Phytochemistry, Vol. 25, No. 10, pp. 2267–2270, 1986.
Printed in Great Britain.

0031-9422/86 $3.00 + 0.00
Pergamon Journals Ltd.

PROPERTIES OF 5′-NUCLEOTIDASE FROM NODULES OF PIGEONPEA (*CAJANUS CAJAN*)

AMARJIT and R. SINGH

Department of Chemistry and Biochemistry, Haryana Agricultural University, Hisar 125004, India

(*Revised received* 13 *December* 1985)

Key Word Index—*Cajanus cajan*; Leguminosae; pigeonpea; nodules; 5′-nucleotidase; properties; intermediate metabolites; regulation.

Abstract—5′-Nucleotidase from pigeonpea nodules has been resolved into two forms, N-I and N-II, having M_rs of 52 000 and 119 000, respectively. Both forms had pH optima in the acidic range (between pH 5.2 and 5.7) with either CMP, GMP, XMP, IMP or AMP as the substrate. Up to pH 6.6, both forms showed higher activity with CMP followed by GMP, XMP, IMP and AMP, respectively. However, the activity changed with pH in the alkaline range making the enzyme relatively more active with purine nucleotides. Neither of the forms had a requirement for any of the metal ions tested. Fe^{3+} inhibited the enzyme activity; the inhibition at 5, 10 and 15 mM concentrations being 11, 43 and 47%, respectively with N-I and 14, 47 and 52%, respectively with N-II. K_m values for AMP, IMP, GMP, CMP and XMP were 0.10, 0.18, 0.40, 0.40 and 0.77 mM, respectively with N-I and 0.12, 0.20, 0.40, 0.40 and 0.99 mM, respectively with N-II. The enzyme was inhibited non-competitively by adenosine and inosine; K_i values being 1.78, 0.25 and 0.30; 3.50, 2.12 and 0.75 mM, respectively with AMP, IMP and XMP as the substrate.

INTRODUCTION

In many of the tropical legumes, including pigeonpea, most of the nitrogen fixed in nodules is translocated in the form of ureides, allantoin and allantoic acid [1–4]. These ureides are synthesized from purines by oxidation and hydrolysis [5–7]. There is evidence that purine biosynthesis in nodules is similar to that in animals and micro-organisms [6, 8–10] with IMP as the end product of the pathway [11, 12]. The reaction sequence leading to the formation of ureides from IMP is also well established [13–17] and a few of the enzymes from this sequence such as xanthine dehydrogenase [18, 19], uricase [20, 21] and allantoinase [22] have been purified and characterized. However, very little is known about the rest of the enzymes which include IMP dehydrogenase, 5′-nucleotidase and nucleosidase [10, 11]. Here we describe some of the properties of 5′-nucleotidase (EC 3.1.3.5) isolated from nodules of pigeonpea.

RESULTS AND DISCUSSION

Developmental pattern

Total nucleotidase activity in the nodules was low during the initial stages of plant growth. After day 60, the activity increased sharply attaining a peak at day 90 and then decreased continuously till the end of plant growth (Fig. 1). The peak of nucleotidase activity coincided very well with that of total N_2ase activity and total ureide content of the nodules [17].

Partial purification

The enzyme fraction obtained between 30 and 70% ammonium sulphate saturation, when loaded onto a DEAE-cellulose column, was resolved into two major peaks of activity (Fig. 2). The peak I enzyme was not adsorbed on DEAE-cellulose, whereas the peak II enzyme was eluted with a 0.1 M sodium chloride gradient. No further enzyme activity was eluted from the column even when the concentration of sodium chloride in elution buffer was increased to 0.4 M. The pooled peak I and peak II enzyme fractions were devoid of non-specific acid and alkaline phosphatases, as neither fraction showed any activity with *p*-nitrophenyl phosphate as the substrate. They were designated as N-I and N-II 5′-nucleotidase, respectively. The degree of purification after DEAE-cellulose chromatography was about 19- and 15-fold for N-I and N-II 5′-nucleotidase, respectively. The specific activity of N-I and N-II with XMP was 3.8 and 3.0 μmol min^{-1} mg^{-1} protein, respectively. Soybean nodule enzyme on purification also revealed two forms of 5′-nucleotidase [10]. Both the enzyme forms were quite stable and did not lose any activity after two weeks storage at 4°.

From Sephadex G-100 chromatography, the relative mobility of N-I nucleotidase was equivalent to an M_r of 52 000 and that of N-II nucleotidase corresponded to an M_r of 119 000. These M_r are very similar to those reported earlier for F-I and F-II nucleotidases of wheat germ cytosol [23].

Effect of pH

Both N-I and N-II nucleotidases had pH optima in the acidic range (between pH 5.2 and 5.7) with either CMP, GMP, XMP, IMP or AMP as the substrate (Fig. 3a, b). Up to pH 6.6, both the forms had higher activity with CMP, followed by GMP, XMP, IMP and AMP, respectively. However, the activity changed with pH in the alkaline range making the enzyme relatively more active towards purine nucleotides AMP, IMP and GMP. Both forms again exhibited almost identical behaviour. At pH 9.0, the highest rate was observed with IMP as the

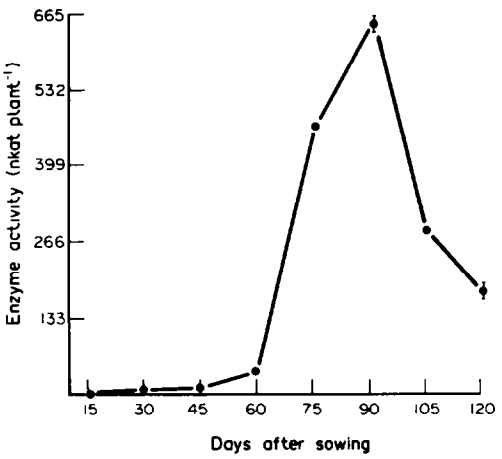

Fig. 1. 5'-Nucleotidase activity in nodules at different days of plant growth.

substrate. It seems to be a general property of the nodule enzyme as the soybean nucleotidase also exhibited a change in specificity with pH [10]. The *in vivo* significance of this observation remains a matter of conjecture in view of the fact that there is no definite information available on pH changes in the nodule cytosol accompanying N_2 fixation and ammonia assimilation [24].

Effect of ions

The effect of various chloride salts of cations such as K^+, Na^+, NH_4^+, Ba^{2+}, Ca^{2+}, Ni^{2+}, Cd^{2+}, Sr^{2+}, Mg^{2+}, Cu^{2+} and Fe^{3+} was studied at 5, 10 and 15 mM each under standard assay conditions. Both forms showed appreciable activity without any of the cations, indicating that the enzyme did not have a requirement for any of the metal ions tested. Fe^{3+} inhibited the enzyme activity; the inhibition at 5, 10, 15 mM concentrations being 11, 43 and 47%, respectively with N-I and 14, 47 and 52%, respectively with N-II. These results indicate that the nodule enzyme is relatively insensitive to metal ions compared to other plant [23] and animal 5'-nucleotidases [25].

Substrate specificity

The enzyme at pH 5.3 followed the normal Michaelis–Menten kinetics and both the forms exhibited comparable K_m values with different substrates. Using N-I enzyme, the K_m values calculated from Lineweaver–Burk plots were 0.10, 0.18, 0.40, 0.40, and 0.77 mM for AMP, IMP, GMP, CMP and XMP, respectively. The corresponding K_m values for N-II enzyme were 0.12, 0.20, 0.40, 0.40 and 0.99 for AMP, IMP, GMP, CMP and XMP. These results indicate that at pH 5.3, the enzyme had a greater affinity for purine nucleotides especially AMP and IMP. Similar indications were obtained when the V_{max}: K_m ratios were calculated for different substrates. For N-I enzyme, the ratios for AMP, IMP, XMP, GMP and CMP were 21.80, 18.31, 4.92, 9.90 and 10.00, respectively. For N-II enzyme, the corresponding values were 13.30, 13.25, 3.03, 7.90 and 8.15. Both the forms had the poorest affinity for XMP. This creates doubt about the role of this enzyme in purine catabolism leading to the formation of xanthosine from XMP in nodules. Similar results have earlier been reported for the soybean nodule enzyme [10]. In view of these observations, it is suggested that the step catalysed by 5'-nucleotidase may be one of the rate limiting steps in the sequence of purine catabolism leading to the formation of ureides. How the reaction is controlled at this step still remains a problem. However, as suggested earlier, the pH in the cytosol may be one of the factors regulating the affinity of the enzyme. Alternatively, IMP and/or AMP may be the true substrates of the enzyme in nodules.

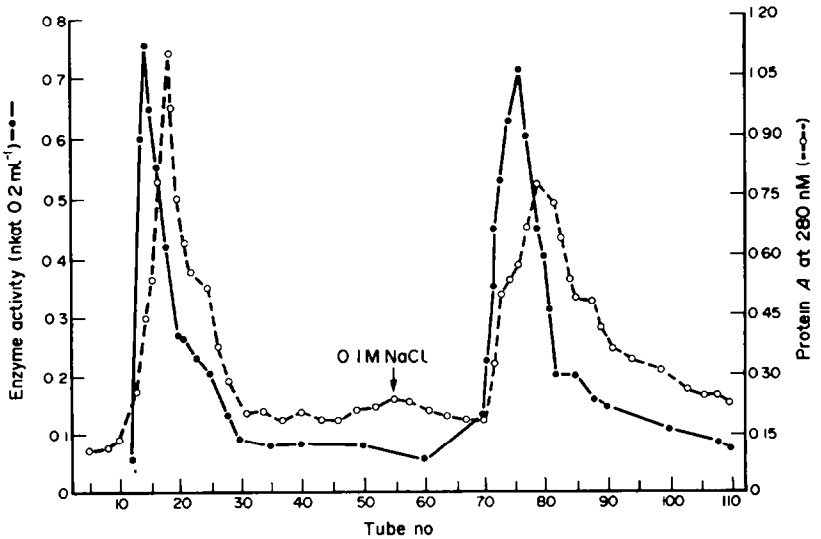

Fig. 2. DEAE-cellulose column chromatography of 5'-nucleotidase. For details see Experimental.

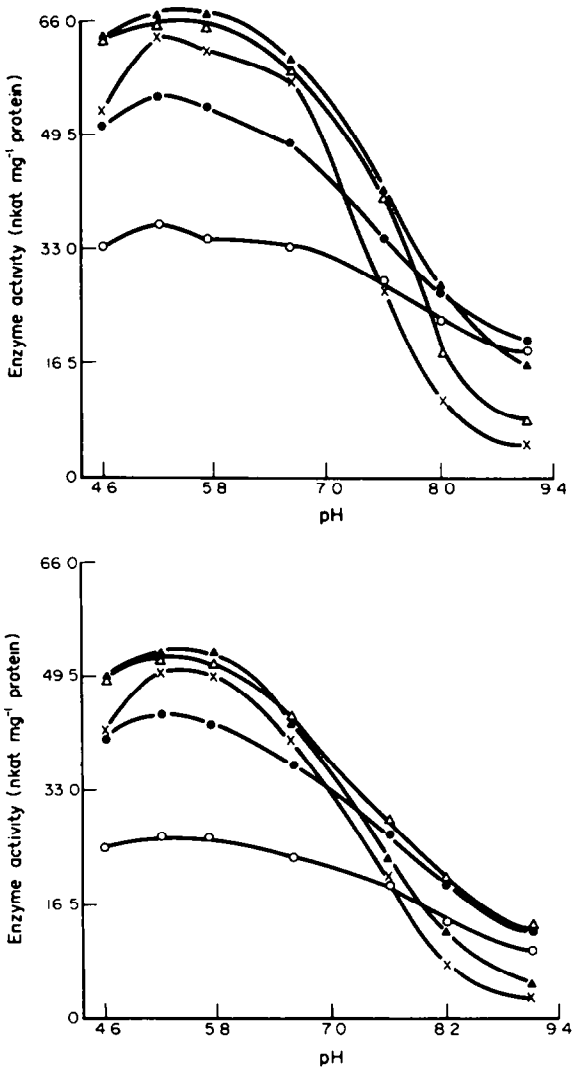

Fig. 3. (a) Effect of pH on the activity of N-I fraction of 5'-nucleotidase in presence of different substrates. AMP (—○—) IMP (—●—), XMP (— × —), GMP (—△—) and CMP (—▲—). (b) Effect of pH on the activity of N-II fraction of 5'-nucleotidase in presence of different substrates. AMP (—○—) IMP(—●—), XMP (— × —), GMP (—△—) and CMP (—▲—).

Table 1. Effect of adenosine and inosine on nucleotidase activity in presence of different substrates

Metabolite (mM)	N-I AMP	N-I IMP	N-I XMP	N-II AMP	N-II IMP	N-II XMP
	(μmol $min^{-1}mg^{-1}$ protein)					
Adenosine						
Control	2.15	3.21	3.60	1.48	2.20	2.82
0.4	1.46	1.79	1.59	1.03	1.24	0.98
	(32)	(44)	(56)	(30)	(44)	(65)
1.0	0.98	1.09	0.81	0.70	0.74	0.59
	(54)	(66)	(78)	(53)	(66)	(79)
2.0	0.86	0.81	0.54	0.52	0.56	0.45
	(60)	(75)	(85)	(65)	(75)	(84)
Inosine						
0.4	1.78	2.30	2.00	1.25	1.63	1.53
	(17)	(28)	(44)	(16)	(26)	(46)
1.0	1.46	1.72	1.27	1.03	1.29	1.07
	(32)	(46)	(65)	(30)	(41)	(62)
2.0	1.09	1.27	0.92	0.74	0.90	0.70
	(49)	(60)	(74)	(50)	(59)	(75)

The values in parentheses indicate % inhibition of control.

Effect of metabolites

ATP, ADP, UTP, UDP, CTP, CDP, GTP, GDP, uric acid, allantoin and allantoic acid had no effect on enzyme activity. However, the enzyme was strongly inhibited by adenosine and inosine, the end products of the reaction with either AMP, IMP or XMP as the substrate (Table 1). The maximum inhibition was observed with XMP as the substrate. The pattern and extent of inhibition was almost identical for both the forms of the enzyme. Xanthosine compared to adenosine and inosine was a very poor inhibitor. Even at the highest concentration (2 mM), it inhibited the enzyme activity by 25–30% and that only when XMP was used as the substrate. The nature of the inhibition in each case as determined by a Dixon's plot was found to be non-competitive and the K_i values for adenosine and inosine were calculated to be 1.78, 0.25 and 0.30 and 3.50, 2.12 and 0.75 mM, respectively with AMP, IMP and XMP as the substrate. These results indicate that for the 5'-nucleotidase to function efficiently, inosine and/or adenosine must not accumulate in nodule cells. However, at this stage, it is not known with certainty as to by which route IMP is ultimately converted to xanthine for onward conversion to ureides.

EXPERIMENTAL

Chemicals. All the biochemicals used here were purchased from Sigma (St. Louis, MO).

Plant material. Seeds of pigeonpea (*Cajanus cajan* L.), cv UPAS-120, after surface sterilization with sodium hypochlorite, were inoculated with *Rhizobium* culture (Strain IPH-159) and sown during summer (July 20, 1984) in a naturally lighted net house as described earlier [26]. Nodules were harvested from 75–90-day-old plants and stored at −20° until required.

Enzyme extraction. Unless stated otherwise, all steps involved in enzyme extraction, purification and M_r determination were carried out at 4°.

Nodules (20 g) were hand homogenized in 160 ml ice-cold 50 mM Tris–HCl buffer (pH 7.5) containing 10 mM 2-mercapto-ethanol and 10 mM KCl. The homogenate was squeezed through four layers of cheesecloth and centrifuged at 12000 *g* for 30 min. The supernatant obtained was referred to as the enzyme extract.

$(NH_4)_2SO_4$ *fractionation.* Solid $(NH_4)_2SO_4$ was added to the extract to 30% satn. After 1 hr, the precipitate was removed by centrifugation at 15000 g for 30 min and the supernatant brought to 70% satn by adding a further amount of $(NH_4)_2SO_4$. The ppt collected by centrifugation at 15000 *g* for 30 min was redissolved in the minimum amount of the above buffer and dialysed against the same buffer for 18 hr.

DEAE-cellulose chromatography. The above fraction obtained between 30 and 70% $(NH_4)_2SO_4$ satn was applied onto a DEAE-cellulose column (2.5 × 30 cm) previously equilibrated with the above buffer. The column was eluted with 1.5 bed vols of the

buffer followed by a stepwise NaCl gradient. Fractions (3 ml each) containing 5'-nucleotidase activity were pooled and concd by $(NH_4)_2SO_4$ precipitation.

Determination of molecular weight. The M_r of the enzyme was estimated from the gel permeation data (Sephadex G-100 column). Alcohol dehydrogenase, BSA, albumin and trypsinogen were used as marker proteins for calibration.

Enzyme assay. The enzyme was assayed as described by Christensen and Jochimsen [10] with minor modifications. The assay mixture in a total vol. of 1.5 ml contained: 100 mM Tris–maleate buffer (pH 5.3), 12 mM $MgCl_2$, 3 mM XMP, 1 mg BSA and the enzyme extract. After 15 min of incubation at 37°, the reaction was terminated by adding 1 ml 10% TCA. The precipitated protein was removed by centrifugation and the Pi estimated in the supernatant [27]. While studying the effect of Fe^{3+} on enzyme activity, suitable controls were run where Fe^{3+} was incubated with different amounts of Pi in an otherwise complete reaction mixture lacking enzyme preparation. The decrease in Pi values obtained in the presence of Fe^{3+} was subtracted from the corresponding experimental values. This was done in view of the fact that Fe^{3+} removes Pi as insoluble iron phosphate.

Protein determination. Protein in column fractions was determined by recording the absorbance at 280 nm. In all other cases, it was determined according to ref. [28]. All estimations were done in triplicate.

Acknowledgements—The authors wish to thank Drs. B. S. Dahiya and F. C. Garg for supplying the seed material and *Rhizobium* culture, respectively. One of the authors (A.J.) is thankful to the Council of Scientific and Industrial Research, New Delhi for providing financial help in the form of fellowships.

REFERENCES

1. Herridge, D. F., Atkins, C. A., Pate, J. S. and Rainbird, R. M. (1978) *Plant Physiol.* **62**, 495.
2. McClure, P. R. and Israel, O. W. (1979) *Plant Physiol.* **64**, 411.
3. Luthra, Y. P., Sheoran, I. S. and Singh, R. (1981) *Curr. Sci.* **50**, 270.
4. Sheoran, I. S., Luthra, Y. P., Kuhad, M. S. and Singh, R. (1982) *Plant Physiol.* **70**, 917.
5. Atkins, C. A., Pate, J. S., Griffiths, G. J. and White, S. T. (1980) *Plant Physiol.* **66**, 978.
6. Schubert, K. R. (1981) *Plant Physiol.* **68**, 1115.
7. Boland, M. J. and Schubert, K. R. (1982) *Arch. Biochem. Biophys.* **213**, 486.
8. Reynolds, P. H. S., Boland, M. J., Blevins, D. G., Randall, D. D. and Schubert, K. R. (1982) *Plant Physiol.* **69**, 1334.
9. Reynolds, P. H. S., Blevins, D. G., Boland, M. J., Randall, D. D. and Schubert, K. R. (1982) *Physiol. Plant.* **55**, 255.
10. Christensen, T. M. I. E. and Jochimsen, B. U. (1983) *Plant Physiol.* **72**, 56.
11. Shelp, B. J. and Atkins, C. A. (1983) *Plant Physiol.* **72**, 1029.
12. Shelp, B. J., Atkins, C. A., Storer, P. J. and Canvin, D. T. (1983) *Arch. Biochem. Biophys.* **224**, 429.
13. Woo, K. C., Atkins, C. A. and Pate, J. S. (1980) *Plant Physiol.* **66**, 735.
14. Triplett, E. W., Blevins, D. G. and Randall, D. D. (1980) *Plant Physiol.* **65**, 1203.
15. Atkins, C. A., Ritchie, A., Rowe, P. B., McCairns, E. and Sauer, D. (1982) *Plant Physiol.* **70**, 55.
16. Amarjit and Singh, R. (1984) *J. Biosci.* **6**, 185.
17. Amarjit and Singh, R. (1985) *J. Biosci.* **7**, 375.
18. Boland, M. J. (1981) *Biochem. Int.* **2**, 567.
19. Triplett, E. W., Blevins, D. G. and Randall, D. D. (1982) *Arch. Biochem. Biophys.* **219**, 39.
20. Rainbird, R. M. and Atkins, C. A. (1981) *Biochim. Biophys. Acta* **659**, 132.
21. Lucas, K., Boland, M. J. and Schubert, K. R. (1983) *Arch. Biochem. Biophys.* **226**, 190.
22. Amarjit and Singh, R. (1985) *Phytochemistry* **24**, 415.
23. Chen, C. M. and Kristopeit, S. M. (1981) *Plant Physiol.* **67**, 494.
24. Raven, J. A. and Smith, F. A. (1976) *New Phytol.* **76**, 415.
25. Naito, Y. and Tsushima, K. (1976) *Biochim. Biophys. Acta* **438**, 159.
26. Luthra, Y. P., Sheoran, I. S., Rao, A. S. and Singh, R. (1983) *J. Exp. Botany* **34**, 1358.
27. Ozbun, J. L., Hawker, J. S., Greenberg, E., Lammel, C. and Preiss, J. (1973) *Plant Physiol.* **51**, 1.
28. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.