Phytochemistry, Vol. 20, No. 9, pp. 2127–2129, 1981.
Printed in Great Britain.

0031–9422/81/092127–03 $02.00/0

THE PURINE NUCLEOSIDASES OF JERUSALEM ARTICHOKE SHOOTS

F. LE FLOC'H and J. LAFLEURIEL

Laboratoire de Phytomorphogenèse Associé au C.N.R.S., 4, rue Ledru, 63038 Clermont-Ferrand, Cédex, France

(*Revised received* 11 *March* 1981)

Key Word Index—*Helianthus tuberosus*; Compositae; Jerusalem artichoke; adenosine nucleosidase; inosine–guanosine nucleosidase.

Abstract—Extracts from Jerusalem artichoke shoots exhibited adenosine and inosine–guanosine nucleosidase activities. The results suggest the existence of two distinct enzymes with some similar properties: high stability, optimal pH from 5 to 7, independence towards phosphate and metallic ions, high affinity for substrates. The Michaelis constants were 17 μM for adenosine, 8.5 μM for guanosine, 2.5 μM for inosine, respectively. The two enzymes were distinguished by their substrate specificity; the inosine–guanosine nucleosidase alone was inhibited by 6-mercaptopurine riboside which was a substrate for this enzyme. The physiological role of these two enzymes is considered.

INTRODUCTION

Treatment of shoots of Jerusalem artichoke (*Helianthus tuberosus*) with [8-^{14}C]adenosine brings about a rapid expansion of the adenyl nucleotide pool [1]. The conversion of adenosine into nucleotides might be expected to take place either by phosphorylation of adenosine to give AMP or by its hydrolysis to adenine and ribose followed by transfer of a phosphoribosyl group from PRPP to adenine. In the second case, conversion of adenosine to AMP requires the presence of two enzymes: adenine phosphoribosyltransferase, already shown to be present in Jerusalem artichoke extracts and of which certain properties have been studied [2]; and adenosine nucleosidase shown to be present in other plant species [3–9]. The initial aim of the present work was to determine whether Jerusalem artichoke extracts also exhibited an adenosine nucleosidase activity, in order to ascertain whether adenosine could be converted to nucleotides by the second of the two pathways proposed above. However, in the course of this study, it was discovered that these extracts were able to hydrolyse not only adenosine, but also inosine and guanosine. The latter activity has been reported in micro-organisms [10] and animals [11], and as regards higher plants has been detected in *Pisum sativum* whole seedlings [12]. The results reported here suggest that these hydrolytic activities are due to the presence of two separate enzymes: adenosine nucleosidase (EC 3.2.2.7) and inosine–guanosine nucleosidase (EC 3.2.2.2).

RESULTS

Nucleosidase stability

Measurement of these activities in a crude protein extract filtered on Sephadex G 25, gave the following specific activities: 4×10^{-4} kat/kg with adenosine as substrate; 1.1×10^{-6} kat/kg with inosine as substrate; and 0.7×10^{-6} kat/kg with guanosine as substrate. Subsequent chromatography of the crude extract on DEAE–Sephacel was found to increase its specific activity towards guanosine and inosine (2.3×10^{-6} kat/kg) but failed to separate adenosine, inosine and guanosine activities which were always eluted together.

All three activities proved to be highly stable, both at +4° and under deep-freeze conditions. The presence of glutathione in the extraction medium was found to be irrelevant to this stability, and indeed neither glutathione nor the more powerful reducing agent dithiothreitol affected reaction rate. The great stability of adenosine nucleosidase has already been reported [4, 6, 8].

Stoichiometry. Influence of pH *and various ions*

The substrates, [8-^{14}C]adenosine, [8-^{14}C]inosine and [U-^{14}C]guanosine were incubated with a sample of the extract at 25° at pH 7 or 7.5. Reaction products and unreacted substrate could be rapidly separated by TLC on cellulose with M ammonium acetate as developer. After prolonged incubation it was determined whether the radioactivity of the three substrates had appeared on the corresponding bases. In addition, hydrolysis of inosine was monitored by UV spectrophotometry by adding xanthine oxidase to the reaction mixture and measuring the increase in absorbance at 293 nm resulting from immediate oxidation of hypoxanthine to uric acid.

Activities for all three substrates were found to be optimal between pH 5 and 7. At pH 4, adenosine nucleosidase activity was 80% of the optimal value in accordance with similar reported findings [4, 6–9] which indicate optimal activity in an acidic medium. However, it is noteworthy that all three activities were still 50% of the optimum value at pH 9. In a further series of experiments the presence of various concentrations of phosphate was found to have no effect whatever on the rate of hydrolysis of any of the three substrates compared to controls, thus ruling out the possibility of a pathway involving phosphorolysis. Similarly, reaction rates remained unaffected after addition of 10^{-3} M NaF or metal ions (Mg^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}, Ca^{2+}, K^+ and Na^+).

Substrate specifity. K_m *inhibition by reaction products*

If with adenosine as substrate cold guanosine or inosine was subsequently added at the same equivalent concentration, no change in hydrolysis rate occurred

compared to controls. Similarly, with either inosine or guanosine as substrate, subsequent addition of adenosine had no effect on rate (Table 1). However, addition of 6-mercaptopurine riboside strongly inhibited hydrolysis of inosine and guanosine but did not affect the rate of hydrolysis of adenosine. 6-Mercaptopurine riboside was itself hydrolyzed and thus constitutes a potential substrate of inosine–guanosine nucleosidase. A plot of the Michaelis-type kinetics of the inosine–guanosine hydrolysis using inverted co-ordinates according to Lineweaver and Burk showed a typical competitive inhibition when cold guanosine was added to the reaction medium with [8-^{14}C]inosine as substrate, and vice versa.

These results indicate therefore that Jerusalem artichoke extracts contain two well-differentiated nucleosidase activities, one specific to adenosine, the other specific to inosine and guanosine. Determination of Michaelis constants gave the following values: 17 μM for adenosine; 8.5 μM for guanosine; and 2.5 μM for inosine. The value obtained for adenosine nucleosidase is of the same order of magnitude as those reported elsewhere [4, 6, 8, 9], and inosine–guanosine nucleosidase was similarly inhibited by hypoxanthine, one of two reaction products of the hydrolysis of inosine. On the other hand, even high concentrations of ribose had no inhibiting action.

DISCUSSION

Jerusalem artichoke tissue extracts possess the property of hydrolysing the main purine nucleosides, adenosine, inosine and guanosine. Adenosine nucleosidase activity is quantitatively much higher than inosine–guanosine nucleosidase activity. These activities share several common features: high stability; similar pH sensitivity; insensitivity to phosphate and metal ions; and specific affinity for their respective substrates. However, intersubstrate competition experiments and inhibition by 6-mercaptopurine riboside and by reaction products indicate the presence of two types of catalytic site. The 6-carbon substituent on the purine nucleus thus seems to be an essential factor in the formation of the enzyme–substrate active complex. The present results thus suggest the existence either of a single enzyme with two separate catalytic sites, or of two separate enzymes of similar structure and properties, one specific to adenosine, the other to inosine and guanosine. The second hypothesis seems the more likely given the very different specific activities of the crude extract (about 400 times higher for adenosine than for inosine) and the specificity of adenosine nucleosidase reported by different workers [4, 6, 8, 9].

Adenosine and inosine–guanosine nucleosidases undoubtedly play an essential part in nucleoside recycling. No adenosine kinase or adenosine or adenine aminohydrolase activity has been detected in Jerusalem artichoke tissues. Thus hydrolysis of adenosine may only occur via adenosine nucleosidase which has not been shown to be present in Jerusalem artichoke. AMP-incorporation of adenine may subsequently occur via adenine phosphoribosyltransferase which exhibits a high affinity for this substrate, as previously shown [2]. The fact that no PRPP deficiency has been found *in situ* further supports such a hypothesis (unpublished results). Moreover, Jerusalem artichoke tissues seem to be devoid of inosine and guanosine kinase activity. Hydrolysis would thus seem to be the sole metabolic pathway available to these nucleosides, via the inosine–guanosine nucleosidase reported here.

EXPERIMENTAL

Plant material. Non-dormant tubers of Jerusalem artichoke (*Helianthus tuberosus* L. var. D19) were scrubbed and rinsed, placed in vermiculite and left for several days at 28° in the dark. The 2–8 cm-long shoots were removed and weighed.

Extraction. The shoots were macerated in a mortar with a small amount of sand and the following extraction medium (1 g shoots/10 ml medium): 0.2 M Tris–HCl buffer pH 7.5 or Tris–phosphate buffer pH 7, 2×10^{-3} M glutathione. The resulting mixture was centrifuged for 1 min at 7000 *g*. The supernatant liquid was filtered in a No. 2 sintered-glass filter and passed through a column of Sephadex G 25 treated with 0.05 M buffer (Tris–HCl or Tris–phosphate). The protein fraction obtained constituted the crude extract.

Chromatography on DEAE-Sephacel. For the study of inosine–guanosine nucleosidase, the crude extract was purified

Table 1. Action of different substances on the adenosine and inosine–guanosine nucleosidase activities as % of controls

Tested substances (10^{-4} M)	AR nucleosidase activity Substrate 10^{-4} M [8-^{14}C]adenosine Radioactivity appearing in adenine (cpm)	% control	IR–GR nucleosidase activity Substrate 10^{-4} M [8-^{14}C]inosine Radioactivity appearing in hypoxanthine (cpm)	% control
Control	31 700	100	12 200	100
Adenosine	—	—	12 230	100
Inosine	31 750	100	—	—
6-Mercaptopurine ribosine	31 750	100	2 450	20
Guanosine	31 700	100	6 700	55
Adenine	29 200	87	12 250	100
Hypoxanthine	31 730	100	10 850	89

Incubation medium (50 μl) was as follows: (AR nucleosidase) 5×10^{-2} M Tris–HCl buffer pH 7.5, 10^{-4} M [8-^{14}C]adenosine, 10^{-4} M tested substance, 10 μl of enzyme solution and water; (IR nucleosidase) 2×10^{-2} M Tris–phosphate buffer pH 7, 10^{-4} M [8-^{14}C]inosine, 10^{-4} M tested substance; 20 μl of enzyme solution and water. Duration 10 min in the first case, 60 min in the second, at 25°.

by chromatography on a column of DEAE-Sephacel (1.5 × 3 cm) treated with 0.05 M Tris–phosphate buffer pH 7. Elution was performed using a 0–0.5 M NaCl linear concentration gradient. Total eluant volume was 50 ml. The most active fractions were combined and constituted the protein fraction used for subsequent experimentation. Solutions were divided up into small fractions and kept at −25°. Protein assay was carried out according to Lowry *et al.* [13] using albumin of ox serum as standard.

Measurement of enzyme activity. Incubation conditions were as follows: 25° pH 7.5 duration 10 min (adenosine nucleosidase), 25° pH 7 duration 60 min (inosine–guanosine nucleosidase). The incubation medium (50 μl) was as follows; for adenosine nucleosidase, 5×10^{-2} M Tris–HCl buffer pH 7.5, 10^{-4} M [8-^{14}C]adenosine (48 mCi/mM), 10 μl of enzyme solution and water; for inosine–guanosine nucleosidase, 2×10^{-2} M or 2.5×10^{-2} M Tris–phosphate buffer pH 7, 10^{-4} M [8-^{14}C]inosine (48 mCi/mM) or 10^{-5} M [U-^{14}C]guanosine (395 mCi/mM) 20 or 25 μl of enzyme solution and water. At the end of the incubation period, a 10-μl aliquot was taken and loaded onto a 0.1 × 25 × 100 mm cellulose TLC plate in a 20 mm strip. Further reaction was arrested by immediate drying in hot air after prior loading of a tracer mixture. Upward development of the plates was carried out using M ammonium acetate solution. Nucleosides and their corresponding bases were rapidly separated: adenine R_f 0.29, adenosine R_f 0.50, hypoxanthine R_f 0.54, inosine R_f 0.77, guanine R_f 0.36, guanosine R_f 0.59. The tracers were located by UV (λ 254 nm) and the corresponding areas scraped off into a scintillating mixture (5 g PPO and 0.3 g dimethylPOPOP in toluene). Radioactivity was measured with an Intertechnique SL40 liquid scintillation counter.

For spectrophotometric monitoring, the incubation medium (600 μl) contained 10^{-4} M inosine, 0.020 M Tris–phosphate buffer pH 7, 10 μl xanthine oxidase (EC 1.2.3.2) Sigma, 200 μl of enzyme solution and water. Recording of the rise in absorbance at 293 nm began from the moment of addition of inosine to the reaction medium.

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