It was documented that yeast isotypes of CK1 were autophosphorylated on tyrosine and were able to phosphorylate poly(Glu<sub>4</sub>Tyr<sub>1</sub>), (Hoekstra *et al.*, 1994). However, substrates of casein kinases, namely phosvitin and the peptide RRKDLHDDEEDEAMSITA (Marin *et al.*, 1994), were not phosphorylated by the maize kinase. Lack of phosphorylation of this peptide which seems to be relatively specific for CK1 (Pulgar *et al.*, 1996), the opposite effect

of poly L-lysine, which is a stimulator of CK2 activity, and a significantly different affinity for heparin and staurosporine, suggest that the 57 kDa maize protein kinases does not belong to the any of the CK classes of enzymes. Thus, our results provide biochemical data indicating that maize protein kinase phosphorylating Tyr is a novel plant dual specificity protein kinase.

Enolase is a candidate as an endogenous substrate of the maize kinase. Such suggestion is supported by the facts that enolase copurified with the maize kinase and that this kinase phosphorylates Tyr and Ser in rabbit muscle enolase.

The partly purified enzymatic preparation of 57 kDa protein kinase after endogenous phosphorylation was resolved by two-dimensional electrophoresis into several phosphorylated species. Different isoelectric points of three proteins (marked as "a" on Fig. 3) may suggest that a single protein undergoes differential phosphorylation. There are indications that maize enolase of apparent molecular masses of 55 and 56 kDa can be resolved into three isoforms upon two-dimensional electrophoresis (Lal *et al.*, 1994). The phosphorylation at tyrosine residues of two enolase isoenzymes from chicken fibroblast has revealed different forms of pI 5.2–6.7 (Eigenbrodt *et al.*, 1983). Because the amino-acid sequence of enolase from different species is highly conserved e.g. maize enolase shows 82% identity with human enolase, it is possible that in maize enolase, like in vertebrates, tyrosine undergoes phosphorylation. Moreover, the *A. thaliana* protein kinase APK1, which is a dual specificity kinase, is able to phosphorylate enolase (Hirayama & Oka, 1992).

Further investigation will focus on molecular characterization of the maize PK phosphorylating Tyr and elucidation of the role of endogenous substrates of the maize DSKs.

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