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Leucine Aminopeptidase and Ferricyanide Reductase Activities in Radish Microsomes

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Abstract. A NADH–ferricyanide reductase activity found in radish microsomes isolated from germinated seeds has been shown to be stimulated by pCMB and pCMBS which are both strong nactivators of many plant proteolytic enzymes. In the same preparation a leucine aminopeptidase was found while endoprotease and carboxypeptidase activities were not detected using exogenous substrates. The aminopeptidase, highly active at the same optimal pH–condition of FeCN reductase, was stimulated by $CoCl_2$ and non-polar detergents (Triton X–100 and Brij 35). It was inhibited by sulphydryl reagents. By gel filtration of microsomal detergent extract two peaks of activity were separated: red I coeluted with LeuAPase and red II, free of aminopeptidase. Red I, a protein, was inhibited by sulphydral reagents and stimulated by duroquinone. Red II, stimulated by pCMB, is not a protein because of the small size and the noninfluence of heating treatment on catalytic activity.

Additional index words: Raphanus sativus L.; proteolytic enzyme – plasma membrane; endoplasmic reticulum; NADH–dependent ferricyanide reductase

The microsomal fraction contains plasmalemma and endoplasmic reticulum membranes. The plasma membrane has cytochromes of the b-type, flavoproteins and quinones, while NADPH-cyt P–450 and NADH-cyt b_5 reductases are present in ER membranes. These enzymes, like all cellular proteins, are continously broken down and, for the most part, renewed. It is now evident that proteolytic reactions play a key role not only in the regulation of intracellular protein turnover, but also in the control of many other physiological functions (*e.g.*, translocation of proteins and enzyme regulation). A considerable number of plant proteolytic enzymes has been described so far, but only in rare cases have the catalysts been associated with a clear-cut function.

Recently a NADH-ferricyanide oxidoreductase activity present in radish microsomes has been shown to be strongly stimulated by pCMB and pCMBS (Vianello and Macri' 1989); moreover, plasma membrane NADH-oxidases are generally inhibited by pCMB or similar compounds (Askerlund *et al.* 1987). Plant proteases

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are mainly thiol enzymes, and numerous accounts involving a variety of plant genera suggest that sulphydryl enzymes are synthesized as one of the initial events of germinating seeds (Ryan and Walker–Simmons 1981). Few data are available on the proteolytic activities present in the microsomal membrane of plants and nothing is known about plant membrane-bound APases (Mikola 1986) although LeuAPase may be an ubiquitous component of all plant tissues. Golgi-ER enriched fraction of spinach callus contains a membrane-bound protease, assayed on casein as substrate with 7.5 of pH optimum, that is strongly inhibited by pCMB and stimulated by detergent deoxycholate (Satoh and Fujii 1985). The only data available on the plasmalemma proteolytic enzymes present in the plant kingdom concern the unicellular alga *Poterioochromonas malhamensis* (Kauss 1986). In the plasma membrane of this alga a proteinase capable of activating the enzyme α-galactosyl-(1→1)-glycerol-3-phosphoric acid synthase has been found. A preliminary experiment showed that 1 mM pCMB totally inhibited autolysis in radish microsomes (data not shown). The limited knowledge about the proteolytic system in microsomal membranes and the possible influence of radish microsomal protease (s) on ferricyanide reductase activity stimulated the present study.

MATERIAL AND METHODS

Material

Radish seeds (*Raphanus sativus* L., cv. Tondo Rosso Quarantino) were germinated for 24 h in the dark in distilled water (5 g of seeds with 75 ml water in 500 ml bottle) renewed after 12 h, in an agitated bath at 27 °C.

Preparation of Microsomes

One day seedlings, decoated and washed with water, were ground by mortar and pestle with 5 volumes of 0.4 M mannitol, 20 mM Hepes-Tris (pH 7.6), 5 mM $MgCl_2$, 1 mM DTT, 1 mM Na-EDTA, 0.1 % BSA and filtered through eight layers of cheesecloth. The suspension was centrifuged for 10 min at 13 000× *g*. Supernatant was then centrifuged for 30 min at 80 000× *g*. Pellets were resuspended in 20 mM Hepes-Tris (pH 7.5), 0.4 M mannitol with a glass homogenizer. Microsomes were stored at –40 °C. Marker enzyme tests described in Vianello and Macri' (1989) were used to determine the posible contamination of the microsomal fraction with other cellular components.

Abbreviation used: APase, aminopeptidase; BSA, bovine serum albumine; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FeCN, ferricyanide; HEPES, 4-(2-hydroxyethyl)-1-piperazineerhanesulfonic acid; LeuAPase, leucine aminopeptidase; MES, 4-morpholineethane-sulfonic acid; NA, β-naphthylamide; NEM, N-ethylmaleimide; pCMB, p-chloromercuribenzoic acid; pCMBS, *p*-chloromercuribenzenesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; Z-, carbobenzoxy-;

Gel Filtration

The microsomal proteins were solubilized by stirring for 30 min, on an ice bath, with 1 % Triton X–100. The suspension was then centrifuged at 12 000× *g* for 20 min and 2 ml of soluble proteins were applied to a column of Sephadex G–100 (1.5 × 22 cm) equilibrated with Na-phosphate 20 mM (pH 7), 1 mM DTT and O.1 % Triton X–100. The gel filtration was done at 4 °C and at 5 ml h^{-1} flow rate. Fractions of 1.5 ml were collected.

Enzymatic Assay

Oxidation of NADH and reduction of ferricyanide was measured by following loss of absorbance at 340 and 420 nm, respectively. The reaction mixture contained 20 mM Hepes-Tris pH 7 (or 20 mM MES–NaOH, pH 5), 0.4 M mannitol, 1 mM EDTA, 0.25 mM NADH, 0.25mM $K_3Fe(CN)_6$ and an aliquot of sample contained 50–100 μg of protein. LeuAPase and other proteolytic activities were assayed as described in Scalet *et al.* (1984). The method of Feller and Erismann (1978) was followed when azocasein was used as substrate.

Protein Determination

Protein was measured by the method of Lowry *et al.* (1951) using BSA as standard. The protein present in the fractions eluted from Sephadex columns was estimated by absorbance at 280 nm.

RESULTS

The microsomal fraction obtained from germinated seeds of *Raphanus sativus* contained little or no functional mitochondrial membranes. These microsomes possessed a NADH-dependent activity inducing the reduction of ferricyanide. The basal FeCN-reduction was strongly stimulated by the addition of pCMB which is a common inhibitor of thiol enzymes. The microsomes were treated with 1 % Triton X–100 and ferricyanide reductase activity was mostly recovered in the soluble fraction. The gel filtration on Sephadex G–100 of soluble microsomal fraction resulted in the separation of two reductase activities, red I and red II (Fig. 1). Both activities used NADH as electron donor and ferricyanide as acceptor, but they showed different characteristics. The largest, with a molecular size close to bovine serum albumine and more active at neutral pH, was inhibited by pCMB and stimulated by duroquinone (Table 1). By contrast, red II was inhibited by duroquinone and stimulated by pCMB. Moreover, this activity was less dependent upon pH and preferentially active at acidic pH. Being eluted at the end of the chromatog-

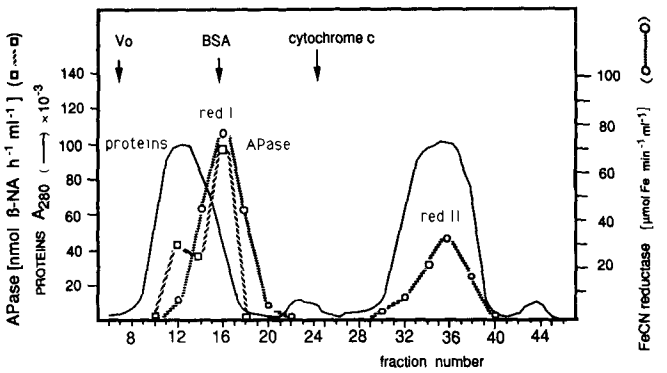

Fig. 1 – Gel filtration by Sephadex G-100 column of leucine aminopeptidase and ferricyanide reductase activities present in the detergent extract of radish microsomes.

TABLE 1.

Activity of two peaks of ferricyanide NADH-reductase in presence of various compounds.

Reagents	mM	RED I	RED II
control		100 (21.25)	100 (9.8)
pCMB	0.5	62	164
	1	57	254
Mersalyt	0.5	54	–
	1	48	–
PMSF	0.5	95	–
	1	80	–
Duroquinone	0.25	150	0–50
	0.5	161	0–30
Coumaric acid	0.25	100	100
	0.5	100	100
	1	100	100

The activity is expressed as % of control. In the controls, activity given as µmol Fe^{3+} min^{-1} protein is in brackets. (–) not tested.

raphy and unaffected by heating treatment, red II did not contain a protein but a small molecule and it cannot be considered an enzymatic activity.

Autolytic protein degradation occurring in microsomes was totally inhibited by pCMB (data not shown). Different substrates commonly used to detect proteases and peptidases were incubated with the radish microsomes (Table 2). Microsomal endoproteolytic and carboxypeptidase activities did not digest various exogenous substrates. In fact, the amount of alpha-amino-N liberated during autolysis – incubation of the microsome without any exogenous substrate – was the same or

TABLE 2.

Autolysis and proteolytic activity on different substrates present in radish microsomal fraction.

Substrate	Activity [nmol h^{-1} mg^{-1} (protein)]	
autolysis	11.8	(± 1.4)
azo-casein	0	
hemoglobin	10.3	(± 0.7)
Z-Phe-Ala	7	(± 1.2)
Z-Phe-Leu	9.7	(± 0.4)
Z-Ala-Leu	12.3	(± 3.4)
Leu-β-NA	110	(± 15)

Experiments have been repeated three times each with three replications. The standard deviation is indicated in brackets. TCA has been immediately added in the controls. For Leu-β-naphthylamide the activity was measured as nmol β-NA h^{-1} mg^{-1} of protein; the controls are incubated in an ice bath.

higher than in the presence of substrate. Only aminopeptidase activity, LeuAPase, was detected by incubation with artificial substrate LeuNA under condition as for pCMB-stimulated NADH-ferricyanide oxidoreductase. Therefore all subsequent experiments were carried out on the aminopeptidase activity.

The column chromatography by Sephadex G–100, shown in Fig. 1, resulted in the

TABLE 3

Effect of various compounds and ions on leucine aminopeptidase present in radish microsomes

Reagents	mM	Activity [% of control]	
none		100	(± 0.8)
pCMB	1	18	(± 0.5)
pCMBS	1	25	(± 0.7)
PMSF	1	28	(± 5.0)
NEM	1	64	(± 3.3)
Mersalyl acid	1	42	(± 1.5)
$CoCl_2$	1.25	129	(± 0.4)
$CoCl_2$	10	158	(± 3.1)
$CuSO_4$	1.25	112	(± 0.4)
$MnCl_2$	1.25	110	(± 0.5)

The microsomes were preincubated with chemicals for 10 min at 27 °C with agitation, and then incubated for 30 min at 27 °C with McIlvaine buffer, pH 6.5 and substrate. The activity of the APase in the control was 145 nmol h^{-1} mg^{-1} (protein) (= 100). The standard deviation is indicated in brackets.

coelution of LeuAPase and red I FeCN NADH-dehydrogenase. LeuAPase activity was clearly inhibited by triol reagent (expecially by pCMB and pCMBS) and also by PMSF (Table 3) which can affect thiol as well as serine-proteases. The aminopeptidase was higly active (with a pH optimum of 6) (Fig. 2) at the optimal neutral pH and temperature of FeCN-reductase assay. The anionic detergents sodium dodecyl sulfate (SDS) and deoxycholate inhibited microsomal LeuAPase, while Triton X-100 and Brij 35, non-ionic detergents, showed a moderate stimulation of LeuNA-degrading activity (Table 4). Some divalent cations seem to stimulate the aminopeptidase. Particularly, cobalt stimulate the aminopeptidase. Particularly, cobalt stimulated the breakdown of substrate (average 30 % more than control).

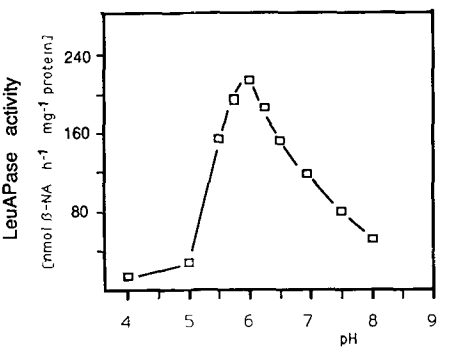

Fig. 2 Effect of pH on leucine aminopeptidase activity.

TABLE 4

Effect of detergents on microsomal APase of radish seedlings

Detergent	Concentration [%]	Activity [% of control]	
none		100	(± 0.8)
deoxycholate	0.05	91	(± 1.1)
SDS	0.05	14	(± 0.2)
SDS	0.5	3	(± 0.4)
Triton X-100	0.05	102	(± 0)
Triton X-100	0.5	123	(± 2.6)
Brij 35	0.05	112	(± 2.6)
Brij 35	0.5	121	(± 3.0)

Microsomes were incubated with McIlvaine buffer, pH 6.5, at 27 °C for 30 min. The activity of the APase in the control (no detergent) was 157 nmol h^{-1} mg^{-1} (protein) (= 100). Standard deviation is in brackets.

DISCUSSION

Radish red I NADH–dehydrogenase resembles the plant redox activities normally present in microsomes. In fact duroquinone–sensitive NAD (P) h dehydrogenases appear as a common redox component of plant microsomes. Plasma membranes and endoplasmic reticulum from cauliflower and mung bean possess duroquinone--stimulated NADH–oxidase activities (Asard *et al.* 1987). The quinone also enhances NADH oxidation by corn root protoplasts (Lin 1984) and two duroquinone–dependent NAD (P) H dehydrogenases are present in Cucurbita microsomes (Pupillo *et al.* 1986).

Radish aminopeptidase showed a molecular size very close to that of pea (Elleman 1974) and spinach proteases (Satoh and Fujii 1985) and it falls in the molecular mass range (70–86 000) of most plant APases (Ryan and Walker–Simmons 1981). No other data is available on proteolytic activities in microsomal membranes of plants.

Most of the studies on function of plant proteolytic enzymes have been focused on nitrogen mobilization during seed germination and leaf senescence. Plant aminopeptidase apparently does not play a major role in mobilization of nitrogen for embryo growth (Ryan and Walker–Simmons 1981). The role of radish APase in microsomal membranes should be related to the chain shortening of proteins. In fact the study of protease function in organelles like ER and Golgi body has focused attention on co– and post-translational proteolytic modification of proteins (Strauss *et al.* 1979). On the other hand the plasma membrane proteases are very specific for substrate indicating a distinct regulatory role (Bond and Butler 1987): the membrane might function in giving specificity to the turnover system. Current knowledge comes from animal plasma membranes that generally contain largely endopeptidases but also some aminopeptidases. A Ca^{2+}–activated neutral protease bound to plasma membrane has been recently isolated and characterized from the vegetative mycelia of the aquatic fungus *Allomyces arbuscula*. The role of this enzyme is not clear. However, it may be involved in the mechanism of intracellular signalling or processing of proteins interacting with cell membranes (Ojha 1989).

The first source of NADH–FeCN reductase of radish microsomes eluted as red I has been characterized in the presence of LeuAPase. Because pCMB inhibited both activities, reductase and aminopeptidase, while it only stimulated the non-enzymatic activity red II, it is possible to conclude that radish microsomes containg a non-enzymatic FeCN reductase stimulated by thiol-blocking compounds like pCMB and pCMBS. On the other hand, the enzymatic FeCN reductase was not influenced by the proteolytic action of microsomal aminopeptidase. The pCMB–activation of ferricyanide reductase activity found in microsomal fraction of radish seedling (Vianello and Macri' 1989; this paper) concerns the activity of red II that may be a quinone compound but not the true enzymatic reductase activity. Further biochemical characteristics are necessary to understand the possible role of LeuAPase localized in radish plasma membranes.

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