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Aminopeptidase P from *Drosophila melanogaster.*

Kulkarni, Gauri V., and Deepti D. Deobagkar[1]. Molecular Biology Research Laboratory, Department of Zoology, University of Pune, Ganeshkhind, Pune-411007. Maharashtra, India. [1]To whom correspondence should be addressed.

Summary

Aminopeptidase-P (AP-P; X-Pro aminopeptidase; EC 3.4.11.9) has the unique ability to cleave off the N-terminal amino acid residue from the peptides having proline as a penultimate amino acid residue. Several biologically active peptides and proteins have the Xaa-Pro motif at the N terminus, which confers resistance to them against cleavage by nonspecific proteases. Here, we report the purification of a cytosolic aminopeptidase P from *D. melanogaster* by immunoprecipitation and its biochemical characterization using the peptide substrates,
substance P and bradykinin.

Aminopeptidase-P (AP-P; X-Pro aminopeptidase; EC 3.4.11.9) was first identified in pig kidney as an exopeptidase capable of releasing the N-terminal amino acid residue from peptides with a penultimate proline (Dehm *et al.,* 1970). Several biologically active peptides including hormones, neuropeptides, neurotransmitters and mediators of immune response escape non-specific protease degradation by having Xaa-Pro motif at their amino termini (Yaron, 1987; Yaron, 1993). AP-P activity is ubiquitous and has been found in many different organisms including bacteria, yeast and vertebrates. Mammalian AP-Ps exists as a membrane-bound and cytosolic form, which represent two distinct gene products (Orawski *et al.,* 1987; Hooper *et al.*, 1990; Rusu and Yaron, 1992; Simmons and Orawski, 1992). The cytoplasmic (soluble) form of mammalian aminopeptidase-P is found in human leukocytes and rat brain (Rusu and Yaron, 1992; Harbeck and Mentlein, 1991). Both forms of AP-P can hydrolyze several peptides including Bradykinin (a vasodilator) and the neurotransmitters like sub-stance P, Peptide YY, and neuropeptide Y. Leucocyte AP-P was demonstrated to cleave Interleukin-6 (Rusu and Yaron, 1992). The insect AP P was reported in *Drosophila melanogaster* (Kulkarni and Deobagkar) which represen-ted the cytosolic form of AP-P. Previous studies have demonstrated the functional

Table 1. Effect of Manganese on the activity of Drosophila AP P: Purified Drosophila aminopeptidase P (100ng) was incubated with 0.1mM each of bradykinin and substance P in presence of various concentrations of MnCl2 in 0.1M HEPES, pH 7.6 at 370C. The activity was analyzed and expressed as percent of maximum activity observed. Results are the mean values of triplicate determinations for each concentration in a single experiment, which do not differ by more than 5%.

$MnCl_2$ Conc. (M)	Substance-P	Bradykinin
0	63.5	100
10^{-5}	72.35	72.60
10^{-4}	79.80	60.50
10^{-3}	100	51.00
10^{-2}	15.70	18.20

expression and biochemical characterization of recombinant *Drosophila* AP-P, which was expressed and purified from *E. coli* (Kulkarni and Deobagkar). Here, we report the purification and characterization of AP-P from *Drosophila* pupae.

Table 2. Effect of divalent metal ions on substance P hydrolysis by *Drosophila* AP-P: The purified *Drosophila* enzyme (100ng) was incubated with the indicated concentration of divalent cation for 15min at 37°C. On addition of 0.1mM substance P, the samples were incubated at 37°C for 3hrs. The activity was analyzed and is expressed as percent of the activity observed in the absence of added metal ions. Results are the mean values of duplicate determinations for each concentration which differ by less than 8%.

Divalent ion	Concentration (mM)	Relative activity (%)
None	-	100
Mn^{2+}	0.01	103
	0.1	185
	1	211
Co^{2+}	0.01	91
	0.1	74
	1	41
Mg^{2+}	0.01	112
	0.1	89
	1	76
Ca^{2+}	0.01	146
	0.1	151
	1	75
Cu^{2+}	0.01	63
	0.1	42
	1	10
Ni^{2+}	0.01	41
	0.1	19
	1	0
Zn^{2+}	0.01	48
	0.1	9
	1	0

The cytosolic AP-P was immunoprecipitated from the soluble extract of pupae using the polyclonal antibodies raised in rabbit against the recombinant DAP-P expressed in *E. coli* (Kulkarni and Deobagkar). The purity was confirmed by SDS-PAGE analysis and western blotting. A single immunoreactive band of DAP-P of the expected size (69kDa) could be detected on the western (Data not shown). The activity of the purified enzyme was assayed using a fluorimetric assay adapted from the method of Simmons and Orawski (Simmons and Orawski, 1992). All the enzyme activity measurements were carried out as described previously by Kulkarni and Deobagkar (Kulkarni and Deobagkar). The *Drosophila* enzyme could hydrolyze the Xaa-Pro bond of the natural peptides, bradykinin and substance P which are typical known substrates for mammalian AP-Ps. Cheating agent, EDTA at the concentration of 100µM inhibited 80% of the enzyme activity (Data not shown). This confirmed the metalloenzyme nature of *Drosophila* AP-P. The enzyme could hydrolyze substance P in the pH range of 7.2-8.0, showing the highest activity at pH 7.4-7.6 (Data not shown).

Effect of divalent metal ions on the enzyme activity was also studied. When, the effect of Mn^{2+} ions on hydrolysis of substance P and bradykinin by *Drosophila* AP-P was analyzed, the enzyme showed a substrate dependent activity (Table 1). Mn^{2+} stimulated hydrolysis of substance P at micromolar range, while no effect on the enzyme activity was observed towards bradykinin hydrolysis. The higher Mn^{2+} concentrations inhibited the enzyme activity towards both the substrates. On the basis of Mn^{2+} dependence, substrates for pig kidney membrane bound AP-P were divided into two groups (Lloyd *et al.,* 1996). The hydrolysis of Group I substrates (β-casomorphin, Gly-Pro-Hyp and substance P) were substantially stimulated by $MnCl_2$, whereas, there was no effect on the metabolism of Group II substrates (bradykinin, Arg-Pro-Pro) at low concentrations of Mn^{2+}. Unlike the human cytosolic AP-P, which showed stimulatory effect of Mn^{2+} on the bradykinin hydrolysis (Cottrell *et al.,* 2000), the activity of *Drosophila* AP P towards bradykinin was not affected by Mn^{2+}.

Divalent ions like Ca^{2+} and Mg^{2+} stimulated the hydrolysis of substance P at micromolar (10µM -100µM) concentrations, but were inhibitory at 1mM concentration. 90% DAP-P activity was inhibited by 1mM concentration of Cu^{2+}. Co^{2+} had no considerable effect on the enzyme activity at

lower concentrations, while 1mM concentration inhibited 59% of the activity. 1mM concentrations of Ni^{2+} and Zn^{2+} completely inhibited the enzyme. It was reported that Zn^{2+} activated the porcine membrane bound form of AP-P to some extent, in addition to Mn^{2+} (Lloyd *et al.,* 1996), while human cytosolic form of AP-P was inhibited by Zn^{2+} (Cottrell *et al.,* 2000). *Drosophila* AP-P activity was found to be inhibited by Zn^{2+} ions.

Thus, the *Drosophila* AP-P showed a manganese dependent activity with an optimum pH of 7.4-7.6 and is inhibited by Zinc. Both these properties reflect similarities with the mammalian cytosolic form of AP-P. Ca^{2+} and Co^{2+} could stimulate the *Drosophila* AP-P activity in contrast to human AP-P, which is inhibited by these metal ions. The enzyme activity was stimulated by Mn^{2+} ion in a substrate dependent manner. The purified soluble *Drosophila* enzyme is thus metallopeptidase with the ability to cleave Xaa-Pro bond from peptides like substance P and bradykinin. Presence of N-terminal Xaa-Pro containing peptides have been documented in D*rosophila* (Nassel *et al.,* 1990). Since, the physiological significance and the precise role of cytosolic AP-P in mammals have not yet been elucidated, further study of *Drosophila* AP-P will be a powerful addition to approaches available for the elucidation of structure-function relationships of this important enzyme.

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