Atti Accademia Nazionale dei Lincei

Classe Scienze Fisiche Matematiche Naturali

Rendiconti

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ATPase, ADPase and p-nitrophenylphosphatase activities in microsomal preparations from various plant materials. II. ATPase, ADPase and acid phosphatase activity and effects of inhibitors and solubilizing treatments

Atti della Accademia Nazionale dei Lincei. Classe di Scienze Fisiche, Matematiche e Naturali. Rendiconti, Serie 8, Vol. **74** (1983), n.2, p. 99–108.

Accademia Nazionale dei Lincei

<http://www.bdim.eu/item?id=RLINA_1983_8_74_2_99_0>

Atti della Accademia Nazionale dei Lincei. Classe di Scienze Fisiche, Matematiche e Naturali. Rendiconti, Accademia Nazionale dei Lincei, 1983.

Biochimica vegetale. — *ATPase, ADPase and p-nitrophenylphosphatase activities in microsomal preparations from various plant materials.* II. *ATPase, ADPase and acid phosphatase activity and effects of inhibitors and solubilizing treatments* (*). Nota di PIERANTONIO PESCI e NICOLETTA BEFFAGNA, presentata (**) dal Corrisp. E. MARRÈ.

RIASSUNTO. — L'inibizione da DES dell'attività ATPasica microsomiale raggiunge valori (sino all'80%) molto più alti di quelli ottenuti con DCCD. Il trattamento con EDTA 10 mM solubilizza con maggior efficacia questa attività enzimatica rispetto al trattamento con NaCl 0.1 M ed in entrambi i casi si rileva in genere un incremento di attività. L'attività ADPasica è largamente presente in tutte le specie esaminate, dove spesso supera quella ATPasica. Ciò vale anche per l'attività *p*–NPPasica (fosfatasi acida) che però è presente in pisello e fava in quantità molto ridotta (circa il 10% dell'ATPasica). I rapporti tra le attività ATPasiche e quelle rispettivamente ADPasiche e *p*–NPPasiche subiscono, in alcuni tra i materiali considerati, variazioni a seconda della presenza del magnesio nel mezzo di omogenizzazione impiegato nella preparazione dei microsomi.

Questi risultati suggeriscono che nelle preparazioni microsomiali di piante superiori sono presenti almeno tre classi di fosfoidrolasi, vale a dire un enzima specifico per l'idrolisi dell'ATP, un enzima idrolizzante ATP e ADP ed una fosfomonoesterasi aspecifica. Il livello di attività ATPasiche, che sono rese solubili con blandi trattamenti, presenti nei microsomi è in alcuni materiali influenzato dalla composizione del mezzo nella fase di omogenizzazione.

INTRODUCTION

Some characteristics (protein yield, ATPase activity and its dependence upon the presence of Mg^{2+} in the assay medium) of microsomal fractions prepared in the presence or not of 10 mM Mg^{2+} in the grinding medium were previously reported in a first note [1]; in this note the considered microsomal preparations were obtained from 11 plant materials and, namely: horse bean (*Vicia faba* L.), pea (*Pisum sativum* L. cv. Alaska), chick pea (*Cicer arietinum* L.), lentil (*Lens esculenta* L.), kidney bean (*Phaseolus vulgaris* L. cv. Borlotto), azuki bean (*Vigna angularis* L.) zucchini squash (*Cucurbita pepo* L.), radish (*Raphanus sativus* L.), barley (*Hordeum vulgare* L.), oat *Avena sativa* L.) and corn (*Zea mays* L. cv. Dekalb XL 640). This second note deals with the sensitivity of the ATPase activity of the different considered plant materials to DES

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(**) Nella seduta dell'8 gennaio 1983.

Abbreviations: DES, diethylstilbestrol; DCCD, N,N′-dicyclohexylcarbodiimide; EDTA, ethylenediaminetetraacetic acid; *p*-NPP, *p*-nitrophenylphosphate; Hepes, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid.

and DCCD, and with the effects of solubilizing treatments, such as 0.1 M NaCl and 10 mM EDTA treatment, on the microsomal ATPase; the capability of these microsomal preparations to hydrolyze ADP and *p*-nitrophenylphosphate was also tested.

Materials and methods

The growth conditions and the preparation of the microsomal fractions were the same as previously described [1].

ATPase and ADPase activity assay.

For the evaluation of the ATPase and of the ADPase activity the routine incubation mixture, the assay conditions and the colorimetric procedure to measure the released phosphate were the same as for the ATPase assay previously described [1]. When the effect of diethylstilbestrol (DES) or of dicyclohexylcarbodiimide (DCCD) was tested, the inhibitors were added at the 3×10^{-4} M concentration in the presence of 10 mM $MgCl_2$ and the mixture was allowed to stand for 10 min at room temperature before adding the substrate.

p-nitrophenylphosphatase activity assay.

The incubation mixture was routinely composed of 100 μl of extract (containing an amount of active preparation three times higher than for the ATPase activity assay), 50 μmol KCl, 10 μmol $MgCl_2$ and 3 μmol substrate in a final 1 ml volume of 250 mM sucrose, 10 mM Hepes buffer (pH 6). The assay conditions and the colorimetric technique to evaluate the hydrolysis of phosphate were the same as for the ATPase and ADPase assay.

Enzyme solubilization.

The solubilizing treatments were performed by resuspending the microsomal preparations in a 250 mM sucrose, 5 mM Hepes (pH 7) medium containing or not 100 mM NaCl or 10 mM EDTA; the ratio between medium and microsomes was of 6 ml buffer/g initial tissue fresh weight. The suspensions were allowed to stand at 0 °C for 30 min, and then centrifuged at 80000 × g for 20 min; the enzyme activities were assayed on both the supernatants and the resuspended sediments.

Results and discussion

I - *Sensitivity to DES and DCCD.*

Fig. 1A shows the effect of 3×10^{-4} M DES on the ATP hydrolyzing activity of the different tested species. The maximum inhibition by DES is observed in microsomes from horse bean (more than 90%), corn (ca. 80%), pea, Azuki

bean and kidney bean (ca. 70%), while a very low inhibitory effect is detected in radish shoots (ca. 10%) and in chickpea roots (ca. 20%). It is of interest to note that the ATP hydrolyzing activities both of chickpea and of pea and horsebean microsomes, which have such a different sensitivity to DES, show the same behaviour towards Mg^{2+}, when added to the incubation mixture of the ATPase test; in fact the cation strongly activates all of these activities [1].

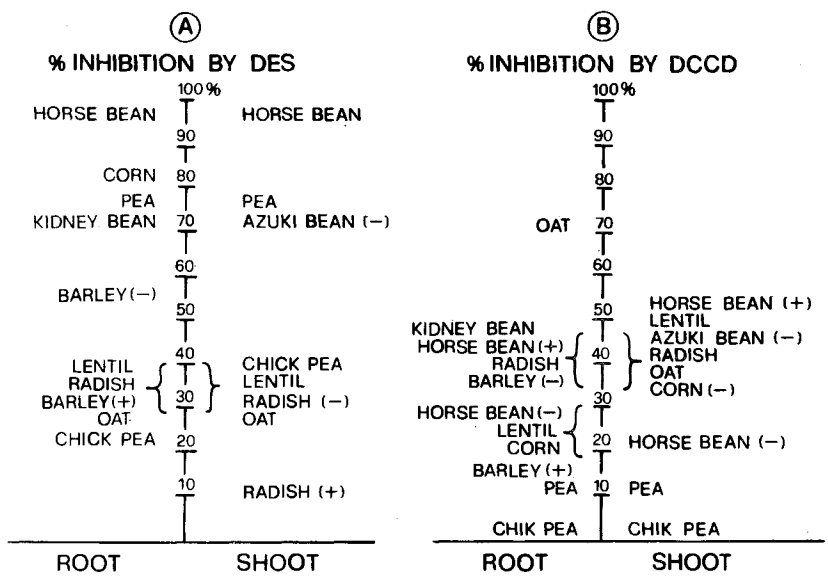

Fig. 1. – Effects of DES and DCCD on ATPase activity.
(—) and (+) indicate the condition of absence or presence of 10 mM Mg^{2+} in the homogenization medium. The lack of any sign indicates that the values are almost equal in the two conditions.

Generally the ATPase activity does not show any change of sensitivity to DES within the same species when microsomal preparations are obtained in the presence or not of Mg^{2+} in the grinding medium, however some change in the effect of the inhibitor is observed in barley roots and radish shoots in the two different homogenization conditions.

Fig. 1B shows that the sensitivity to DCCD of the microsomal ATPase activity is generally lower than that to DES for all of the tested species; in fact only in the case of oat roots a 70% inhibition is achieved, while for all the other plant preparations the inhibition is lower than 50 %. A difference between shoot and root ATPase sensitivity to the inhibitor is detectable only again in the case of oat microsomal preparations, being practically negligible for all the other species. The different composition of the grinding medium induces a different sensitivity to DCCD only in horse bean (both shoot and root) and in barley root.

TABLE I

% *solubilization of microsomal ATPase activity by NaCl and EDTA treatments.* Experimental conditions as described in methods. The values are the average of three experiments run in triplicate.

	Root microsomes resuspended in:			Shoot microsomes resuspended in:		
	plain buffer	plus 0.1 M NaCl	plus 10 mM EDTA	plain buffer	plus 0.1 M NaCl	plus 10 mM EDTA
Horse bean	20	47	77	10	30	80
Pea	39	48	64 (—) 86 (+)	7	45	90
Chick pea	40	45	84	53	50	72
Lentil	50	50	58	38	40	48
Kidney bean	54	68	70	—	—	—
Azuki bean	—	—	—	20	58 (—) 37 (+)	55
Zucchini squash	55	67	70	—	—	—
Radish	38	45	53	20	61	62
Barley	32	53	64	30(—) 14(+)	66 (—) 38 (+)	67 (—) 44 (+)
Oat	23(—) 36(+)	45	56	30	46	52
Corn	55	66	70	—	—	—

(—) and (+) indicate the condition of absence or presence of 10 mM Mg^{2+} in the homogenization medium.

The lack of any sign indicates that the values are almost equal in the two conditions.

II – *Stability of the binding of the ATPase activity to the microsomes.*

Mild treatments such as simple washings of the microsomes with buffer induce the release of variable amounts of ATP hydrolyzing activities from the membranes, the maximum solubilization (50%) being observed in chick pea shoots (Table I). The release of ATPase due to these treatments is, in some case, greater in roots than in shoots. When a 0.1 M NaCl treatment is per-

TABLE II

% increment of ATPase activity induced by NaCl and EDTA solubilizing treatments. Experimental conditions as described in methods. The control is the microsomal preparation resuspended in plain buffer.

		Root		Shoot	
		0.1 M NaCl	10 mM EDTA	0.1 M NaCl	10 mM EDTA
Horse bean	(—)	+ 19	+ 5	+ 14	— 8
	(+)	— 3	— 20	0	— 11
Pea	(—)	+ 53	— 9	+ 10	+ 40
	(+)	+ 77	+ 50		
Chick pea	(—)	+ 51	+ 43	+ 15	+ 16
	(+)	+ 220	+ 182	+ 3.5	— 11
Lentil	(—)	+ 19	+ 50	+ 31	+ 47
	(+)	+ 115	+ 78	+ 45	+ 39
Kidney bean	(—)	+ 10	+ 42	—	—
	(+)	+ 13	+ 30	—	—
Azuki bean	(—)	—	—	+ 14	+ 15
	(+)	—	—	— 23	— 22
Zucchini squash	(—)	—	—	0	+ 43
	(+)	—	—	+ 45	+ 146
Radish	(—)	+ 39	+ 81	+ 61	+ 126
	(+)	+ 5	+ 83	+ 5	+ 134
Barley	(—)	— 1	+ 3	+ 3	+ 8.4
	(+)	— 9	— 2	+ 8	+ 16
Oat	(—)	+ 75	+ 167	— 11	+ 5
	(+)	+ 73	+ 157	+ 39	+ 88
Corn	(—)	+ 21	+ 40	—	—
	(+)	+ 37	+ 58	—	—

(+) and (—) indicate the microsomal preparations obtained in the presence or absence of 10 mM Mg^{2+} in the grinding medium.

formed on the membranes, a higher solubilization is achieved with the exception of the microsomes from chick pea shoots and from lentil roots which release the same amount of ATPase as when they are submitted to a simple washing with buffer. When membranes of shoots and roots are treated with 10 mM EDTA a very large part of ATPase is shifted to the soluble form; in fact this agent is generally the most efficient in solubilizing the enzymes from the microsomes, even if in some case it leads to a release of ATPase very close to that obtained with a NaCl treatment.

As a rule the presence or absence of Mg^{2+} in the grinding medium does not cause any change, within the same species, in the stability of the binding of the ATPase to the membranes except for barley, where a higher release of ATPase is observed when the microsomes are prepared in the absence of magnesium.

Table II shows that both NaCl and, at a greater extent, EDTA treatments induce an increase of the microsomal ATPase activity. Very high activity increments are obtained in chick pea and oat roots by treatment with NaCl or EDTA, and in radish roots and shoots and in oat shoots (only when EDTA is used). No increase of ATPase activity, or a very scarce one, is observed in horse bean and barley shoots and roots treated with both the solubilizing agents, and only minor changes are observed for the other tested species.

III – *ADPase activity.*

ADPase activity, which is largely present in all of the examined microsomal preparations, is usually higher than ATPase activity (Fig. 2). The maximum difference between these two activities is observed in corn root preparations

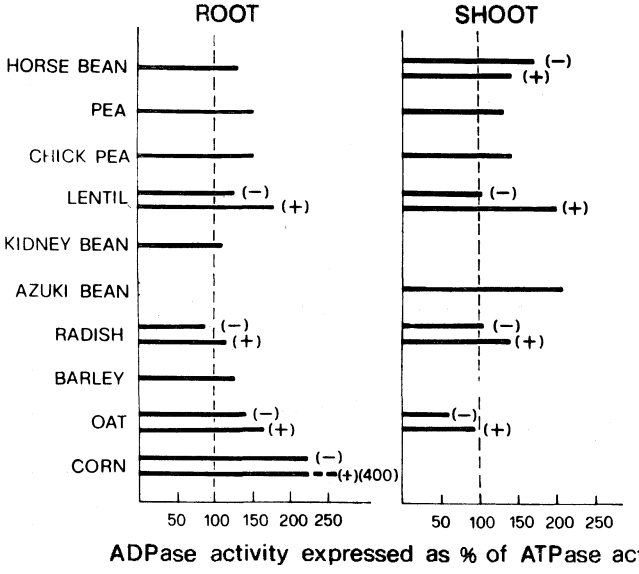

Fig. 2. – Comparison between ADPase and ATPase activity. (—) and (+) indicate the condition of absence or presence of 10 mM Mg^{2+} in the homogenization medium. The lack of any sign indicates that the values are almost equal in the two conditions.

where the activity towards ADP is four times higher than that toward ATP, when the microsomes are prepared in the presence of Mg^{2+} in the grinding medium. Only in microsomes from radish roots, oat shoots and from corn shoots prepared in the presence of Mg^{2+} ADPase activity is lower than ATPase activity or very close to it.

The presence or absence of magnesium in the grinding medium mainly influences the activities from corn roots and from lentil shoots and roots, whereas only a slight difference in activity is observed in microsomes from horse bean shoots. No change in activity is detected in microsomal preparations from chick pea and pea.

IV – *p-nitrophenylphosphatase activity.*

A large variability is observed in the *p*-nitrophenylphosphate (*p*-NPP) hydrolyzing activity: the microsomes from both shoots and roots of some species show activity values even double those of the activity toward ATP, while the *p*-NPPase activities of the microsomes from horse bean and pea amount only to 10% of it, or even to lower values (Fig. 3).

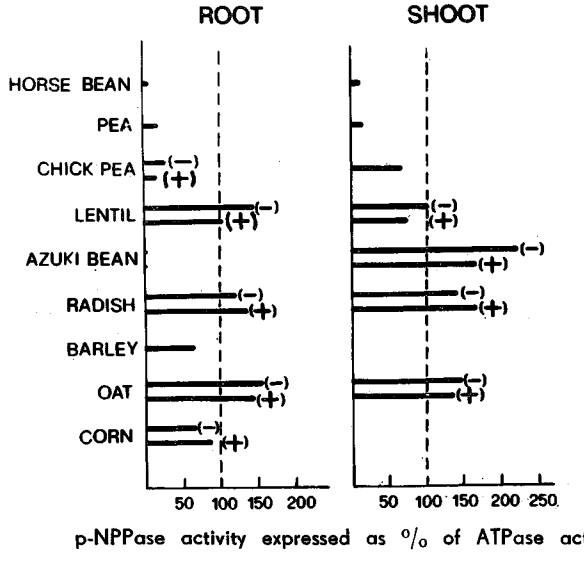

Fig. 3. – Comparison between *p*–NPPase and ATPase activity.
(—) and (+) indicate the condition of absence or presence of 10 mM Mg^{2+} in the homogenization medium. The lack of any sign indicates that the values are almost equal in the two conditions.

The presence or absence of Mg^{2+} in the grinding medium induces only small effects on the *p*-NPPase activity, and no sensitivity to the Mg^{2+} when added to the incubation medium for the enzyme test, is observed (data not shown).

DCCD and DES show practically no inhibitory effect on the *p*-NPPase activity of our preparations: in fact, the maximum inhibitions, observed only in some case (chick pea, oat and radish), amount to 10–20%.

Conclusions

The results reported above show a marked heterogeneity of the microsomal preparations obtained from the 11 species here investigated. This heterogeneity comprises: *a*) the sensitivity to the ATPase inhibitors DES and DCCD; the tendency of the ATPase activity to shift from the microsomal to the soluble form following relatively mild treatments; *c*) the relative activities against ADP and *p*-nitrophenylphosphate as substrates. In particular:

I – *Inhibitors.*

DES and DCCD, two widely used (although not very specific), inhibitors of plasmalemma ATPase [2, 3, 4, 5] are practically inactive on the *p*-NPPase activity of our preparations while they show consistent differences in inhibiting the ATPase activities of the microsomal preparations from the different plant materials. Horse bean, corn, pea, azuki bean, kidney bean and barley microsomal ATPase is more sensitive to DES than to DCCD, while the opposite is true for oat (roots). Large differences are also observed between the microsomes from shoot or, respectively, root tissue of the same species, depending on the presence or absence of Mg^{2+} in the homogenization medium.

II – *Stability of the binding of the ATPase activity in the microsomal particles.*

The ATPase, ADPase and *p*-NPPase activities reported in this paper are those detectable at pH 6 in "crude" microsomal preparations, namely the fractions precipitating in 0.250 M sucrose buffer between 13000 and 80000 × g. This activities can be considered as particle-bound at least in a very preponderant proportion, as the contamination from the homogenate soluble fraction and from soluble enzymes sequestrated inside sealed vesicles during the preparation of the homogenate should be almost negligible. However, the problem of the strength of the bond between the microsomal enzyme activities here considered and the membrane is open to investigation. The data presented in this paper show that a very mild treatment (washing with a low ionic strength buffer) can remove from 30 to 50% of the ATPase activity in 13 of the 18 materials investigated. Less mild treatment such as that with 0.1 M NaCl or with 10 mM EDTA can rise in many cases the ATPase solubilization value to more than 50%, and in pea, chick pea and horse bean to more than 80%.

III – *ADPase activity.*

A remarkable ADPase activity is present in the microsomal preparations from all of the plant materials investigated, with values (at pH 6) similar or, often, higher than those of the ATPase activity. The highest ATPase/ADPase activity ratios are seen in oat (shoot), radish (root), barley (root). This ratio

is increased by the presence of Mg^{2+} in the homogenization medium, in all cases with the exception of horse bean; moreover it shows large differences, within a same species, depending on whether the microsomes have been obtained from the root or, respectively from the shoot (see f.e. corn and oat in Fig. 2).

IV – *p-nitrophenylphosphatase.*

The microsomal preparations from all the plant materials here investigated also show a *p*-NPPase activity, which is comparatively very low in horse bean, pea and chick pea, wereas it reaches values similar or even higher than those of the ATPase activity on the microsomes from the other investigated species. The ATPase/*p*-NPPase ratio appears little influenced by the presence of Mg^{2+} in the homogenization medium.

For the purpose of the main aim of the present research, namely a preliminary guide to the identification of materials suitable for the characterization of cell membrane ATPase activities, these results lead to the following conclusions:

1 – the microsomal preparations from practically all of 20 materials here investigated contain phosphohydrolases able to attack ATP, ADP and *p*-NPP. The results of the experiments here reported, in agreement with previous more detailed works on some of the materials here investigated [6, 7, 8] strongly suggest that at least three classes of phosphohydrolases are present in the "microsomal" preparations of most higher plants: *i*) ATP specific phosphohydrolase; *ii*) relatively aspecific nucleosidephosphohydrolase attacking nucleosides di and triphosphates but not other phosphate esters; *iii*) largely aspecific acid monophosphoesterase. The relative ratios between these enzyme activities vary very widely from plant to plant species.

2 – The procedure followed in preparing the microsomes, and in particular the ionic composition of the incubation medium, markedly influences the recovery of ATPase activity in the microsomal fraction, and the relative ratios among the ATPase, ATP-ADPase and acid phosphomonoesterase activities.

3 – The binding of the ATPase activities (and that of other microsomal phosphohydrolases) to the microsomal membranes appears relatively loose, and mild treatments such as washing the microsomes with NaCl or EDTA can, in many materials, shift a very large enzyme activity fraction to the free soluble form.

It seems that the consideration of these general features of the phosphohydrolases situation of microsomal preparations from plant materials may provide a useful basis for further work aimed at the identification of the best material for studies on plant membrane ATPases.

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