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Phytochemistry 67 (2006) 1476–1485
PHYTOCHEMISTRY
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Calcium-stimulated guanosine–inosine nucleosidase from yellow lupin (*Lupinus luteus*)

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Received 3 February 2006; received in revised form 15 May 2006
Available online 3 July 2006

Abstract

Guanosine–inosine-preferring nucleoside *N*-ribohydrolase has been purified to homogeneity from yellow lupin (*Lupinus luteus*) seeds by ammonium sulfate fractionation, ion-exchange chromatography and gel filtration. The enzyme functions as a monomeric, 80 kDa polypeptide, most effectively between pH 4.7 and 5.5. Of various mono- and divalent cations tested, Ca^{2+} appeared to stimulate enzyme activity. The nucleosidase was activated 6-fold by 2 mM exogenous $CaCl_2$ or $Ca(NO_3)_2$, with $K_a = 0.5$ mM (estimated for $CaCl_2$). The K_m values estimated for guanosine and inosine were 2.7 ± 0.3 μM. Guanosine was hydrolyzed 12% faster than inosine while adenosine and xanthosine were poor substrates. 2′-Deoxyguanosine, 2′-deoxyinosine, 2′-methylguanosine, pyrimidine nucleosides and 5′-GMP were not hydrolyzed. However, the enzyme efficiently liberated the corresponding bases from synthetic nucleosides, such as 1-methylguanosine, 7-methylguanosine, 1-N^2-ethenoguanosine and 1-N^2-isopropenoguanosine, but hydrolyzed poorly the ribosides of 6-methylaminopurine and 2,6-diaminopurine. $MnCl_2$ or $ZnCl_2$ inhibited the hydrolysis of guanosine with $I_{50} \approx 60$ μM. Whereas 2′-deoxyguanosine, 2′-methylguanosine, adenosine, as well as guanine were competitive inhibitors of this reaction (K_i values were 1.5, 3.6, 21 and 9.7 μM, respectively), hypoxanthine was a weaker inhibitor ($K_i = 64$ μM). Adenine, ribose, 2-deoxyribose, 5′-GMP and pyrimidine nucleosides did not inhibit the enzyme. The guanosine–inosine hydrolase activity occurred in all parts of lupin seedlings and in cotyledons it increased up to 5-fold during seed germination, reaching maximum in the third/fourth day. The lupin nucleosidase has been compared with other nucleosidases.

Keywords: *Lupinus luteus* L.; Fabaceae; Yellow lupin; Guanosine–inosine nucleosidase; Enzyme purification; Calcium as the enzyme stimulator; Purine metabolism in plants

1. Introduction

Among the enzymes involved in purine salvage are purine nucleoside phosphorylases (EC 2.4.2.1), found in animals, some bacteria (Bzowska et al., 2000) and parasitic protozoa (Miller et al., 1984; Estupiñán and Schramm, 1994), and purine nucleoside hydrolases found in plants (Guranowski and Schneider, 1977; Chen and Kristopeit, 1981; Le Floc'h and Lafleuriel, 1981; Guranowski, 1982; Stasolla et al., 2003), some protozoa (Miller et al., 1984) and some fungi (Abdel-Fatah et al., 2003). These enzymes release adenine, guanine and hypoxanthine from the corresponding nucleosides: adenosine, guanosine, and inosine, respectively. Moreover there exist specific phosphorylases (EC 2.4.2.28) in animals (Subhi et al., 2003), fungi (Guranowski and Paszewski, 1982; Marchitto and Ferro, 1985), archaebacteria (Appleby et al., 2001; Cacciapuoti et al., 2003) and protozoa (Miller and Toorchen, 1988), as well as specific hydrolases (EC 3.2.2.16) in plants (Guranowski et al., 1981) and bacteria (Gianotti et al., 1990; Sekowska and Danchin, 1999) that release adenine from 5′-deoxy-5′-methylthioadenosine. The liberated bases can be utilized by the adenine- and guanine/hypoxanthine phosphoribosyltransferases for the synthesis of corresponding nucleotides; 5′-AMP and 5′-GMP or 5′-IMP, respectively. Moreover, the nucleic bases, as smaller and less polar molecules than their pertinent nucleosides, can be easily trans-

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doi:10.1016/j.phytochem.2006.05.021

ported from such a storage organ as cotyledon to other fast growing organs of the developing seedling: hypocotyl, root and leaves. Generally, the catabolism of nucleosides in higher plants has been studied less than in other phyla. For a quarter of a century, many enzymes involved in the metabolism of purine nucleotides and nucleosides in the yellow lupin (*Lupinus luteus*) have been studied in this laboratory. Among these are three specific hydrolases: methylthioadenosine nucleosidase (Guranowski et al., 1981) and inosine nucleosidase (EC 3.2.2.2) (Guranowski, 1982) found in seeds, and adenosine nucleosidase (EC 3.2.2.7), detected in the cotyledons only during seed germination (Guranowski and Pawełkiewicz, 1978). Yellow lupin seeds are a rich source of many enzymes involved in the metabolism of nucleotides and nucleosides and meal of the seeds is a convenient material for the enzyme isolation. It has low fat content and no tannins or other secondary metabolites interfering in the purification.

The lupin inosine nucleosidase, tested with 0.5 mM concentration of potential substrates, catalyzed the hydrolysis of adenosine 10-fold, and guanosine *100-fold* more slowly than the cleavage of inosine (Guranowski, 1982). For this reason, we wondered if lupin seeds possess another enzyme that can effectively hydrolyze guanosine. Using a low pH buffer in the reaction mixture, we have found a new guanosinase activity in low-ionic-strength extracts of yellow lupin seed meal. Electrophoretically homogeneous protein was produced using conventional purification techniques and during enzyme characterization we serendipitously noticed that Ca^{2+} was the only cation to stimulate the hydrolysis of guanosine to guanine and ribose. This unusual stimulation, previously unreported for any other nucleosidase, prompted our further study of this enzyme, the first homogeneous plant guanosine–inosine-preferring nucleosidase to be described. In this paper we present, in addition to the purification procedure and the effect of calcium, general characterization of the lupin enzyme and compare it with other purine nucleosidases.

2. Results and discussion

2.1. Purification of guanosine–inosine nucleosidase from yellow lupin seeds

The purification procedure was carried out at 4 °C. Yellow lupin seed meal (0.5 kg) was extracted with 1.5 l of buffer A (10 mM potassium phosphate, pH 6.8, containing 5% glycerol and 1 mM 2-mercaptoethanol) for 60 min and the slurry centrifuged (20,000*g* for 20 min). The guanosinase was precipitated from the supernatant (crude extract) with ammonium sulfate (50–70% saturation). The protein precipitate was suspended in buffer A, dialyzed against this buffer and insoluble material removed by centrifugation. Clear supernatant was applied on to a DEAE-Sephacel column (5 × 35 cm) equilibrated with buffer A. The column was washed with 3.5 l of buffer A and a linear gradient (0–0.5 M KCl in buffer A, total 7 l) was applied. The nucleosidase eluted at 80–120 mM KCl. It was precipitated with ammonium sulfate (70% saturation), dissolved in a small volume of buffer B (50 mM potassium phosphate, pH 6.8, containing 5% glycerol and 1 mM 2-mercaptoethanol) and chromatographed on a Sephadex G-100 column (2.6 × 90 cm) equilibrated with buffer B. The hydrolase eluted at $V_e/V_0 = 1.15$. The preparation was kept frozen (−20 °C) and used for further characterization. A summary of the purification is given in Table 1.

2.1.1. Comments on the purification

In preliminary experiments it was found that the concentration of potassium phosphate buffer had no effect on the extraction of the guanosine–inosine nucleosidase from lupin seed meal. The rationale for checking if the ionic strength of the extraction buffer affected the yield of nucleosidase was based on two earlier observations: first, that the higher the concentration of extraction buffer was, the higher the protein concentration in the extract, and second, that some enzymes are extracted only at appropriately high ionic strength (Jakubowski and Pawełkiewicz, 1974). Thus, enzymes can be selectively extracted from lupin seed meal. In the case of the guanosine–inosine nucleosidase we got higher specific activity at low (10 mM) than at higher (20, 50 or 100 mM) buffer concentrations. Therefore, this enzyme was routinely extracted with 10 mM potassium phosphate, pH 6.8. After three conventional purification steps, highly purified (over 1700-fold) enzyme protein was obtained with a reasonable 6.3% yield relative to the crude extract. Particularly useful was the gel-filtration step, which separated the peak of the enzyme activity from several larger species and from the majority of proteins of lower molecular mass (Fig. 1). The final preparation appeared at least 95% pure according to SDS–polyacrylamide gel electrophoresis (Fig. 2). Other chromatographic steps, such as hydroxyapatite and dye-linked agaroses, did not augment the purity of the nucleosidase preparation.

Table 1
Purification of guanosine–inosine nucleosidase from meal of yellow lupin seeds (0.5 kg)

Step	Total volume (ml)	Total activity (pkat[a])	Total protein (mg)	Specific activity (pkat/mg)	Yield (%)
Crude extract	1110	16,600	63,825	0.32	100.0
Ammonium sulfate (50–70%)	55	3520	1403	2.56	21.2
DEAE-Sephacel	688	3040	140	21.76	18.3
Sephadex G-100	35	1056	1.9	555.68	6.3

[a] 1 pkat is the amount of enzyme that converts 1 pmol of guanosine per second.

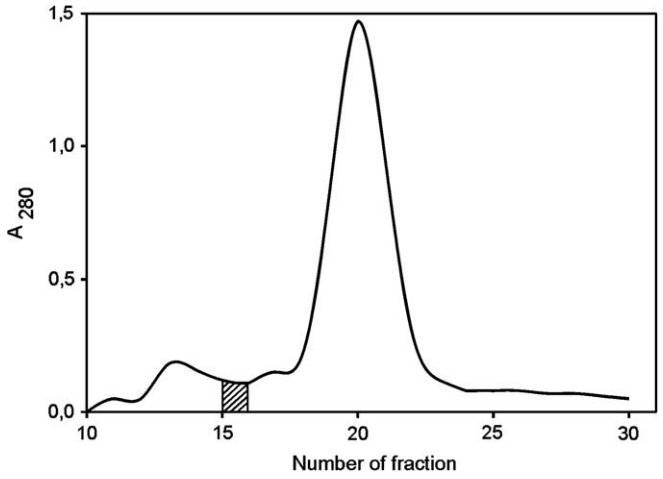

Fig. 1. Chromatography of the lupin guanosine–inosine nucleosidase on Sephadex G-100. Resolubilized ammonium sulfate precipitate of the active fractions eluted from DEAE-Sephacel step was applied to the Sephadex column (2.5 × 88 cm) equilibrated with 50 mM potassium phosphate buffer (pH 6.8) containing 5% glycerol, and 5-ml fractions were collected. Active fractions are depicted by the shaded area.

2.2. Purity, molecular mass and subunit structure of the nucleosidase

When the purified enzyme (peak fraction) was subjected to disc electrophoresis in a 7% polyacrylamide gel (Davis, 1964) and stained with Coomassie Brilliant Blue, a single band was observed that migrated between the monomers and dimers of bovine serum albumin (not shown). On SDS gels (Laemmli, 1970), the denatured enzyme yielded a band of 80 kDa (Fig. 2). Since the molecular mass of the native enzyme estimated by gel filtration on a Superdex 200 column was also 80 kDa, one can conclude that the lupin nucleosidase functions as a single polypeptide chain. The standards used for calibration of the latter column were: dimer (134 kDa) and monomer (67 kDa) of bovine serum albumin, inosine nucleosidase from yellow lupin seeds (62 kDa) (Guranowski, 1982) and diadenosine tetraphosphatase (18 kDa) (Jakubowski and Guranowski, 1983). In comparison with other purine nucleosidases, the lupin guanosine–inosine nucleosidase is relatively large. Moreover, the other lupin nucleosidases, such as methyl-thioadenosinase (Guranowski et al., 1981) and adenosinase (Abusamhadneh et al., 2000), differ from the guanosine–inosine nucleosidase in that they are homodimers.

The yellow lupin guanosine–inosine nucleosidase is the first plant guanosine–inosine-preferring glycohydrolase to be purified to homogeneity. Molecular activity calculated from the data shown in Table 1 is very low; 0.044 s^{-1}. The preparation of the lupin enzyme was free of other nucleoside- or nucleotide-metabolizing enzymes. Another plant nucleosidase with this substrate specificity was partially purified from Jerusalem artichoke but its molecular mass was not determined (Le Floc'h and Lafleuriel, 1981). So far, the only other report of a homogeneous guanosine–inosine nucleosidase is of an enzyme purified from the protozoan *Crithidia fasciculata*. Its molecular activity was 234 s^{-1} (Estupiñán and Schramm, 1994).

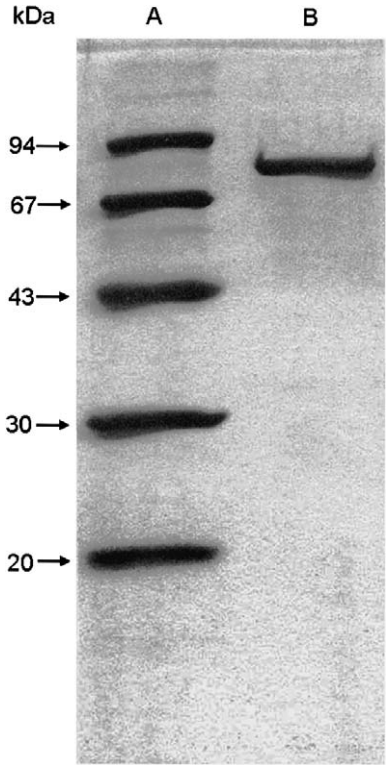

Fig. 2. Sodium dodecyl sulfate-gel electrophoresis of yellow lupin guanosine–inosine nucleosidase, Sephadex G-100 fraction. Electrophoresis was performed according to Laemmli (1970) and the gel stained with Coomassie Brilliant Blue. (A) Standard proteins from top to bottom are: phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa) and soybean trypsin inhibitor (20 kDa). (B) Yellow lupin guanosine–inosine nucleosidase (peak fraction of the Sephadex G-100 step).

2.3. Characterization of reaction products

With guanosine, inosine, adenosine and other nucleosides as substrates, one of the two products of enzymatic degradation was the corresponding purine base. When analyzed by TLC, it co-migrated with the reference base, e.g. guanine, hypoxanthine or adenine, the base always having a higher R_f value than the parent nucleoside (see Fig. 3). The other product co-migrated with a ribose standard and gave the yellow color typical of a reducing sugar when complexed with copper ions and 2,9-dimethyl-1-10-phenanthroline (Perkin, 1996).

2.4. Effect of pH

The rate of guanosine hydrolysis was measured in the following 100 mM buffers: sodium acetate, potassium phosphate, Mes/KOH and Ches/KOH, covering the pH range from 2.7 to 9.1. The velocity versus pH dependence exhibited a symmetrical bell-shaped curve. Optimal activity

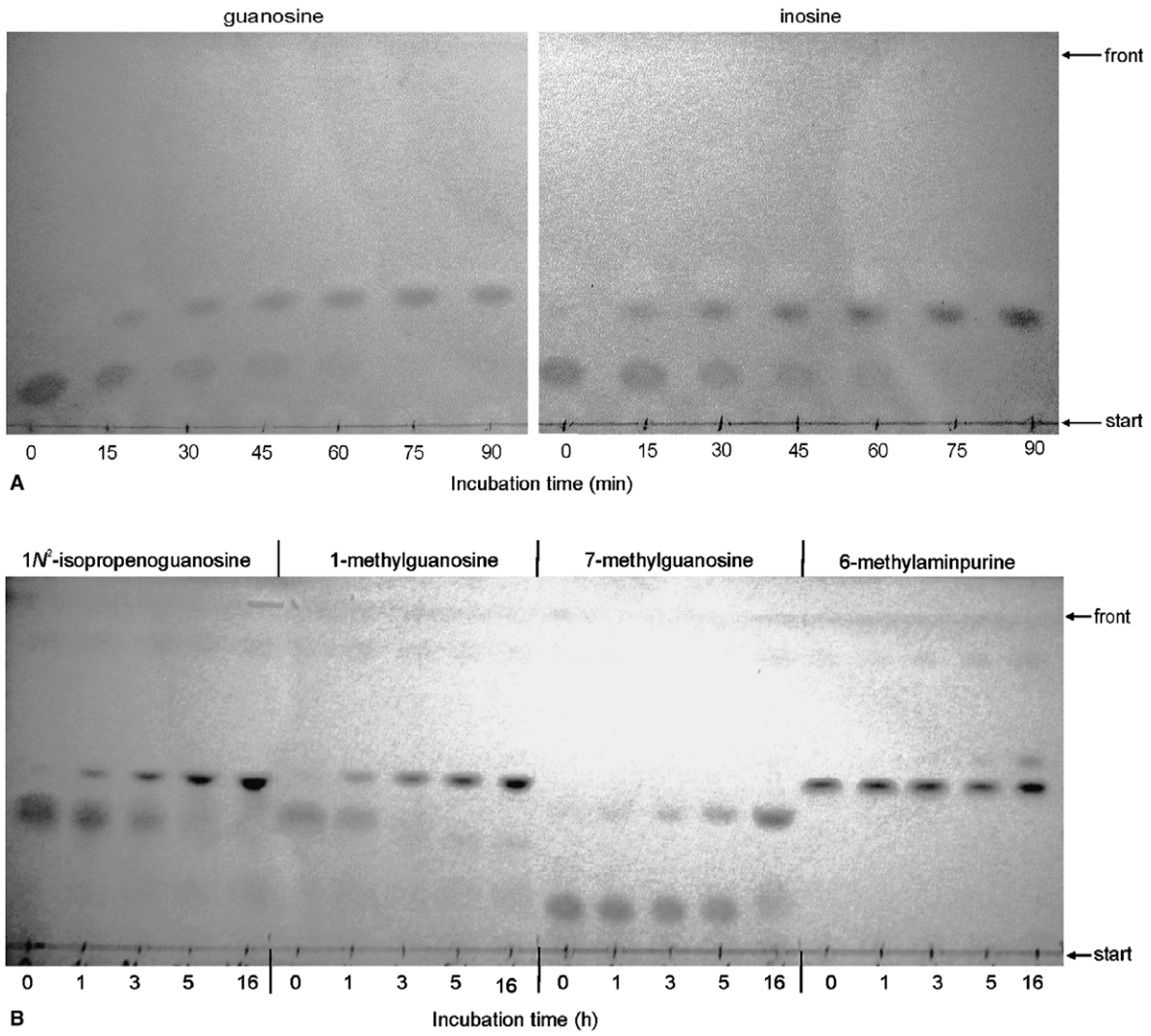

Fig. 3. Hydrolysis of different nucleosides by the yellow lupin guanosine–inosine nucleosidase, demonstrated on thin layer chromatograms. Indicated substrates (1 mM) were incubated as described under Enzyme assays. At the indicated times, 3 μl aliquots were spotted on to silica gel plates containing fluorescent indicator, developed for 60 min in ethyl acetate:propan-2-ol:ammonia;water (27:23:5:3, by volume) and photographed under short-wave UV light. (A) shows the hydrolysis of the preferred substrates and (B) of the substrates which are hydrolyzed at lower rates than guanosine or inosine.

was obtained between pH 4.7 and 5.5, with half maximal activity at pH 4.3 and 6.4. Sodium acetate buffer, pH 4.75, was routinely used in reaction mixtures.

Acidic pH optima were previously reported for the adenosine nucleosidases from wheat germ (Chen and Kristopeit, 1981), Jerusalem artichoke shoots (Le Floc'h and Lafleuriel, 1981), and spinach beet (Poulton and Butt, 1976), barley (Guranowski and Schneider, 1977), tea (Imagawa et al., 1979), tomato (Burch and Stuchbury, 1986) and coffee-tree (*Coffea arabica*) leaves (Campos et al., 2005). Also, the adenosine nucleosidase isolated by us from the cotyledons of 5-day yellow lupin seedlings according to the procedure of Abusamhadneh and co-workers (2000) had a definite acidic pH optimum rather than the optimum at pH 7.5 reported by these authors. The pH optima estimated for two other purine nucleosidases, methylthioadenosinase (Guranowski et al., 1981) and inosinase (Guranowski, 1982) were 8.0–8.5 and 8.0 respectively.

2.5. Effects of metal cations

Chlorides of monovalent (Li^+, Na^+, K^+, NH_4^+) and divalent (Mg^{2+}, Ca^{2+}, Co^{2+}, Mn^{2+}, Zn^{2+}) cations were tested as effectors of the hydrolase. Of these, Ca^{2+} alone appeared to stimulate the hydrolysis of guanosine though the enzyme was also active in the presence of 1 mM EDTA. The effect of Ca^{2+} depended upon the cation concentration, maximum stimulation (over 5-fold) being reached at 2 mM, with no further change up to 10 mM $CaCl_2$ (Fig. 4). The calculated activation constant K_a was 0.5 mM. $Ca(NO_3)_2$ exerted the same effect as $CaCl_2$. Calcium also stimulated the enzymatic hydrolysis of other nucleosides (see below). After recognition of its stimulatory effect, we incorporated 2 mM $CaCl_2$ in all reaction mixtures used for further characterization of the enzyme.

To the best of our knowledge, the effect of metal cations on other purine nucleosidases has either not been tested or, as reported for the barley adenosine nucleosidase

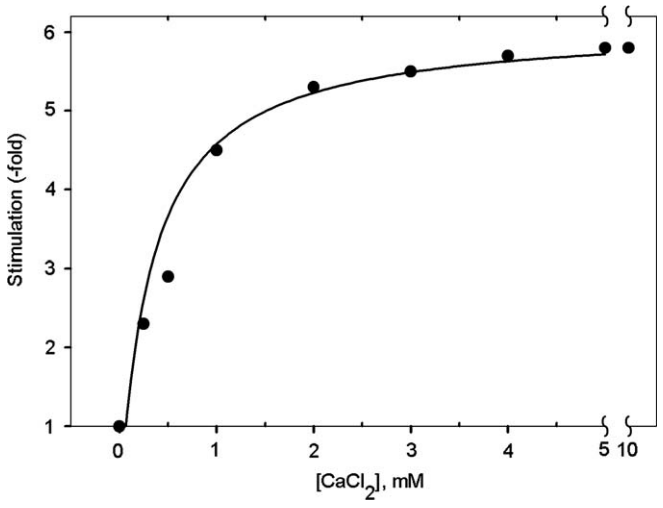

Fig. 4. Stimulation of the hydrolysis of guanosine catalyzed by the yellow lupin guanosine–inosine nucleosidase by calcium chloride.

(Guranowski and Schneider, 1977) and the adenosine and guanosine–inosine nucleosidases from Jerusalem artichoke (Le Floc'h and Lafleuriel, 1981), these cations exerted no effects on catalysis. During this study we also tested the effect of calcium on the lupin adenosine-, inosine- and methylthioadenosine nucleosidases and none of them was affected by 5 mM $CaCl_2$. Although the observed calcium stimulation has not been reported for any other nucleosidase, it is worth noting that tightly bound Ca^{2+} has been identified as a catalytic site ligand of the nonspecific nucleosidase from *C. fasciculata* (Degano et al., 1998). Whether this is mere coincidence or whether Ca^{2+} is always involved in a mechanism common to different nucleosidases requires further investigation.

Of the cations investigated, Mn^{2+} and Zn^{2+} appeared to act as potent inhibitors of the lupin guanosine–inosine hydrolase activity with estimated I_{50} values of 60 μM for each of them (not shown).

2.6. Substrate specificity

Of the natural nucleosides, the lupin nucleosidase only hydrolyzed inosine to any extent in addition to guanosine (Fig. 3A). Adenosine and xanthosine were poor substrates. Using tritium-labeled guanosine, inosine or adenosine at 0.1 mM, we compared the rates of hydrolysis of these nucleosides. Inosine was hydrolyzed only slightly (12%), and adenosine over 50-fold more slowly than guanosine. The enzyme did not release the corresponding base from 2′-deoxyguanosine, 2′-deoxyionosine, 5′-GMP or from pyrimidine nucleosides, such as uridine, cytidine, thymine riboside or 2′-thymine deoxyriboside (thymidine). Of different synthetic nucleosides tested, 1-methylguanosine, 7-methylguanosine, 1-N^2-ethenoguanosine and 1-N^2-isopropenoguanosine were quite good (Fig. 3B), and the ribosides of 6-methylaminopurine and 2,6-diaminopurine rather poor substrates. Purine riboside (nebularine) and 2′-methylguanosine were refractory to enzymatic hydrolysis. Nucleosides that have been demonstrated to be substrates of the lupin guanosine–inosine hydrolase are shown in Fig. 5. The general conclusion of these studies

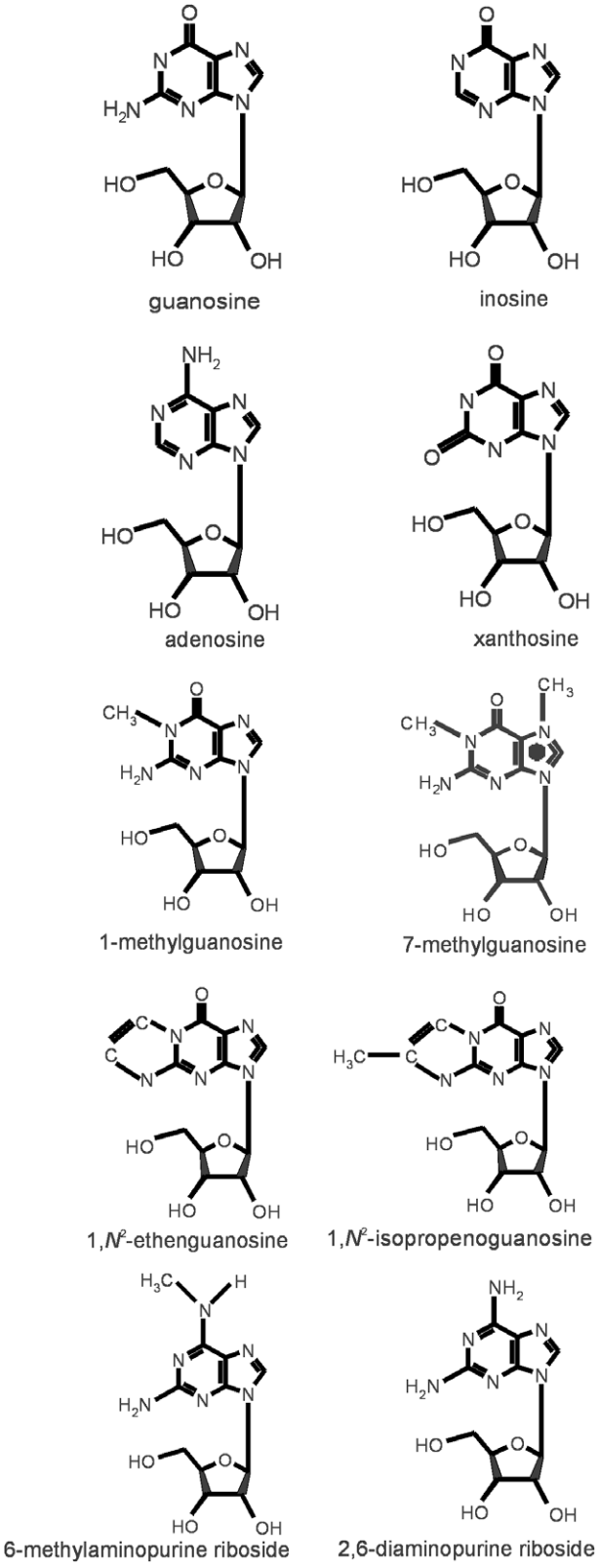

Fig. 5. Structures of the nucleosides that are substrates of the yellow lupin guanosine–inosine nucleosidase.

is that the preferred substrates are ribosides of 6-oxopurines. Even small modifications of the substrate (guanosine) in its sugar moiety, such as substitution of one hydrogen with a methyl group (2′-methylguanosine), deletion of oxygen (2′-doxyguanosine) or addition of a phosphate group (5′-GMP), are not tolerated by the enzyme. With respect to substrate specificity the lupin enzyme resembles mostly the guanosine–inosine nucleosidase from *C. fasciculata* (Estupiñán and Schramm, 1994) for which these two nucleosides were definitely the preferred substrates.

No other lupin enzyme exhibits such a strong preference for guanosine as the guanosine–inosine nucleosidase. Comparative studies performed earlier at 0.5 mM concentration of the potential substrates showed that the inosine nucleosidase partially purified from yellow lupin seeds hydrolyzed guanosine 100-fold more slowly than inosine (Guranowski, 1982). The lupin adenosine nucleosidase isolated by us according to the method of Abusamhadneh and co-workers (2000) did not degrade guanosine even during prolonged incubation. We should mention here that this result contrasts with that reported by those authors who found that their preparation of yellow lupin adenosinase hydrolyzed guanosine 4-fold and inosine 14-fold more slowly than adenosine. This discrepancy can be explained by contamination of the latter adenosinase preparation isolated from 4-day-old seedling cotyledons by the guanosine–inosine hydrolase whose occurrence has been demonstrated by us in those plant organs and which reaches the highest activity just in that developmental stage (see below).

2.7. Michaelis constants

Similar K_m values for guanosine and inosine of 2.7 ± 0.3 μM were estimated under optimal conditions, i.e. in 0.1 M sodium acetate buffer (pH 4.75) containing 2 mM $CaCl_2$ and labeled nucleoside at concentrations between 1 and 12 μM. In this respect the lupin nucleosidase resembles most closely its counterpart from Jerusalem artichoke for which the K_m constants were 8.5 μM for guanosine and 2.5 μM for inosine. The corresponding values estimated for the *C. fasciculata* nucleosidase were 77 μM and 16 μM (Estupiñán and Schramm, 1994), for the nucleosidase from *T. cruzi* 18 and 13 μM (Miller et al., 1984) and for the enzyme from *T. brucei brucei* 46 and 18 μM (Parkin, 1996). The K_m values of other plant nucleosidases for their preferred substrates are summarized in Table 2.

2.8. Inhibition by some nucleosides and nucleic bases

Among various compounds tested as potential inhibitors of guanosine hydrolysis, all the aforementioned pyrimidine nucleosides and GMP had no effect on the reaction but nucleosides that differ only slightly from the preferred substrates of the enzyme, such as 2′-deoxyguanosine, 2′-methylguanosine and adenosine, did inhibit. These compounds acted as competitive inhibitors with estimated K_i values of 1.5, 3.6 and 21 μM, respectively. Of the reaction products, ribose was not inhibitory (neither was 2-deoxyribose) but guanine inhibited the reaction with $K_i = 9.7$ μM. Interestingly, hypoxanthine was a less potent inhibitor ($K_i = 64$ μM). Also, when we tested guanine and hypoxanthine as potential inhibitors of the hydrolysis of inosine, the former base was a moderate inhibitor ($K_i = 29$ μM) while the latter practically did not inhibit. These data suggest that a natural nucleoside such as 2′-deoxyguanosine may control the turnover of guanosine preventing its degradation to free base and ribose. This inhibitory nucleoside apparently binds strongly to the enzyme active site via its guanine moiety and, not being converted, occupies it for a longer time than would deoxyribose and guanine.

Table 2
Kinetic and molecular properties of plant hydrolases that degrade purine nucleosides

Enzyme (EC number)	Source	K_m (μM), substrate	pH optimum	Molecular mass (kDa), (subunit structure)	References
Inosine nucleosidase (EC 3.2.2.2)	Yellow lupin seeds	65, Inosine	8	62, (n.d.)	Guranowski (1982)
Adenosine nucleosidase (EC 3.2.2.7)	Barley leaves	0.8–2.3, Adenosine	4.7–5.4	66, (dimer)	Guranowski and Schneider (1977)
	Wheat germs	1.4, Adenosine	4.7	68, (n.d.)	Chen and Kristopeit (1981)
	Yellow lupin seedling cotyledons	4.8, Adenosine	7.5	72, (dimer)	Abusamhadneh et al. (2000)
	Coffea arabica young leaves	6.3, Adenosine	6	72, (dimer)	Campos et al. (2005)
	Yellow lupin seedling cotyledons	n.d.	5	n.d.	Szuwart et al. (this paper)
Methylthioadenosine nucleosidase (EC 3.2.2.9)	Yellow lupin seeds	0.4, Methylthioadenosine	8–8.5	62, (dimer)	Guranowski et al. (1981)
Guanosine–inosine nucleosidase (EC does not exist)	Yellow lupin seeds	2.7, Guanosine, inosine	4.7–5.5	80, (monomer)	Szuwart et al. (this paper)

n.d., not determined.

2.9. Activity of guanosine–inosine nucleosidase in extracts from yellow lupin cotyledons during seedling development and in extracts from other parts of the seedling

Development of yellow lupin seedlings grown at room temperature under natural light conditions is shown in Fig. 6B. Enzyme activity was tested in the optimal reaction mixture (0.1 M sodium acetate buffer, pH 4.75, 2 mM $CaCl_2$, 0.1 mM [^{3}H]guanosine) incubated with equal aliquots of the extracts prepared from yellow lupin cotyledons, collected first after 4 h and then every day of seed germination, and the extracts of 6-day-old seedling hypocotyls, roots and emerging leaves. As shown in Fig. 6A, the level of the nucleosidase activity increased dramatically from 122 pkat per pair of cotyledons removed from the water imbibed seed up to 620 pkat per pair of cotyledons from a 3-day-old seedling. The activity then declined, approaching the level of 147 pkat per pair of cotyledons at the seventh day of germination. This profile of guanosine nucleosidase activity in lupin seedling cotyledons differs from that reported earlier for the adenosine nucleosidase. The latter is absent in seeds, appears only

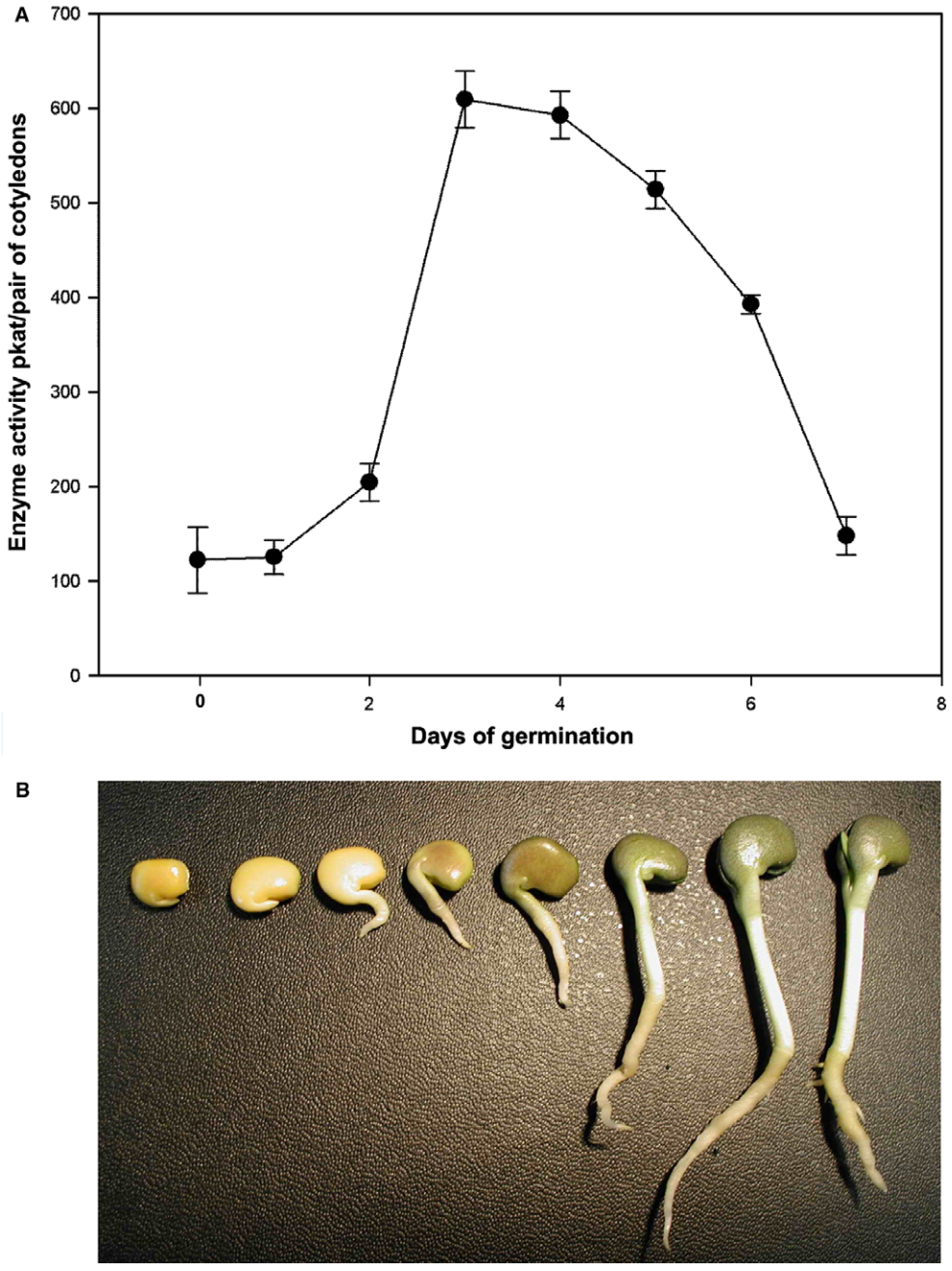

Fig. 6. Changes in the activity of guanosine nucleosidase in the cotyledons during first week of their development (A) and the yellow lupin seedlings during first week of germination (B). (Brownish-grey black-spotted shells that cover the cotyledons up to the fifth day of germination have been removed.)

in the cotyledons of 2-day-old seedlings and then increases dramatically reaching more than 22 nkat per pair of cotyledons (Guranowski and Pawełkiewicz, 1978). Two other purine nucleosidases, inosine (Guranowski, 1982) and methylthioadenosine (Guranowski et al., 1981) nucleosidases, are present both in dry yellow lupin seeds and in the seedling cotyledons but changes in their activities during germination have not been determined. We have estimated also total activity of the guanosine nucleosidase in other parts of yellow lupin seedlings. Whereas pair of cotyledons of 6-day-old seedling contains 415 pkat of the nucleosidase, the hypocotyls, root and emerging leaves have 21.6, 18.2 and 3.3 pkat, respectively. (The values are means of three independent estimations; standard errors did not exceed 10%).

Since the guanosine–inosine nucleosidase occurs in different plant tissues and at different developmental stages, it can be considered as a house-keeping enzyme. Its 5-fold increase in the cotyledons during seedling development reflects acceleration of basic metabolism in these storage organs at that stage of plant ontogenesis.

3. Concluding remarks

This work extends our knowledge of purine metabolism in higher plants. Basic molecular and kinetic properties of the most thoroughly studied plant purine-nucleoside hydrolases are summarized in Table 2.

Our data suggest that the guanosine–inosine nucleosidase contributes to liberation of guanine from guanosine that originates from different guanine nucleotides which ceased their functioning as building blocks of nucleic acids or coenzymes. Then, the free base (guanine) can be easily transported from cotyledons to other developing plant organs and reutilized there by the guanine/hypoxanthine phosphoribosyltransferase (EC 2.4.2.8). The latter enzyme has been demonstrated in yellow lupin seeds (Guranowski and Barankiewicz, 1979). Inosine nucleosidase also present in these seeds barely hydrolyzes guanosine (Guranowski, 1982). Thus it has to be the guanosine–inosine nucleosidase that plays the main role in releasing of guanine.

The simple purification procedure developed here has allowed a homogeneous preparation of plant guanosine–inosine hydrolase to be obtained for the first time. Generation of larger amounts of enzyme may be necessary for those who would like to pursue an investigation into the stimulatory effect of Ca^{2+} observed during this study and which, to the best of our knowledge, has not been reported for any other combination of nucleosidase and cation.

Calcium is an essential plant nutrient and well known intracellular messenger (White and Broadley, 2003). The cytosolic [Ca^{2+}] is a pivotal element of complex network of plant cell signal transduction. As has been noticed, calcium signaling involves a separation of different concentrations of Ca^{2+} by membranes, and the signals must be regulated (Buchanan et al., 2000). Whereas cells maintain very low resting concentrations of cytosolic Ca^{2+} (100–200 nM), cell wall, mitochondria, chloroplast, and even the nucleus may store Ca^{2+}, and its concentration may vary from 0.1 to 1 mM. In plant vacuoles [Ca^{2+}] can reach 10 mM. Thus one can speculate that the lupin guanosine–inosine nucleosidase functions most effectively in the vacuole; firstly because of its low-pH optimum, and secondly due to millimolar calcium concentration occurring in that cell compartment.

4. Experimental

4.1. Plant material

Seeds of yellow lupin (*L. luteus* var. Juno) were purchased from The Plant Breeding Station in Wiatrowo (near Poznań). Before extraction, the seeds were pulverized. Germination of the seeds was performed on wet cellulose wool under natural light conditions at room temperature. The extracts of seedling cotyledons and other organs of 6-day-old seedling were obtained with the use of an Omnimixer in 10 mM potassium phosphate buffer containing 5% glycerol and 1 mM EDTA.

4.2. Enzymes

The purification of the guanosine–inosine nucleosidase has been described in Section 2. The methylthioadenosine nucleosidase (Guranowski et al., 1981) and inosine nucleosidase (Guranowski, 1982) were purified from the meal of yellow lupin seeds as described previously. Adenosine nucleosidase from yellow lupin seedlings was purified as described by Abusamhadneh and co-workers (2000). Adenosine deaminase from calf intestine, which was used for the conversion of labeled adenosine into inosine, was from Sigma (St. Louis, MO, USA).

4.3. Chemicals

Unlabelled common nucleosides, nucleic acid bases, ribose and 2-D-deoxyribose were from Sigma. The other guanosine analogues were kindly donated by Professor Jerzy Boryski (Institute of Bioorganic Chemistry, Polish Academy of Sciences, Poznań, Poland). Radioactive [8-^{3}H]guanosine (15 Ci/mmol) was from Moravek Biochemicals, Brea CA, USA and [2,8-^{3}H]adenosine (40 Ci/mmol) was from MP Biomedicals, Inc., Irvine, CA, USA. Labelled inosine was obtained by enzymatic conversion of the [^{3}H]adenosine with adenosine deaminase.

4.4. Chromatographic systems

Gel filtration was performed either on Sephadex G-100 or Superdex 200 and ion-exchange chromatography on DEAE-Sephacel, which were purchased from Pharmacia. TLC was performed on silica gel aluminum plates containing fluorescent indicator (Merck, Darmstadt, Germany). The

chromatograms were developed in ethyl acetate:propan-2-ol:ammonia:water (27:23:5:3, by volume).

4.5. Enzymes assays

The nucleosidase was assayed quantitatively in a reaction mixture (50 μl) containing 0.1 M sodium acetate buffer (pH 4.75), 2 mM $CaCl_2$, 0.1 mM [^{3}H]-labelled substrate (300 000 cpm), other additions (e.g. salt) and a rate-limiting quantity of enzyme fraction. The reaction was carried out at 30 °C. To estimate reaction rates, 3 μl aliquots were spotted on to TLC plates, usually at four time intervals, unlabelled standards added and the plates developed for 30–40 min to separate the labeled nucleoside from corresponding product base. The compounds were visualized under short-wave UV light, spots of the products excised, immersed in scintillation cocktail and the radioactivity measured.

During enzyme purification, the activity of guanosine nucleosidase was monitored by TLC analysis of the conversion of 0.5 mM guanosine into guanine. This qualitative approach was sufficient for making quick decisions about which fractions from one step of enzyme purification could be collected and used in a following step. The same qualitative assay was applied for testing unlabelled guanosine analogues as potential substrate of the lupin nucleosidase (for example see Fig. 3).

4.6. Kinetic parameters

The K_m values for guanosine and inosine were estimated in the standard reaction mixture with substrate concentrations ranging between 1 and 12 μM. The values were computed from the Eadie-Hofstee plot (v versus $v/[S]$). K_i values were calculated according to the method of Dixon and Webb (1964).

4.7. Protein quantification

Protein concentration was determined by the turbidimetric tannin method (Mejbaum-Katzenellenbogen, 1955) using bovine serum albumin as standard. For column eluates the absorbance at 280 nm was monitored.

Acknowledgement

Financial support from the Agricultural University in Poznań, within grant 264/R/81/W, is gratefully acknowledged.

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