Determination of 5'-Nucleotidase Activity of Higher Plants by Using an Improved Method*

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Abstract: The presence of relatively high non-specific phosphatase activities are known in various plant tissues. These enzymes also catalyze the removal of inorganic phosphate from nucleoside monophosphates. According to published reports, so far, the experimental investigations, concerning the 5'-nucleotidase activity, have been mostly carried out without consideration of the non-specific phosphatase activities in crude enzymic extracts. However, few researchers have been included some inhibitors of these phosphatases, such as $NaMoO_4$ or KF, in assay mixtures. But phosphatases are not completely inhibited by these inhibitors and, therefore, interfere the activity of 5'-nucleotidase. Thus, the non-specific phosphatase activities were determined in crude enzymic extracts by using *p*-nitro-phenyl phosphate as substrate and the apparent 5'-nucleotidase activity was, first time, corrected accordingly in this present work.

Key Words: 5'-Nucleotidase, nucleoside 5'-monophosphate, phosphatase, specific activity, pyrimidine.

Yüksek Bitkilerde 5'-Nükleotidaz Aktivitesinin Geliştirilmiş Bir Yöntemle Tayini

Özet: Çeşitli bitki dokularında nispeten yüksek, spesifik olmayan fosfataz aktivitelerinin varlığı bilinmektedir. Bu enzimler aynı zamanda nükleosit monofosfatlardan da inorganik fosfatın uzaklaştırılmasını katalize ederler. Şimdiye kadar yayınlanan raporlara göre, saflaştırılmamış enzim ekstraktlarında 5'-nükleotidaz aktivitesiyle ilgili deneysel araştırmalar çoğunlukla spesifik olmayan fosfatazların aktiviteleri dikkate alınmadan yapılmıştır. Bununla birlikte bazı araştırıcılar, fosfatazların $NaMoO_4$ ve KF gibi bazı inhibitörlerini assay karışımlarına ilave etmişlerdir. Fakat fosfatazlar bu inhibitörlerle tamamen inhibe edilmeden 5'-nükleotidazın aktivitesine katılmışlardır. Bu yüzden mevcut çalışmada, saflaştırılmamış enzim ekstraktlarında spesifik olmayan fosfataz aktiviteleri, substrat olarak *p*-nitrofenil fosfatın kullanılmasıyla tayin edilmiş ve dolayısıyla 5'-nükleotidaz aktivitesi de, ilk defa, fosfatazların aktivitesinin de dikkate alınmasıyla doğru olarak belirlenmiştir.
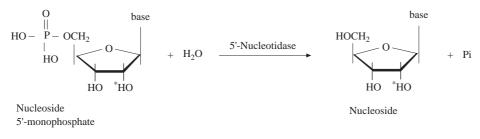
Anahtar Sözcükler: 5'-Nükleotidaz, nükleosit 5'-monofosfat, fosfataz, spesifik aktivite, pirimidin.

Introduction

5'-Nucleotidase (EC 3.1.3.5) hydrolyses nucleoside 5'-monophosphates to their corresponding nucleosides and releases inorganic phosphate (Figure 1).

*There is not oxygen at 2'-deoxy forms.

Figure 1.   Hydrolysis of nucleoside 5'-monophosphates by 5'-nucleotidase activity.

The published investigations concerning the 5'-nucleotidase activities from higher plant sources have been relatively few and those that have appeared have been largely in relation to purine metabolism. Polya (1,2) described 5'-nucleotidase activities from wheat seedling leaves and potato, and suggested an involvement of this enzyme with a cyclic nucleotide regulatory system. The regulation of cytokinin metabolism in wheat germ by a 5'-nucleotidase has been suggested by Chen and Kristopeit (3). Carter and Tipton (4) purified and described a 5'-nucleotidase from *Zea mays* microsomes. The activity of the enzyme have been also investigated in relation to the formation of ureides, allantoin and allantoic acid, as a result of purine *de novo* synthesis of inosine 5'-monophosphate (IMP) followed by oxidative degradation (5-7). This present work, which was carried out by an improved method, represents the first comparative study of the specific activity of 5'-nucleotidase of plants with pyrimidine nucleoside 5'-monophosphates as substrates.

Material and Methods

Plant material. Seeds of *Pisum sativum* L. *cv*. Meteor were from Sharpes Intl. Ltd., Sleaford, Lincs., *Albizzia julibrissin* Durazz. seeds were from Thompson & Morgan, Ipswich, Suffolk. *Lathyrus tingitanus* L.seeds were supplied by the University of Wales Swansea Botanic Garden and those of *Phaseolus aureus* Roxb. and *Glycine max* (L.) Merr. seeds were purchased locally in Swansea, UK. In all cases, dry seeds were well washed and before sowing separately allowed to imbibe for 15 hr in the dark in distilled water. *Albizzia* seeds were chipped before imbibition. The imbibed seeds were set to germinate in plastic trays 26 cm x 22 cm x6 cm depth, containing presoaked vemiculite, obtained from Vitagrow Ltd., Stoneferry, Hull., UK. Each tray, which had drainage holes in the bottom, was watered daily with distilled water. Seedlings were grown in a constant temperature room at 25 °C in a light cycle of 16 hr light (6 klx) and 8 hr dark.

Chemicals. Analytical grade chemicals and solvents were from British Drug Houses Ltd., Poole, Dorset, UK. Pyrimidines, *p*-nitrophenyl phosphate, *p*-methylaminophenol sulfate, ammonium molibdate, tris-base, copper sulfate pentahydrate and other chemicals were purchased from Sigma (London) Chemical company Ltd., Surrey, UK.

Enzyme extraction and assay. Seedlings (10-13 days old) were homogenised, using a prechilled mortar and pestle, in 0.2 M Tris-HCI buffer (pH 7.0) containing 2 mM dithioerithritol allowing 1 ml buffer gr^{-1} tissue. The homogenate was pressed through two layers of cheese-cloth and the filtrate centrifuged at 12000 *g* for 20 min. at 4 °C. The supernatant was dialysed overnight at 4 °C against the same buffer and used as the crude enzymic preparation.

The enzymic activity was assayed in a reaction mixture comprising 0.1 M Tris-HCI buffer (pH 7.0) containing 2 mM $MgCl_2$, 5 mM $NaMoO_4$ and 10 mM of the appropriate pyrimidine-nucleoside 5'-monophosphate (UMP, CMP, TMP). Correction was made for non-specific phosphatase activity, which was determined in the same preparation in a similar manner except that nucleoside 5'-monophosphates were replaced by 10 mM *p*-nitrophenyl phosphate. For both assays, 0.1 ml of enzymic extract was used in a final reaction volume of 1 ml. The enzymic preparation was omitted in controls and the substrates were omitted in blanks. The reactions were allowed to proceed for 30 min. at 37 °C and terminated by adding 0.1 ml of 75 % (w/v) trichloroacetic acid. Precipitated proteins were removed by centrifuging at 3000 *g* for 3 min. The inorganic phosphate released was determined by the colorimetric method of LeBel *et al*. (8). This method is based on the reduction of a phosphomolybdate complex by *p*-methyl-aminophenol sulfate in a copper acetate buffer. To make a standard curve of inorganic phosphate, 1.0 mg of P*i*/ml was prepared by dissolving anhydrous KH_2PO_4 (0.4387 gr/100 ml) in distilled water.

The specific activity of the enzyme was expressed as nkatal mg^{-1} of protein.

Protein determination. The method employed to estimate the protein concentration of the enzymic extracts was that described by Bradford (9). This is a colorimetric assay which involves the binding of Coomassie Brilliant Blue (G-250, Bio-Rad) to protein.

Results and Discussion

The results show that the specific activity of the 5'-nucleotidase is quite similar with each of the pyrimidine-nucleoside 5'-monophosphates, except that is almost twice higher with UMP in *P. sativum, L. tingitanus* and *A. julibrissin* (Table 1). There have been several published investigations that seedlings of *P. sativum* synthesize and accumulate willardiine and isowillardiine (10-12), seeds and seedlings of *L. tingitanus* accumulate lathyrine (13,14) and that seedlings of *A. julibrissin* produce albizziine (15, 16). All these compounds have been shown to originate from uracil as their precursor and known as pyrimidine-derived secondary products. It was shown by our earlier investigation that (17) *P. aureus* and *G. max* do not produce and accumulate such compounds. The relatively greater activity of 5'-nucleotidase with UMP in the group of pyrimidine-derived secondary compound producers, which are *P. sativum*, *L. tingitanus,* and *A. julibrissin*, is most probably because of the produce more uridine and subsequentially uracil as the precursor of these compounds.

Table 1.   The specific activity of 5'-nucleotidase in the experimental plants.

Substrate	Enzyme source	Specific activity (nkatal x 10^2 mg^{-1} protein)
Uridine 5'-monophosphate	*Pisum sativum*	9.2
(UMP)	*Lathyrus tingitanus*	9.5
	Albizzia julibrissin	8.6
	Phaseolus aureus	5.4
	Glycine max	4.7
Cytidine 5'-monophosphate	*Pisum sativum*	6.6
(CMP)	*Lathyrus tingitanus*	5.7
	Albizzia julibrissin	6.0
	Phaseolus aureus	6.8
	Glycine max	5.3
Thymidine 5'-monophosphate	*Pisum sativum*	5.1
(TMP)	*Lathyrus tingitanus*	4.6
	Albizzia julibrissin	6.9
	Phaseolus aureus	5.5
	Glycine max	6.2

Details of substrate concentrations and incubation conditions are in Methods. Data are a typical set from replicate experiments showing similar trends.

The step catalysed by 5'-nucleotidase may be suggested as one of the rate-limiting steps in the sequence of pyrimidine metabolism. Because, the end product of orotate pathway, UMP, is not only the source of all the primary products of pyrimidine metabolism but is also the feed-back inhibitor of aspartate transcarbamoylase (ATCase; EC 2.1.3.2) the second enzyme of the pathway. The main restraint on ATCase is removed by the more hydrolysis of UMP and, thus, the pyrimidine production is increased. But the control of the hydrolysis reaction of UMP is not still well known. However, as suggested by Amarjit and Singh (6), the pH in the cytosol may be one of the factors regulating the affinity of 5'-nucleotidase. The relatively lower specific activity of the enzyme was obtained at pH 7.5 in our earlier investigation (15). These findings, therefore, confirm the above suggestion.

So far, the specific activity of 5'-nucleotidase has been calculated by determination of inorganic phosphate that released from nucleoside monophosphates. But it is well known that, the non-specific phophatases also catalyze the removal of inorganic phosphate from nucleoside monophosphates and interfere the 5'-nucleotidase activity in the crude enzymic extracts.

Therefore *p*-nitrophenyl phosphate, which is not an appropriate substrate for 5'-nucleotidase (3,4), was used for non-specific phosphatase activities in our investigation. The correct specific activity of 5'-nucleotidase was calculated by considering the value of non-specific phosphatase activities. Thus, one of the most important results of this present work is, first time, the determination of the specific activity of 5'-nucleotidase correctly in the crude enzymic preparations of higher plants.

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