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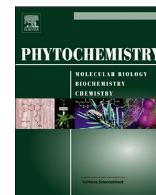

Isolation and bioinformatic analysis of seven genes encoding potato apyrase. Bacterial overexpresssion, refolding and initial kinetic studies on some recombinant potato apyrases

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A B S T R A C T

Here we have isolated seven apyrase encoding cDNA sequences (*StAPY4–StAPY10*) from the potato variety Saturna tuber cDNA library by affecting necessary modifications in the screening protocol. The cDNA sequences were identified with a pair of primers complementary to the most conserved sequences identified in potato variety Desiree apyrase genes. Our data strongly suggest the multigenic nature of potato apyrase. All deduced amino acid sequences contain a putative signal sequence, one transmembrane region at the amino terminus and five apyrase conserved regions (ACRs) (except StAPY6). Phylogenetic analysis revealed that encoded proteins shared high level of DNA sequence identity among themselves, representing a family of proteins markedly distinct from other eukaryotic as well as prokaryotic apyrases. Two cDNA sequences (*StAPY4* and *StAPY6*) were overexpressed in bacteria and recombinant proteins were found accumulated in inclusion bodies, even thought they were fused with thioredoxin-tag. Additionally, we present the first successful *in vitro* attempt at reactivation and purification of recombinant potato apyrase StAPY6. The ratio of ATPase/ADPase hydrolysis of recombinant StAPY6 was determined as 1.5:1. Unlike other apyrases the enzyme lacked ACR5 and was endowed with lower molecular weight, high specificity for purine nucleotides and very low specificity for pyrimidine, suggesting that StAPY6 is a potato apyrase, not described so far.

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1. Introduction

The apyrases (nucleoside triphosphate diphosphohydrolases, NTPDases) are multitasking enzymes involved in pathogen–host interaction, plant growth, lipid and protein glycosylation in the cells, organogenesis such as eye development, and oncogenesis (Knowles, 2011). Their diverse expression levels and their membranous and subcellular localization also hint at their roles in various metabolic processes (Day et al., 2000; Cohn et al., 2001; Steinebrunner et al., 2003; Riewe et al., 2008b; Govindarajulu et al., 2009; Kim et al., 2009; Clark et al., 2010; Knowles, 2011). Therefore, the fact that plant apyrases are being studied for the last 50 years is in order and rather further underscore their significance. Soluble enzymes participate in metabolism regulation affecting the energy status of plant cells (Thomas et al., 1999; Chivasa et al., 2005; Riewe et al., 2008a), in uptake of nucleotide degradation products from apoplast (Thomas et al., 1999; Riewe et al., 2008a), in *Solanum tuberosum* in accumulation of starch and tuber growth (Riewe et al., 2008b), and in *Arabidopsis thaliana* apyrases play an important role in regulation of plant growth, reproduction and stomatal aperture (Steinebrunner et al., 2000, 2003; Wolf et al., 2007; Clark et al., 2011). Furthermore, apyrases are involved not only in production of ADP in leguminous plants but also they seem to be one of the key factors in the initiation of symbiosis with *Rhizobium* bacteria (Etzler et al., 1999; Day et al., 2000; Navarro-Gochicoa et al., 2003; Govindarajulu et al., 2009). Plant apyrases also take part in mechanisms involved in xenobiotic resistance and in regulation of ABC transporter driven activities (Thomas et al., 2000). The other studies suggest that the plant apyrases also participate in regulating or silencing nucleotide signaling processes (Roux and Steinebrunner, 2007; Kim et al., 2009; Clark et al., 2011).

Apyrases have been utilized in nucleic acids sequencing, in assay for determining adenylate cyclase activity and in bioluminometric monitoring of ATP (Nourizad et al., 2003). Notably, these enzymes are frequently shown to possess therapeutic significance as these proteins can be used in therapy of certain nervous and circulatory diseases (Marcus et al., 2005).

Till now over 25 genes encoding apyrases have been identified and isolated from various plant species. While, Handa and Guidotti (1996) were the first to isolate cDNA sequence encoding potato apyrase (ATP diphosphohydrolase), the other two sequences of potato apyrase were isolated by Riewe et al. (2008b). Additionally,

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twelve apyrase genes have been identified in leguminous plants (Etzler et al., 1999; Shibata et al., 1999; Day et al., 2000; Cohn et al., 2001), seven in *A. thaliana* during genome analysis (Steinebrunner et al., 2000) and two genes in each *Mimosa pudica* and *Gossypium hirsutum* cells (Clark et al., 2010; Okuhata et al., 2011).

Proteins encoded by the plant apyrase genes are single polypeptide chains with the molecular weights between 45–52 kDa (Shibata et al., 1999; Day et al., 2000; Steinebrunner et al., 2000; Riewe et al., 2008b). However, the apyrases from *M. pudica* weight 36 and 67 kDa (Okuhata et al., 2011). Furthermore, the plant apyrases possess a signal peptide and a single transmembrane domain at the amino-terminal end (Steinebrunner et al., 2000; Kozakiewicz et al., 2008). Most of isolated and described plant NTPDases are soluble proteins, while all known animal enzymes are membrane bound proteins (Valenzuela et al., 1989; Komoszyński and Skalska, 1990; Komoszyński, 1996; Komoszyński and Wojtczak, 1996; Shibata et al., 1999; Robson et al., 2006; Yoneda et al., 2009; Knowles, 2011). ACR regions (*apyrase conserved regions*) that determine binding specificity, affect the substrate hydrolysis and are responsible for divalent cation interactions, are evolutionarily conserved in plant and animal NTPDases (Kirley et al., 2006; Kozakiewicz et al., 2008).

Plant apyrases hydrolyze di- and triphosphate bonds of nucleotides in presence of divalent metal ions according to the scheme I and II: I/ATP + $2H_2O \rightarrow$ AMP + $2P_i$ or II/ADP + $H_2O \rightarrow$ AMP + P_i with AMP or ADP and one or two ortophosphates as the final products (Kettlun et al., 1982; Wujak and Komoszyński, 2011). Apyrases do not hydrolyze ester bonds and inorganic pyrophosphate (Handa and Guidotti, 1996; Komoszyński, 1996; Kettlun et al., 2005). The highest activity of ADP hydrolysis is observed in the presence of calcium and magnesium ions. Previous studies indicated that Ca^{2+} and Mg^{2+} ions stimulate hydrolysis of ADP and ATP by potato apyrase most effectively (Kettlun et al., 2005). NTPDases have a broad substrate specificity and their velocity of ATP hydrolysis is not limited by the concentration of free ADP or source of enzyme (Komoszyński 1996; Robson et al., 2006; Wujak and Komoszyński, 2011). Interestingly, in spite of the high level of amino acid identity, all the eight animal NTPDases also possess unique substrate specificity (Komoszyński, 1996; Vorhoff et al., 2005; Robson et al., 2006).

However, in spite of the fact that many plant apyrases were described much earlier than animal NTPDases, understanding of their full structure and function is still inadequate and requires further investigation. In this back drop our data presented here assume significance. We particularly aimed our studies at identification of genes encoding NTPDases from potato and at attempting their bacterial expression as well as at characterizing some of these recombinant apyrases to better appreciate their evolutionary positioning and significance.

2. Results

2.1. Screening of potato cDNA sub-libraries

The single PCR product of the expected size of 550 bp was obtained from the second PCR reaction. On the basis of PCR analysis we found that sub-libraries 1–8, 10, 11, 13–15, 18–21 and 23 contained cDNA clones encoding potato apyrase (Fig. 1). Nine of them were chosen for further analysis using the PCR technique combined with the dilution screening protocol (Israel, 1993). Seven cDNA sequences with very high identity to already identified potato apyrase genes were isolated and were termed *StAPY4–10* (*StAPY1*, *StAPY2* and *StAPY3* were the apyrase genes cloned previously). *StAPY4–10* demonstrated differences in length and nucleotide composition of the open reading frame (ORF). The length of the open reading frames of *StAPY7–10* was 1362 bp, whereas *StAPY4* lacked a fragment of nine nucleotides at 3′ end and *StAPY5* lacked a fragment of 26 nucleotides near the 3′ end. *StAPY6* is the shortest sequence with 1027 bp demonstrating two 14 nucleotide deletions also near the 3′ end. We identified two additional sequences containing the same open reading frame as *StAPY8*. These three sequences demonstrate the differences only in nucleotide composition of 3′-untranslated region (UTR). It suggests that they may be transcribed from different genes templates (data not shown).

2.2. Bioinformatic analysis

2.2.1. Multiple alignment

Multiple alignment analysis of StAPY4–10 proteins revealed a very high homology at the amino acid level. The amino acid fragments between residues 44 and 212 of these proteins were almost 100% identical or homologous (Supplemental Fig. S1). Three different amino acid residues were only found in StAPY6 protein. The most significant differences between the sequences were close to the N- and C-termini of proteins.

All analyzed proteins (except for StAPY6) contained all amino acid motifs typical of apyrases such as ACRs and an ATP β-phosphate binding motif (Kirley et al., 2006; Robson et al., 2006). The amino acid composition of five ACR regions was identical, with the exception of ACR5 in StAPY4 protein (substitution for tryptophan by phenylalanine). Interestingly, StAPY6 lacked ACR5.

StAPY4–10 proteins showed the highest identity to potato apyrases, described previously (the identity of 88–99%). Most of them demonstrated the highest similarity to StAPY1 (ATP-diphosphohydrolase). For example, StAPY4 differed from StAPY1 in only three amino acid residues. StAPY5 and StAPY6 showed the highest similarity to StAPY3. The level of the identity between StAPY3 and remaining proteins was approximately 90%.

2.2.2. Analysis of amino acid sequences and physicochemical properties

The proteins StAPY7–10 were comprised of 454 amino acids, which corresponded with molecular weight of approximately 50 kDa (Table 1). Whereas, molecular weights of StAPY4 and StAPY5 were 451 and 440 amino acids, respectively. The open reading frame of *StAPY6* sequence encoded a polypeptide consisting of 335 amino acids, which corresponded with molecular weight of 37 kDa. *In silico* analysis of physicochemical parameters of StAPY4–10 revealed differences in calculated isoelectric points values (Table 1). Four proteins (StAPY4, 8, 9, 10) with a pI value of 6.64 were almost in agreement with the apyrase identified in potato var. Desiree having the similar pI value (Kettlun et al., 1982). Interestingly, calculated isoelectric points of remaining proteins were situated in alkaline pH range (7.17 for StAPY5 and StAPY10, 8.41 for StAPY6). Moreover, the isoelectric point of StAPY6 was similar to pI of apyrase purified from potato var. Pimpernel (Kettlun et al., 1982; Mancilla et al., 1984).

Hydropathy analysis of the protein sequences indicated that StAPY4–10 proteins had a single strong transmembrane helix of 24 amino acids at the N-terminus. The transmembrane regions differed in amino acid composition which may affect hydrophobic properties of the transmembrane domain and subcellular localization of potato apyrases. Moreover, the N-termini of StAPY4–10 were characteristic of signal peptides with a putative cleavage site after residue 30 according to data obtained from Peptide Cutter tool.

2.2.3. Phylogenetic analysis

The results of phylogenetic analysis indicate that potato var. Saturna apyrases constitute a family of closely related proteins. Simultaneous phylogenetic analysis of Saturna apyrases and

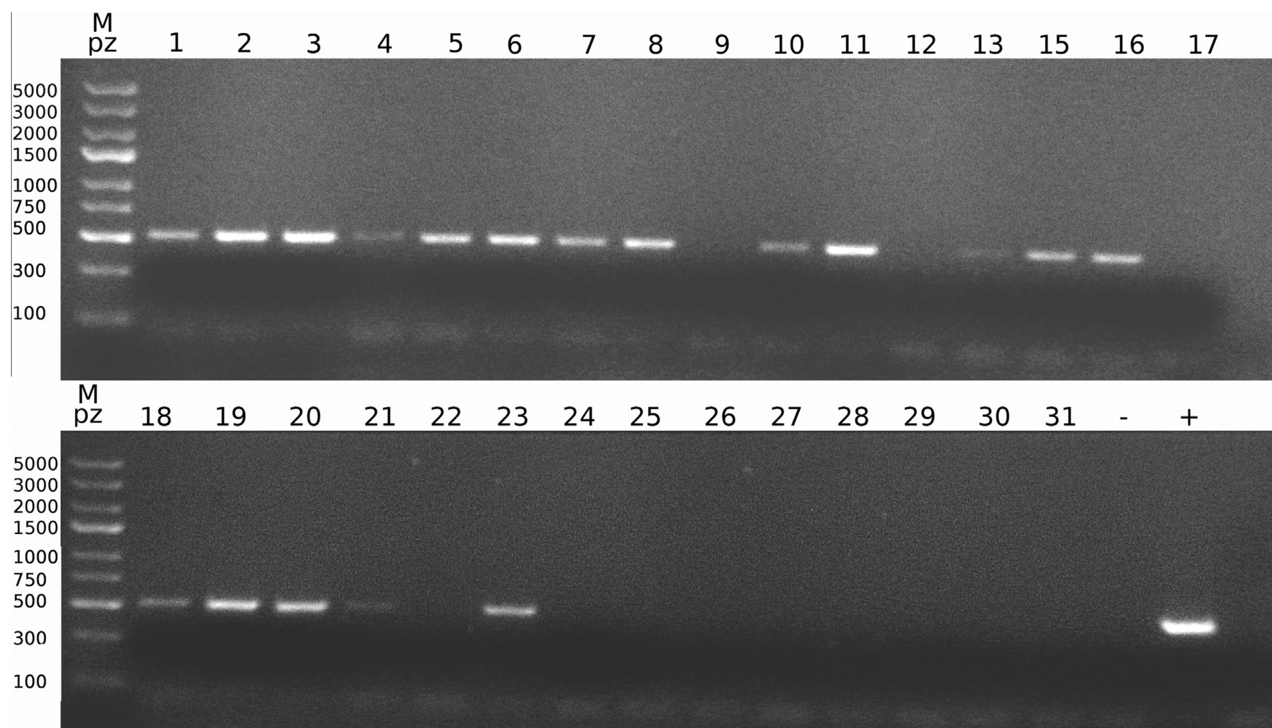

Fig. 1. Electrophoresis of PCR products amplified in the second PCR reaction on 31 sub-libraries as a DNA template. M – DNA mass marker, 1–31 – cDNA sub-libraries, (+) – positive control (1 μL of amplified cDNA library), (−) – negative control (nuclease free water instead of template).

Table 1
Analysis of physicochemical parameters of StAPY4–10 proteins.

Protein	Number of amino acids	Molecular weight (Da)	Theoretical pI
StAPY4	451	49764.6	6.64
StAPY5	440	48748.3	7.18
StAPY6	335	36989.9	8.41
StAPY7	454	50076.8	6.64
StAPY8	454	50031.8	6.64
StAPY9	454	50003.8	6.64
StAPY10	454	50061.8	7.17

NTPDases from different organisms revealed that potato apyrases of different varieties are markedly distinct from other plant apyrases (Fig. 2). StAPY4–10 proteins can be divided into four groups based on their phylogenetic relationships. The first group consists of most closely related StAPY7, StAPY8 and StAPY9 proteins. The second group is represented by StAPY10 and StAPY5 proteins. Meanwhile, StAPY4 and StAPY6 proteins not only demonstrate slightly lower identity to the other proteins, but also differ from each other. StAPY6 is most distinct from all identified apyrases of potato var. Saturna (Fig. 2). This apyrase demonstrates the closest phylogenetic relationship with apyrase from potato var. Desiree (StAPY2) as well as a protein encoded by cDNA sequence isolated from a cDNA library of tomato (Accession Number: AK329087.1). Among the plant apyrases, characterized so far, apyrases from *Glycine soja* demonstrate the closest evolutionary relationship with apyrases identified as StAPY4–10.

2.3. Expression of *S. tuberosum* apyrases

Apyrases StAPY4, StAPY5 and StAPY6 were overexpressed in *Escherichia coli* with fusion tags (thioredoxin-tag, 6xHis-tag and S-tag). Only two apyrase genes from *S. tuberosum* (*StAPY4* and *StAPY6*) were successfully overexpressed in *E. coli* BL21 CodonPlus (DE3) cells (Fig. 3). The protein encoded by *StAPY5* gene was not synthesized in above bacteria strain. The use of different expression system allowed to obtain an active form of this protein (data not shown).

Calculated *in silico* molecular weights of recombinant proteins StAPY4 and StAPY6 amounted to 65 and 54 kDa, respectively. Overexpressed recombinant proteins StAPY4 and StAPY6 demonstrate similar molecular masses (Fig. 3).

Electrophoresis of proteins obtained in bacterial cultures revealed that overproduced enzymes were in insoluble fraction (inclusion bodies), suggesting that presence of thioredoxin-tag did not improve the solubility of recombinant proteins. Moreover, there was no increase in nucleotidase activity in supernatants obtained from bacterial cultures induced to StAPY4 and StAPY6 synthesis in comparison with the non-induced culture. The noticeable increase in ADPase activity in presence of Mg^{2+} ions occurred only in case of StAPY5, however, the SDS–PAGE analysis of bacterial proteins did not reveal the presence of additional band of the recombinant protein StAPY5 (Fig. 3; Fig. 4A and B).

2.4. Refolding and purification of recombinant StAPY6

2.4.1. Refolding of StAPY6

For refolding we used inclusion bodies from bacteria that expressed StAPY4 and StAPY6 proteins. From several examined procedures only one enabled successful *in vitro* refolding of StAPY6. We used 2.5 mg of proteins for *in vitro* reactivation and obtained 1.46 mg as an effect of refolding and purification. Efficiency of refolding was 37%. Dialysis increased refolding yield to 68%. Specific activity of renaturated StAPY6 in presence of Ca^{2+} ions was 30.4 and 19.7 μmol Pi·min^{-1} mg of protein^{-1} for ATP and ADP, respectively (Fig. 5) and ratio of ATPase/ADPase hydrolysis was 1.5:1. The presence of EDTA in incubation mixtures completely inhibited activity for both substrates. We attempted to refold StAPY4 under the similar conditions, but we did not obtain active

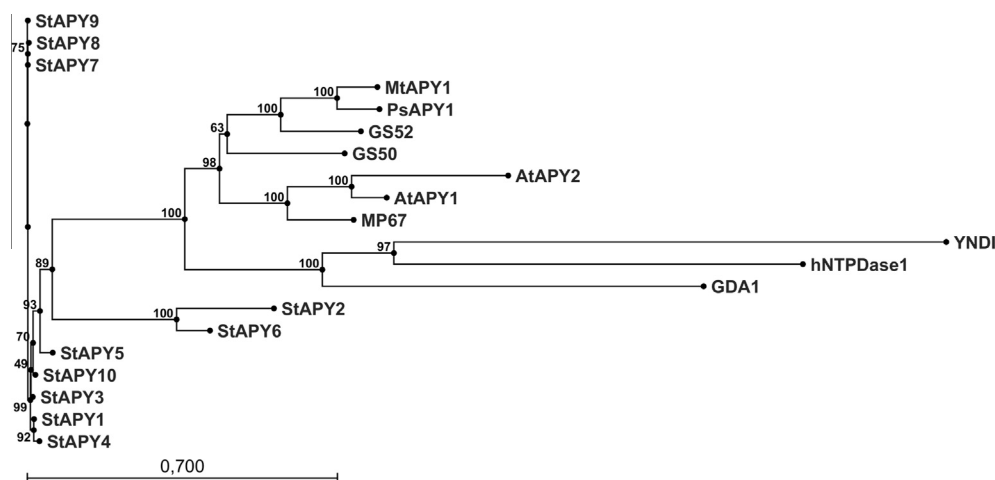

Fig. 2. Neighbor-joning tree representing phylogenetic relations between apyrases from potato var. Saturna (StAPY4–10) and NTPDases from selected sources: StAPY1 – apyrase RROP1 from *S. tuberosum* var. Desiree (AAB02720.1); StAPY2 – apyrase from *S. tuberosum* var. Desiree (ABV26544.1); StAPY3 – apyrase from *S. tuberosum* var. Desiree (ABV26545.1); GS50 and GS52 – apyrases from *G. soja* (AAG32959.1 and AAG32960.1); MtAPY1 – apyrase from *Medicago truncatula* (AAK15160.1); PsAPY1 – apyrase from *Pisum sativum* (BAB85977.1); AtAPY1 and AtAPY2 – apyrases from *A. thaliana* (AAF26805.1 and AAF66599.1); MP67 – apyrase from *M. pudica* (BAK78982.1); ENTPDase1 – human ENTPDase1 (NP_001767.3); YNDI – apyrase from *Saccharomyces cerevisiae* (EDV08832.1); GDA1 – apyrase from *S. cerevisiae* (EDV08832.1). The alignment of the amino acid sequences and the phylogenetic analysis were performed using CLC Sequence Viewer (with 100 bootstrap analyses).

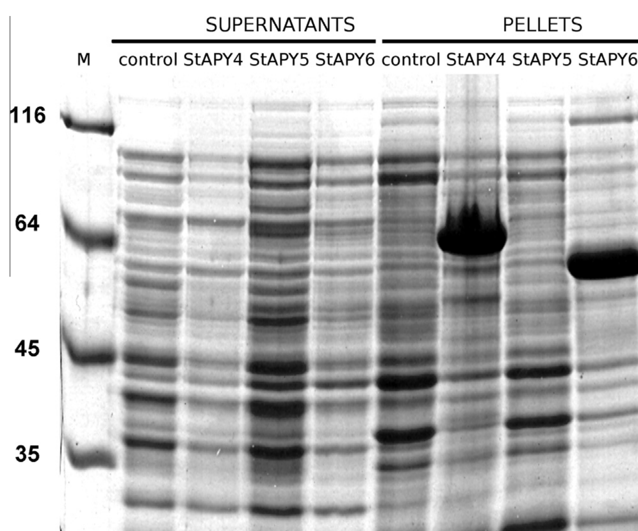

Fig. 3. SDS–PAGE electrophoresis of soluble (supernatants) and insoluble (pellets) bacteria proteins. Line: M – molecular mass marker. C – control (non-induced bacteria culture); StAPY4, StAPY5, StAPY6 – proteins of bacteria cultures induced to synthesis of recombinant StAPY4, StAPY5 and StAPY6, respectively. The molecular weight of fusion tags (thioredoxin-tag, 6His-tag and S-tag) is approximately 13 kDa.

enzyme. This may be caused by differences in length and different amino acid sequences between these two proteins. For this reason we used recombinant StAPY6 for further experiments.

2.4.2. Purification of StAPY6

The active StAPY6 protein was purified 1.25 times on Sephadex G75 column (Fig. 6; Fig. 7; Table 2). For further experiments we used proteins present in fractions 5–7. The efficiency of purification was 86%. The protein obtained in foregoing procedure was homogeneous. The molecular weight of purified recombinant StAPY6 is the same as molecular weight calculated *in silico* (Fig. 7).

2.5. Initial kinetic analysis of recombinant StAPY6

The ratio of ATPase to ADPase activity of purified StAPY6 is similar to the ratio of potato apyrase previously characterized, but the products of ATP hydrolysis were ADP and AMP, whereas ADP was hydrolyzed to AMP (Fig. 8A and B). Refolded enzyme hydrolyzed purine nucleotides efficiently. The hydrolysis rate of pyrimidine nucleotides was equal to 10% of the rate for purine nucleotides (Table 3).

The pH optimum of recombinant StAPY6 was 6.5. Divalent cations activated this enzyme in the following order $Ca^{2+} > Mg^{2+} > Co^{2+} > Mn^{2+}$. EDTA and Cu^{2+} completely inhibited catalytic activity of StAPY6. The removal of thioredoxin-tag from purified apyrase caused 10-times reduction of specific activity (Table 3).

3. Discussion

3.1. Isolation and identification of cDNA sequences encoding potato apyrase

This study describes the isolation and identification of seven cDNA sequences encoding potato apyrases including expression of three of them in *E. coli*. Not the filter hybridization but highly sensitive, specific and efficient PCR technology was utilized to screen the potato tuber cDNA library (Israel, 1993). The modifications that we introduced in choosing optimal dilutions of recombinant phages for each screen enabled the identification and the isolation of pure cDNA clones just after four rounds of screening. The isolated sequences were named as *StAPY4*, *StAPY5*, *StAPY6*, *StAPY7*, StAPY8, *StAPY9*, *StAPY10*. In our approach, *StAPY4–StAPY10* cDNA sequences were isolated utilizing a single pair of primers complementary to the most conserved sequences identified in *StAPY1*, *StAPY2* and *StAPY3*. We show that the cDNA sequence *StAPY6* has the STOP codon at positions 1025 to 1027, which shortens its length. Proteins StAPY2 and StAPY6 share a very high identify (88%). Contrary to Rewie's assumption, our results indicate that apyrase expressed on the *StAPY6* cDNA template results in catalytically active protein after *in vitro* refolding. Though, due to tetraploidy and multiplicity of genes encoding the plant apyrases and various approaches to identify and isolate them, it is very difficult to completely reconcile our results with the existing data, nevertheless, the isolated cDNA of *StAPY2* has a comparable length to *StAPY1* and *StAPY3* sequences (Handa and Guidotti, 1996; Riewe et al., 2008b; Urbany et al., 2011). In addition to the TAA STOP codon at positions 1363 to 1365 found in the other apyrase se-

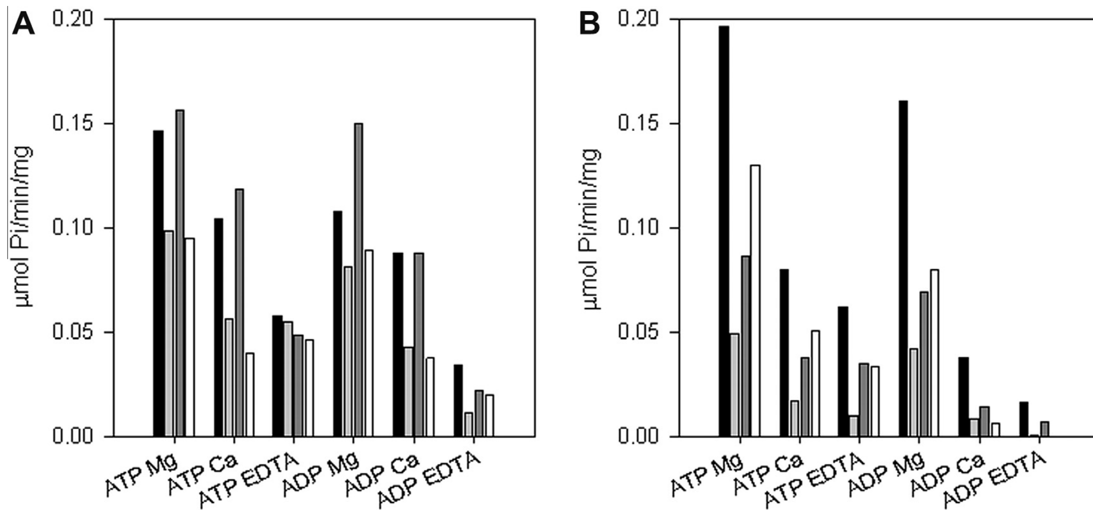

Fig. 4. Nucleotidase activity of bacteria *E. coli* proteins; A – specific activity of soluble bacteria proteins (supernatants); B – specific activity of insoluble bacteria proteins (pellets). ■ control; ■ bacteria culture induced to StAPY4 synthesis; ■ bacteria culture induced to StAPY5 synthesis; □ bacteria culture induced to StAPY6 synthesis. Exemplary results of one representative experiment are presented ($n = 3$).

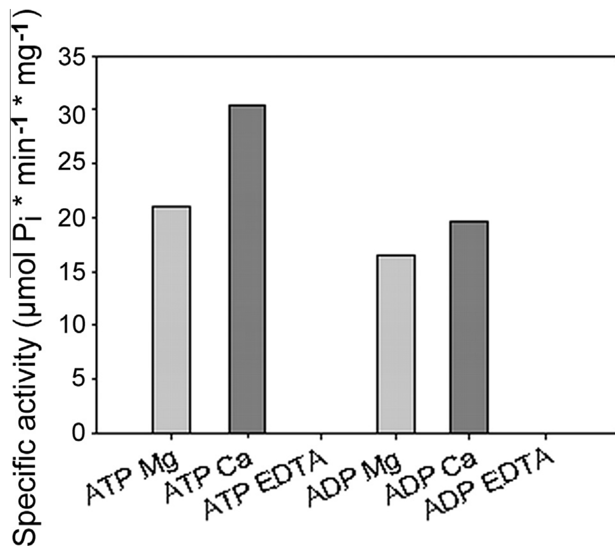

Fig. 5. The influence of divalent cations on ADPase and ATPase activity of renaturated StAPY6. Exemplary results of one representative experiment are presented ($n = 3$).

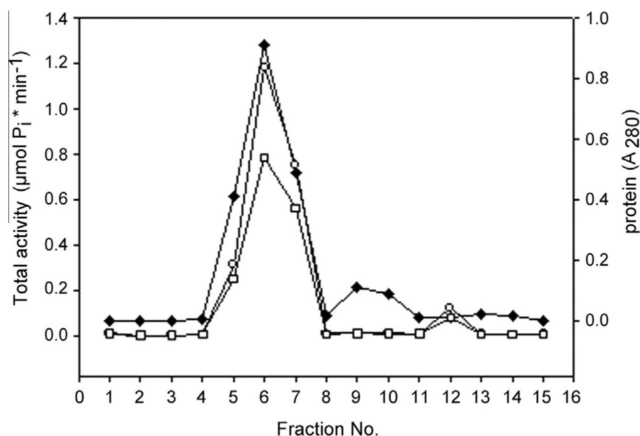

Fig. 6. Chromatogram of renaturated proteins from inclusion bodies on Sephadex G75. ♦ protein concentration, ○ ATPase activity, □ ADPase activity. Exemplary results of one representative experiment are presented ($n = 3$).

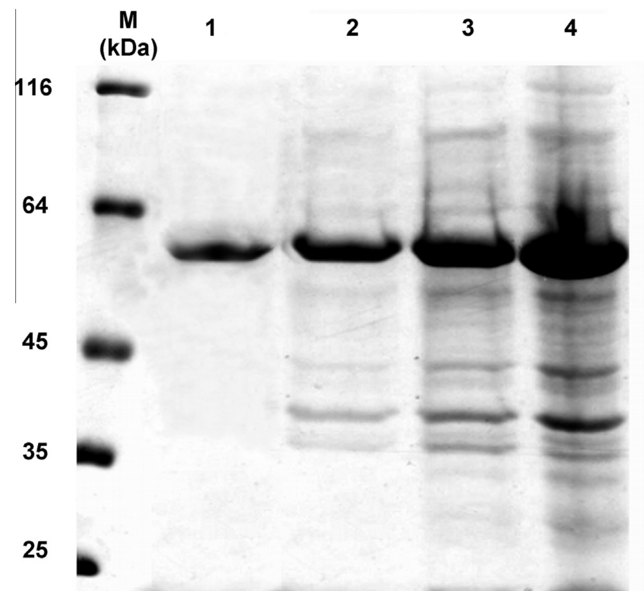

Fig. 7. SDS–PAGE electrophoresis of inclusion bodies proteins and recombinant StAPY6 purified with Sephadex G75. Line: M – molecular mass marker; 1 – purified StAPY6; 2 – inclusion bodies proteins obtained after refolding; 3 – inclusion bodies proteins before refolding (10 µg); 4 – inclusion bodies proteins before refolding (20 µg).The molecular weight of fusion tags (thioredoxin-tag, 6His-tag and S-tag) is approximately 13 kDa.

Table 2
Activity of recombinant StAPY6 after subsequent steps of purification.

Steps of isolation and purification	Total activity (μM min^{-1})		Specific activity (μM min^{-1} mg of protein^{-1})	
	Substrate		Substrate	
	ATP	ADP	ATP	ADP
Inclusion bodies isolation	0.7	0.04	0.014	0.008
In vitro reactivation	152.0	98.5	30.4	19.7
Sephadex G75 purification	130.7	83.7	38.0	25.5

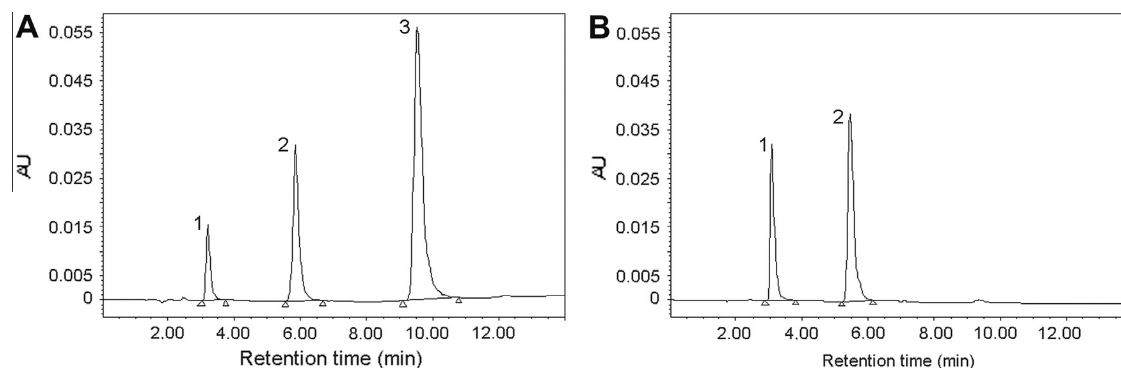

Fig. 8. Products of hydrolysis of ATP (A) and ADP (B) catalyzed by purified StAPY6. 1 – AMP, 2 – ADP, 3 –ATP. Reaction was run for 10 min.

Table 3
Comparison of nucleotidase activity of StAPY6 before and after the removal of thioredoxin-tag (trx). Rates of hydrolysis of purine and pyrimidine nucleotides catalyzed by purified StAPY6 are shown.

Nucleotide	Activity of StAPY6-trx		Activity of StAPY6	The ratio of hydrolysis velocity of StAPY6 and StAPY6-trx (%)
	μmol Pi·(mg of protein)$^{-1}$·min^{-1}	% of nucleotidase hydrolysis	μmol Pi·(mg of protein)$^{-1}$·min^{-1}	
ATP	30.4	100	3.4	8.94
ADP	19.7	68.0	2.1	9.38
GTP	27.3	84.6	2.5	10.9
GDP	21.1	69.2	2.4	8.79
CTP	3.2	12.5	0.8	4.0
CDP	0.8	3.34	0.67	1.19
UTP	7.2	28.2	1.6	4.5
UDP	2.1	6.8	0.45	4.66

quences, there is an additional STOP codon at positions 850 to 852 confirming distinct truncation, validating the observation of Riewe et al. who had suggested that potato cells var. Desiree lack functional StAPY2 (Riewe et al., 2008b). Our data also confirm the results of Southern blot analysis of the potato genome carried out independently by Handa and Guidotti (1996) and Riewe's group (2008b). The potato apyrases represent a large protein family, the members of which share a very high identity among themselves. Moreover,h they are distinct from apyrases found in other plants (Shibata et al., 1999; Day et al., 2000; Steinebrunner et al., 2000; Cohn et al., 2001; Clark et al., 2010; Okuhata et al., 2011).

These homologies are extendable further: The *in silico* calculated molecular weights of StAPY4–StAPY10 proteins do not exceed 50 kDa, and that of STAPY1 and StAPY3 are approximately 52 kDa in size, which concord with similar molecular weights demonstrated for apyrases from other plant species (Valenzuela et al., 1989; Komoszyński, 1996; Shibata et al., 1999; Day et al., 2000; Cohn et al., 2001; Steinebrunner et al., 2000; Riewe et al., 2008b; Govindarajulu et al., 2009; Clark et al., 2010). The StAPY6 having a molecular weight 37 kDa bears close homology with an apyrase from *M. pudica* (Okuhata et al., 2011). The apyrases StAPY4–StAPY10 are characterized by a very high amino acid identity. The presence of ACR1 and ACR4 motifs in identified apyrases with a capacity to bind β- and γ-phosphate moieties suggests that these proteins can be classified as members of the actin/Hsp70/hexokinase superfamily (Smith and Kirley, 1999).

The potato var. Saturna apyrases and animal apyrases demonstrate some similarities in the structure. Neverthless, the presence of QIPLRR motif after the transmembrane domain may suggest that at least StAPY4, 5, 7–10 potato apyrases are soluble or weakly associated with membrane endoapyrases.

3.2. Phylogenetic analysis

Among the analyzed proteins, StAPY4 is closely related with the ATP-diphosphohydrolase (StAPY1), meanwhile StAPY6 shows the closest phylogenetic relationship with StAPY2. In addition, only StAPY6 of seven identified apyrases demonstrates clearly closer relationship with animal NTPDases (especially with NTPDase4 and NTPDase7). Apyrases StAPY7, StAPY8 and StAPY9 are less closely related to animal NTPDases. Interestingly, apyrases isolated from other plant species demonstrate the closest phylogenetic relationship with animal NTPDases than with potato apyrases. This suggests that potato apyrases might have diverged early on from other plant apyrases and evolved along a different evolutionary trajectory. For this reason their functions in potato may be slightly different from other plant apyrases (Lavoie et al., 2004; Kozakiewicz et al., 2008).

3.3. Bacterial overexpression of genes encoding potato apyrase

Our data represent the first successful attempt of bacterial overexpression and *in vitro* reactivation of potato apyrases. This is our unique contribution particularly in light of previously failed attempts at overexpression of active potato apyrase in *E. coli* (Marahisa Handa announcement). Steinebrunner and co-workers (2000) though had overexpressed *AtAPY1* and *AtAPY2* in *E. coli* without transmembrane regions. These enzymes were accumulated in inclusion bodies which required purification under denaturing conditions but authors did not show their refolding (Steinebrunner et al., 2000). Earlier, Ivanenkov et al. (2003) and Murphy-Piedmonte et al. (2005) had indicated that bacterial expression systems can be used for overexpression of NTPDases. Human NTPDase5 and 6 were overexpressed in a similar system (Ivanenkov et al., 2003; Murphy-Piedmonte et al., 2005). Animal NTPDases were expressed and fused with His-tag and purified with IMAC. The *StAPY4*, *StAPY5* and *STAPY6* cDNA sequences were fused with thioredoxin-tag, His-tag and S-tag utilizing pET32a vector. The presence of thioredoxin-tag increases the solubility of some fusion proteins. But in opposition, we show that since the expressed apyrases remained trapped in inclusion bodies, there is least contribution of thioredoxin in their solubility. Unsuccessful apyrases overexpression in *E. coli* is ascribed to their toxicity due to degradation of nucleotides, which results in dramatic changes in total cellular energy charge. Nourizad and co-workers (2003) had used yeast *Pichia pastoris* as a host for ATP-diphosphohydrolase overexpression and obtained active but highly N-glycosylated enzyme. In distinction, using dilution and dialysis technique we show *in vitro* reactivation of recombinant potato apyrases, similar to *in vitro* refolding of animal NTPDase5 and 6 (Ivanenkov et al., 2003; Murphy-Piedmonte et al., 2005). Since, we could recover the catalytic

activity of only StAPY6 under these conditions, we are tempted to suggest that the observed differences in amino acid sequences between STAPY4 and StAPY6 might have been responsible for unique physicochemical properties of these enzymes.

3.4. Kinetic analysis of StAPY6 apyrase

In spite of marked differences in their molecular weights, potato StAPY6 and animal NTPDase3 hydrolyze ATP to ADP and AMP in a similar manner (Vorhoff et al., 2005; Robson et al., 2006). Specific activity of refolded and purified StAPY6 is much lower than activity of native potato apyrase and is similar to the activity of refolded animal NTPDases5 and 6 (Ivanenkov et al., 2003; Murphy-Piedmonte et al., 2005). The hydrolysis rate of pyrimidine nucleotides is nine times lower than the hydrolysis rate of purine nucleotides. These properties of StAPY6 also distinguish this enzyme from human NTPDase3, which hydrolyzes purine and pyrimidine nucleotides with the similar rate (Lavoie et al., 2004; Vorhoff et al., 2005). However, the influence of thioredoxin-tag cannot be excluded. The pH optimum of refolded StAPY6 was at 6.5 and is similar to the pH optimum of apyrase isolated and purified from potato (pH = 6) (Kettlun et al., 2005). Animal NTPDases, contrary to potato apyrases, have pH optimum mostly in alkaline range (near 7.4). The optimum pH for NTPDase8 is more than 8 (Kukulski et al., 2005). StAPY6, similar to native potato apyrase and recombinant NTPDase from soybean, demonstrates higher activity in presence of calcium and magnesium ions (Kettlun et al., 2005; Espinosa et al., 2000). Animal NTPDases1, 2 and 3 have comparable sensitivity to these ions, whereas refolded ecto-domains of animal NTPDases are five to ten times more activated with calcium than magnesium ions (Kukulski et al., 2005; Zebisch and Sträter, 2007).

4. Conclusions

In conclusion, we introduced the modification in screening processes that allowed us the rapid and effective isolation of pure cDNA clones. Our results clearly indicate that potato var. Saturna genome contains at least seven different apyrase encoding genes. The specific sequence QIPLRR contiguous to transmembrane domain is common in all encoded proteins except StAPY6. Contrary to other refolding methods used in animal NTPDase *in vitro* reactivation, the refolding buffer used in our studies contained glycerol and lacked the oxidizing and reducing agents. Nevertheless, the method was equally efficient. Our results indicate that thioredoxin-tag present at the N-terminus of the recombinant StAPY6 may have facilitated *in vitro* refolding and stabilization of the apyrase. The potato apyrase StAPY6 cloned by us, having lower molecular weight and high purine specificity and missing ACR5, is a novel enzyme distinct from other plant apyrases described so far.

5. Experimental

5.1. Construction of cDNA library from potato tubers

5.1.1. RNA isolation

RNA was isolated from eight weeks old potato var. Saturna tubers (received from Zegart-Farms Company, Zegartowice, Poland). A 100 g mass of tubers was homogenized in extraction buffer (100 mM Tris–HCl pH 8.8 buffer containing 1% sodium sarcosine, 10 mM dithiothreitol, 20 mM EDTA, 0.25 M NaCl and 1% phenol) and stirred for 5 min. A volume of 100 mL of phenol saturated with TE buffer (100 mM Tris–HCl containing 10 mM EDTA) was added to the above homogenate with continuous stirring for 10 min. Homogenate was centrifuged (10 min, 10,000g, 4 °C) and pellet was discarded. Aqueous phase was mixed with an equal volume of chloroform and centrifuged as above. RNA was precipitated by adding 8 M LiCl solution to final concentration of 2 M, followed by overnight incubation and centrifugation for 15 min, at 12,000*g*, 4 °C. The supertnatant was discarded and pellet was washed twice with 2 M LiCl solution and then centrifuged as above once again. RNA pellet was redissolved in RNAse free water. RNA was precipitated with 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volume of ethanol for 30 min and centrifuged as above. Pellet was washed twice with 70% ethanol and centrifuged (10 min, 12,000*g*, 4 °C). RNA pellet was dried and resuspended in RNAse free water. Purity and concentration of RNA was assayed spectrophotometrically at 260 and 280 nm and by electrophoresis in an agarose gel containing 5 M urea.

5.1.2. Isolation and purification of poly(A)

Poly(A)-RNA was purified from total potato RNA using the mRNA Purification Kit according to the manufacturer's instructions (Pharmacia). The amount and quality of purified mRNA was measured spectrophotometrically at 260 and 280 nm.

5.1.3. Potato tuber cDNA library construction and amplification

Potato tuber cDNA library was constructed using cDNA Synthesis Kit according to the manufacturer's instructions (Stratagene). The cDNA library was cloned into the Uni-ZAP XR lambda vector and then divided into 31 sub-libraries. Each sub-library was amplified in XL1-Blue MRF' cells according to the manufacturer's instructions (Stratagene). The titer of amplified cDNA library (pfu/mL) was determined taking into account the number of plaques raised on a plate and the dilution of the sub-library. Phage titer increased to ~109 pfu/mL after amplification. The amplified cDNA sub-libraries were stored at −80 °C.

5.2. Screening of potato tuber cDNA sub-libraries

5.2.1. Primer design

Three mRNA sequences encoding potato apyrase: 1/*S. tuberosum* apyrase 2 (Apy2) mRNA (Accession Number AF535135.1); 2/*S. tuberosum* ATP-diphosphohydrolase (RROP1) mRNA (U58597.1); 3/*S. tuberosum* cultivar Desiree apyrase 3 mRNA (EU125183.1) cloned previously by other laboratories were used to design PCR primers for potato tuber cDNA library screening. The sequences were aligned using ClustalW program (Chenna et al., 2003) in order to identify highly conserved DNA regions, and design a pair of primers (forward primer: 5′-GATCTTGGTGGTGGTTCAGTCC-3′ and reverse primer: 5′-CTTTAGCTGCATTTAAGTATTGAATTGG-3′). The designed primers were used for screening of potato tuber cDNA sub-libraries as well as for isolation of single clones encoding potato apyrase.

5.2.2. Identification of cDNA sub-libraries containing cDNA sequences encoding potato apyrase

The identification of cDNA sub-libraries containing cDNA sequences encoding potato apyrase was performed in two separate PCR steps. In the first step, PCR reaction was carried out in a final volume of 50 μL using KOD DNA polymerase according to the manufacturer's instructions (Novagen). T7 primer (5′-GTAATACGACTCACTATAGGGCG-3′) complementary to the left arm of the vector was used to amplify all cDNA inserts. The final concentration of T7 primer was 1 μM. Meanwhile 1 μL of 10-fold diluted cDNA sub-library served as a PCR template. The first PCR reaction was carried out in a thermal cycler (TC-312, Techne) with the following cycling parameters: 94 °C for 3 min followed by 25 cycles, each cycle at 94 °C for 30 s, at 53.5 °C for 20 s and at 72 °C for 30 s and finally for another 5 min at the same temperature. The second PCR reaction was performed in a final volume of 25 μL, containing 12.5 μL of PCR Master Mix 2x (Fermentas) and 50 pmol of each

oligonucleotide primer (forward and reverse). 1 μL of 1000-fold diluted reaction mixture from the first PCR reaction was used as a template. The second PCR reaction was carried out in a thermal cycler (TC-312, Techne) and the cycling temperature ramp comprised 95 °C for 3 min followed by 35 cycles at 94 °C for 60 s, 51 °C for 30 s and 72 °C for 2 min followed by 72 °C for another 10 min. Reaction products (25 μL) were electrophoresed through a 1.2% agarose/TAE gel (40 mM Tris acetate, 2 mM EDTA) and visualized with ethidium bromide staining.

5.2.3. Screening of potato tuber cDNA sub-libraries

cDNA sub-libraries were screened according to the protocol described by Israel with several modifications (Israel, 1993). Bacteria XL1-Blue MRF' and Uni-ZAP XR lambda vectors were grown in a 96-well plate in an 8 × 8 matrix in LB medium containing 10 mM $MgSO_4$. The culture was incubated for 6 h at 37 °C with continuous shaking at 250 rpm to allow amplification of phages. To prepare PCR templates, phages from 8 wells down each column (25 μL/well) were combined in 8 separate tubes. Probes were incubated for 10 min at 95 °C to allow denaturation of capsid proteins. 1 μL of prepared mixture served as a DNA template in PCR reaction, which was performed with forward and reverse primers under conditions described above. PCR products were electrophoresed through a 1.2% agarose/TAE gel and visualized with ethidium bromide staining to identify a column containing cDNA clones encoding potato apyrase (“positive column”). After that 8 separate PCR reactions were performed on each well from a “positive column”. 1 μL of 10-fold diluted culture from each well served as a DNA template. The electrophoresis of PCR products allowed identification of a single well containing cDNA clones encoding potato apyrase. Phages from positively identified well were tittered according to cDNA synthesis Kit instructions (Stratagene) and used to perform the second round of screening. The amount of phages cultured in 96-well plates was reduced during each round according to data depicted in Table 4.

The isolation of single clones was carried out after the fourth round of screening. Phages from a “positive well” were tittered and 10 single plaques were scored from the agar plate and transferred to a sterile microcentrifuge tube containing 250 μL of SM buffer (5.8 g of NaCl, 2 g of $MgSO_4 \cdot 7H_20$, 50 mL of 1 M Tris–HCl, pH 7.5, 5 mL of 2% gelatin (w/v)) and 10 μL of chloroform. Elution of phages was performed according to cDNA Synthesis Kit instructions (Stratagene). Ten separate PCR reactions were performed (under conditions described above) to identify a single cDNA clone encoding potato apyrase. 1 μL of 10-fold diluted phage obtained after elution served as a DNA template. Electrophoresis of PCR products allowed identification of a pure clone encoding potato apyrase.

5.2.4. In vivo excision of the pBluescript SK(-) phagemid and cDNA insert sequencing

Isolated Uni-ZAP XR vectors containing cDNA inserts encoding potato apyrase were converted to the pBluescript SK(-) phagemids using a filamentous helper phage and SOLR strain (cDNA Synthesis Kit, Stratagene). Phagemids were purified using GeneMATRIX Plasmid Miniprep DNA Purification Kit (EURx) and cDNA inserts were sequenced in Laboratory of DNA Sequencing and Oligonucleotides Synthesis (Polish Academy of Sciences, Poland).

Table 4
Number of phages amplified in each round of cDNA sub-library screening.

Screen cycle	Number of phage used to infection of bacteria XL1-Blue MRF' culture	Number of phage given to each well of microtiter plate	Dilution
I	3×10^6	~15625	–
I	150,000	~780	20×
III	7500	~40	20×
IV	375	~2	20×

5.3. Bioinformatic analysis

DNA chromatograms from sequenced cDNA inserts were assembled and analyzed using DNA Baser Assembler. Thus obtained consensus sequences were compared with sequences encoding plant, animal, yeast, and bacterial apyrases deposited in GenBANK using ClustalW program (Chenna et al., 2003). Phylogenetic analysis of the sequences was conducted using Neighbor Joining method. Identified sequences were also analyzed at the amino acid level. Physicochemical properties of encoded proteins were determined using ProtParam tool available in open source package Expasy (www.expasy.org). Transmembrane domain and potential cleavage sites prediction were performed on the SOSUI server (http://bp.nuap.nagoya-u.ac.jp/sosui/sosui_submit.html) and using PeptideCutter tool (www.expasy.org), respectively (von Heijne, 1986; Gasteiger et al., 2003).

5.4. Purification and N-terminal sequencing of commercial soluble potato ATP-diphosphohydrolase (Sigma)

The purification of commercial potato ATP-diphosphohydrolase (Sigma) was carried out using non-denaturing polyacrylamide gel electrophoresis and electroelution. The protein sample was run through 7.5% polyacrylamide gel at a constant voltage of 100 V. After that proteins were electroeluted from the excised gel slices. The recovered protein samples demonstrating ATPase and ADPase activities were concentrated by ultrafiltration in preparation to N-terminal sequencing. N-terminal protein sequence analysis was performed at BioCentrum Ltd. (Kraków, Poland). The sequentially detached phenylthiohydantoin derivatives of amino acids were identified using Procise 491 – automatic sequence analysis system working according to a standard protocol of manufacturer (Applied Biosystems, Foster City, CA, USA). As result of N-terminal sequencing of homogenous soluble potato ATP-diphosphohydrolase we obtained QIPLRR sequence. This specific sequence was found after signal and transmembrane sequences in all proteins encoded by isolated genes (except StAPY6) (Fig. 2). Two NTPDases previously isolated from potato tubers are soluble enzymes (Handa and Guidotti, 1996; Kettlun et al., 1982). Therefore, isolated potato NTPDase genes were prepared without signal and transmembrane regions for bacterial overexpression.

5.5. Overexpression of S. tuberosum apyrases

Three (*StAPY4*, *StAPY5*, *StAPY6*) of seven (*StAPY4–StAPY10*) *in silico* analyzed cDNA sequences were expressed in bacteria expression system. *StAPY4*, *StAPY5* and *StAPY6* sequences encode proteins which differ in: a/molecular mass, b/isoelectric point, c/identity level between themselves and three potato NTPDases identified till now, and d/composition of different amino acids in the transmembrane domain. Each of chosen sequences was cloned into pET32a vector between the EcoRI and XhoI sites in order to fuse the sequence to the thioredoxin-tag, 6xHis-tag and S-tag sequences. Recombinant plasmid pET32a was transformed to *E. coli* BL21 CodonPlus (DE3) bacteria cells using heat shock method. A single colony was used to inoculate LB medium supplemented with ampicillin (50 μg/mL), chloramphenicol (34 μg/mL) and 0.5% glucose. The culture was incubated overnight at 37 °C with shaking. Next day bacteria were transferred to the fresh medium with above components and grown in the same conditions. When an OD_{600} of the culture reached approximately 0.5, the cells were induced to synthesis of apyrases by addition of IPTG (1 mM) and the

cultures were incubated for 3 h. After that the bacterial cultures were cooled on ice and harvested by centrifugation for 15 min at 10000xg. The bacteria pellet was resuspended in 25 mM Tris–Cl, pH 7.0 buffer and stored at −20 °C.

5.6. Electrophoresis under denaturing conditions

SDS–PAGE was performed using Ogita – Markert method (Ogita and Markert, 1979). Proteins were incubated for 5 min at 95 °C in buffer containing 4% SDS and 10% β-mercaptoethanol. Prepared samples were run through 4% stacking gel and 10% separating gel at a constant voltage of 100 V.

5.7. Catalytic activity assay

Activity of expressed enzymes was determined using modified Fiske-Subbarow method as described by Komoszyński and Skalska (1990). Apyrase was incubated with substrate (2 mM ATP or ADP) in the presence or lack of divalent metal ions (3 mM $MgCl_2$, 3 mM $CaCl_2$ or 10 mM EDTA). Nucleotidase activity was measured on the basis of the amount of inorganic phosphate released during the reactions. Additionally, nucleotide products of the reaction were analyzed using HPLC method (Czarnecka et al., 2005). All experiments were repeated minimum of three times, and qualitatively identical results were obtained.

5.8. Refolding and purification of StAPY6

5.8.1. Inclusion bodies isolation

Bacterial pellet was suspended in 50 mM HEPES buffer containing 5% glycerol at pH 6.5 (buffer A) and sonicated three times, each for 20 s. The lysate was centrifuged for 15 min at 25,000g, 4 °C. Supernatant was discarded and pellet containing inclusion body proteins was washed with buffer A containing 0.5% and 1% sodium sarcosine.

5.8.2. Refolding of StAPY6 from inclusion bodies

Inclusion bodies were dissolved in 50 mM Tris–HCl pH 6.5 buffer containing 6 M guanidine hydrochloride and 10 mM β-mercaptoethanol at protein concentration 1.5 mg/mL. Denaturated proteins were diluted 20 times with buffer A containing 20% glycerol at 0 °C and then incubated for 72 h at 13 °C. The sample was dialyzed against buffer A for 16–20 h at 4 °C. Proteins were concentrated by ultrafiltration and stored in buffer A containing 40% glycerol at −20 °C.

5.8.3. Purification of StAPY6

Refolded proteins from inclusion bodies were applied on Sephadex G75 column (750 × 8 mm). 1 mL fractions were collected. Fractions containing ATPase and ADPase activity were concentrated by ultrafiltration.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2013.03.014.

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