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Low molecular weight humic substances stimulate H⁺-ATPase activity of plasma membrane vesicles isolated from oat (*Avena sativa* L.) roots*

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Abstract

The effect of <5 KDa (low molecular weight, LMW) and >5 KDa (high molecular weight, HMW) humic fractions on transport activities of isolated plasma membrane vesicles was studied. The K⁺-stimulated component of the ATP-hydrolyzing activity was considerably increased by LMW humic substances at concentrations ranging from 0.075 mg org CL⁻¹ to 1 mg org CL⁻¹. The stimulation was still evident when the detergent Brij-35 was added in the assay mixture, indicating a direct effect of LMW humic substances on plasma membrane ATPase activity. The LMW humic fraction stimulated ATP-dependent intravesicular H⁺-accumulation with a pattern similar to that recorded for ATP hydrolysis. LMW humic substances induced also an increase in passive membrane permeability to protons, as revealed by following the dissipation of an artificially imposed pH gradient. Membrane permeability to anions, as measured by the anion-dependent active proton accumulation was affected by LMW humic substances. In the presence of NO₃⁻ these molecules clearly enhanced proton transport, while Cl⁻-dependent activity was almost unaffected, thus suggesting a specific action of LMW humic fraction on transmembrane NO₃⁻ fluxes. On the other hand, HMW humic substances decreased the passive permeability to protons and reduced the anion-dependent intravesicular H⁺-accumulation. The results suggest that the stimulatory effect of soil humic substances on plant nutrition and growth might be, at least in part, explained on the basis of both direct action of LMW humic molecules on plasma membrane H⁺-ATPase and specific modification of cell membrane permeability.

Abbreviations: A.O. — acridine orange; BSA — bovine serum albumine; BTP — bis-tris-propane (1,3-bis(tris(hydroxy-methyl)-methylamino)-propane); DDT — DL-Dithiothreitol; EGTA — ethylene glycol bis(β-aminoethyl ether)-N,N'-tetracetic acid; HMW — high molecular weight; IDA — iminodiacetic acid; LMW — low molecular weight; MES — (2(N-morpholino) ethanesulfonic acid); PMSF — phenylmethylsulfonyl fluoride; PVPP — polyvinylpyrrolidone; SDS — sodium dodecyl sulfate

Introduction

The plasma membrane of root cells constitutes the major membrane barrier between cytoplasm

and soil environment. Working strictly in contact with the cytoplasm and the apoplast, it plays a central role in the complex interaction between root and soil solution, contributing to the regulation of solute transport processes and to the modification of the rhizosphere. Humified organic

*In memory to the late Professor Angelo Maggioni.

matter is an important element of soil fertility, improving not only physico-chemical characteristics of soil (Vaughan and Ord, 1985), but also exerting direct effects on plant growth and metabolism, as demonstrated by several investigations (Vaughan and Malcolm, 1985). In the plant-soil system the interaction between root cells and humic substances is possible when humic molecules present in the soil solution are small enough to flow in the apoplast and reach the plasma membrane. The capacity of humic substances to accumulate in the apoplast and, at least in part, enter into the cell has been demonstrated (Vaughan and Ord, 1981). More recently, evidence that organic acids present in root exudates can modulate the size of humic substances has also been presented (Albuzio and Ferrari, 1989).

Several investigations have suggested that humic acids extracted from the soil can affect plant nutrition through an action at the level of cell membranes (Chaminade, 1966). In particular, the interactions between the lipidic matrix of the plasma membrane leading to modifications of membrane permeability and fluidity have been interpreted on the basis of surface-active effect of humic acids (Samson and Visser, 1989). However, humic substances also affect the activity of KCl-stimulated ATPase activity of unfractionated microsomes isolated from oat roots (Maggioni et al., 1987; Nardi et al., 1991). Moreover, Pinton et al. (1992) have shown that low molecular weight humic substances can stimulate the ATP-dependent proton accumulation in tonoplast vesicles. In this paper we compare the effects of two fractions of humic substances characterized by different molecular weight on the transport properties of plasma membrane enriched vesicles isolated from oat roots. Evidences are provided that a LMW humic fraction, conceivably present in the soil solution, can directly stimulate the activity of the proton-translocating ATPase associated with the plasma membrane.

Materials and methods

Extraction of humic substances

The separation and purification of humic sub-

stances was performed as outlined by De Nobili et al. (1986).

Briefly, humic substances were extracted from a soil sample collected from the Ao horizon of a lithic rendoll (Fusine, Italy), by adding 10 mL of 0.1M $\text{Na}_4\text{P}_2\text{O}_7$ at pH 7.1 per gram of the air-dried soil and shaking for 1 h under N_2 atmosphere at room temperature. The suspension was centrifuged at 2500 g for 30 min; the resulting supernatant was filtered on a Whatman WCN 0.45 μm membrane filter and subsequently subjected to ultrafiltration through a YM5 membrane in an Amicon apparatus continuously supplied with 0.1 M $\text{Na}_4\text{P}_2\text{O}_7$, pH 7.1 at a pressure of 4 atm and a flux of 100 mL h^{-1} with continuous stirring under N_2 atmosphere. The collected fractions (<5 KDa and >5 KDa) were then acidified to pH 2.0 by the addition of concentrated H_2SO_4 , loaded onto a cross-linked polyvinylpyrrolidone (Polyclar AT, Serva) column (100 \times 20 mm) and washed with distilled water in order to remove the excess of pyrophosphate, which was reduced to less than 0.2 μM , and contaminating non humic organic compounds. Adsorbed humic material was then eluted from the column with 0.5 N NaOH. The excess of Na in the basic solution was eliminated with the cationic-exchange resin Amberlite IR 120 (H^+ form) and the pH brought to neutrality with 0.2 N NaOH. Humic substances were then frozen and lyophilized. Before use they were dissolved in distilled water (Pinton et al., 1992).

Plant material

Oat seeds (*Avena sativa* L. cv. Ombrone) were germinated and grown over an aerated solution of 1 mM CaSO_4 in the dark at 25°C. Roots were harvested when the seedlings were 4 d old.

Preparation of membrane vesicles

Roots, usually 50 g, were homogenized with a mortar and pestle in a freshly prepared ice-cold medium containing 0.25 M sucrose, 2 mM MgSO_4 , 10 mM sodium glycerol-1-phosphate, 2 mM EGTA, 10% glycerol (v/v), 0.5% BSA (w/v), 6% choline-I (w/v), 1 mM PMSF, 2 mM Na-ATP, 2 mM DTT, 1% PVPP (w/v), 25 mM BTP titrated to pH 7.6 with MES using

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4 mL of medium per g fresh weight root tissue. The composition of the homogenization medium ensured the recovery of a large amount of tightly sealed inside-out plasma membrane vesicles (De Michelis and Spanswick, 1986; Giannini et al., 1987). The brei was filtered through four layers of cheesecloth and the homogenate was subjected to differential centrifugation steps: 1000 g for 5 min (supernatant recovered), 13000 g for 20 min (supernatant recovered) and 82500 g for 30 min (pellet recovered). The pellet (microsomal fraction) was gently resuspended in 5 mL of homogenization medium and layered on 10 mL of 40% sucrose (w/w) prepared in 5 mM BTP-MES pH 7.4 buffer containing all the protectants present in the homogenizing medium, and centrifuged at 95000 g for 60 min. Vesicles banding above the sucrose cushion were collected, diluted with homogenization medium and centrifuged at 120000 g for 30 min. The pellet, resuspended in a medium containing 0.25 M sucrose, 10% glycerol (v/v), 1 mM DTT, 1 mM PMSF, 0.2% BSA (w/v) and 2 mM BTP titrated to pH 7.0 with MES, was quickly frozen in liquid N_2 and stored at -80°C until used (not more than a week after freezing). Before use, vesicles were placed in ice and slowly allowed to reach 0°C . All preparation steps were performed at 4°C .

Vesicles obtained with the above described procedure were enriched in plasma membrane vesicles as tested for each membrane preparation in experiments run in the presence of diagnostic inhibitors. The K^+ -stimulated ATPase activity was more than 80% inhibited by 100 μM vanadate, whereas the activity, measured at pH 8.0, was almost unaffected (about 10%) by 1 mM NaN_3 or 100 mM KNO_3 .

ATPase assay

ATP-hydrolyzing activity was measured by determining the release of inorganic phosphate according to Forbusch (1983). The reaction mixture contained 50 mM MES-BTP (pH 6.5), 5 mM MgSO_4 , 5 mM ATP-BTP (pH 6.5), 0.6 mM Na_2MoO_4 , and, when present, 100 mM K-IDA in a final volume of 0.6 mL. The reaction was started adding substrate and run for 30 min at 38°C . Vesicles were incubated 10 min at 2°C

and then 20 min at 20°C with humic substances before adding the substrate (Maggioni et al., 1987).

In some experiments, the detergent Brij-35 (23 lauryl ether) was added to the reaction mixture at a final concentration of 500 μM .

Proton transport assay

Proton accumulation into inside-out plasma membrane vesicles was measured as absorbance decrease of acridine orange (A.O.) at 492 nm by a double-beam Perkin Elmer 550 SE spectrophotometer at 25°C , following the method described by De Michelis et al. (1983).

The assay medium contained 50 mM MES-BTP (pH 6.5), 7.5 μM acridine orange, 5 mM MgSO_4 and 30 μg protein of membrane vesicles. The addition of 5 mM ATP-BTP to energize proton transport determined only a very slight decrease of A.O. absorbance. After the addition of 10–100 mM KNO_3 or KCl a clear absorbance decrease was recorded and followed until the absorbance remained constant. At this time, the quenched absorbance was completely restored upon addition of 4 μM gramicidin-D. Vesicles were routinely incubated 10 min at 25°C with humic substances before adding KNO_3 or KCl.

The presence of humic substances, even in combination with KNO_3 or KCl, did not modify the absorption spectra of the optical probe in the experimental conditions employed, indicating that the formation of complexes between the polyanionic humic molecules, anions and the dye did not occur.

Assays run in the presence of oligomycin 5 $\mu\text{g mL}^{-1}$, 100 mM NO_3^- and 100 μM vanadate (inhibitors of mitochondrial, tonoplast and plasma membrane ATPase, respectively) confirmed that the membrane fraction utilized in this study was enriched in tightly sealed inside-out plasma membrane vesicles.

pH jumps

The proton release from intravesicular lumen was measured as the absorbance increase of A.O. at 492 nm by a double-beam Perkin Elmer 550 SE spectrophotometer at 25°C as described

by Giannini et al. (1987) with minor changes. The vesicles used for the assay were resuspended in a medium containing 0.25 M sucrose, 10% (v/v) glycerol, 1 mM DTT, 1 mM PMSF, 0.2% (w/v) BSA and 25 mM BTP titrated to pH 6.5 with MES. The buffer used in the assay contained resuspending medium pH 6.5 and 7.5 μ M A.O. The assay mixture was obtained by mixing 5 mM MgSO₄ and 60 μ g protein of membrane vesicles with buffer to a final volume of 1 mL. By adding 1 N NaOH to the assay medium to produce a pH jump of about 1.8 units, a rapid decrease of absorbance was obtained, immediately followed by a slow recovery. The addition of 4 μ M gramicidin-D completely collapsed the remaining pH gradient.

Protein assay

Protein content was determined by the Micro-BCA method using BSA as standard after diluting membrane vesicles 100-fold with ice cold water and centrifuging at 120000 g for 45 min, according to De Michelis and Spanswick (1986).

Results

Effect of humic substances on ATPase activity

In the absence of detergents the Mg²⁺-dependent component, calculated as the difference of enzyme activities in the presence or absence of Mg²⁺, was not affected by addition of LMW and HMW humic fraction at concentrations up to 1 mg org CL⁻¹. On the contrary, K⁺-stimulated ATPase activity (difference between the activity measured in the presence or absence of 100 mM K⁺ supplied as IDA⁻ salt) was substantially stimulated by the LMW humic fraction in the entire range of concentrations tested with the highest stimulation at 0.075 mg org CL⁻¹ (435% increase) (Table 1). The stimulation of ATP-phosphohydrolyzing activity by LMW humic substances was completely abolished by 100 μ M vanadate (not shown). When the experiment was run in the presence of the detergent Brij-35, LMW humic substances weakly stimulated the

Table 1. Effect of LMW humic substances on Mg²⁺-dependent (Δ Mg) and K⁺-stimulated (Δ K) ATPase activity of plasma membrane-enriched vesicles isolated from oat roots, in the absence and in the presence of the detergent Brij-35 at the final concentration of 500 μ M. Data are means from an experiment run in triplicate representative of four independent preparations of plasma membrane vesicles. S.D. did not exceed 5% of the means

Humic substances (mg org CL ⁻¹)	ATPase activity (nmol Pi mg ⁻¹ prot min ⁻¹)			
	-Brij-35		+Brij-35	
	Δ Mg	Δ K	Δ Mg	Δ K
0.000	520	65	1048	198
0.075	498	348	1258	1280
0.150	505	193	1267	952
0.250	512	175	1247	500
0.500	510	148	1237	1032
1.000	515	117	1233	327

Mg²⁺-dependent component, and sharply increased the K⁺-stimulated ATPase activity with a pattern of stimulation showing two peaks, one at 0.075 mg org CL⁻¹ (545% increase) and one at 0.5 mg org CL⁻¹ (420% increase). In the absence of Brij-35, HMW humic substances did not affect the Mg²⁺-dependent component while increased K⁺-ATPase activity by 30% at a concentration of 0.5 mg org CL⁻¹ (Table 2). When the detergent was present in the assays HMW

Table 2. Effect of HMW humic substances on Mg²⁺-dependent (Δ Mg) and K⁺-stimulated (Δ K) ATPase activity of plasma membrane-enriched vesicles in the absence and in the presence of the detergent Brij-35 at the final concentration of 500 μ M. Statistics as in Table 1

Humic substances (mg org CL ⁻¹)	ATPase activity (nmol Pi mg ⁻¹ prot min ⁻¹)			
	-Brij-35		+Brij-35	
	Δ Mg	Δ K	Δ Mg	Δ K
0.000	520	65	1048	198
0.075	505	70	1035	212
0.150	505	73	997	228
0.250	488	73	1080	190
0.500	468	85	1080	190
1.000	498	63	1058	193

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humic substances did not affect either the Mg^{2+} -dependent or K^+ -stimulated component of ATPase activity.

Effect of humic substances on H^+ transport

Figure 1 shows the effects of LMW and HMW humic fractions on the initial rate of intravesicular acidification driven by plasma membrane-bound H^+ -ATPase activity measured in the presence of 100 mM KNO_3 . LMW humic molecules stimulated the rate of H^+ accumulation at any concentration assayed with a pattern of stimulation showing two distinct peaks, one at 0.075 mg org CL^{-1} and one at 0.5 mg org CL^{-1} . On the contrary, HMW fraction determined a progressive inhibition of H^+ transport which was reduced approximately by 35% at 1 mg org CL^{-1} .

Proton transport can be quantitated in terms of both initial rate of quenching of A.O. absorbance ($\Delta A_{492} \times \text{min}^{-1} \text{mg}^{-1} \text{prot}$) and ionophore reversible quench (Giannini et al., 1987). In this

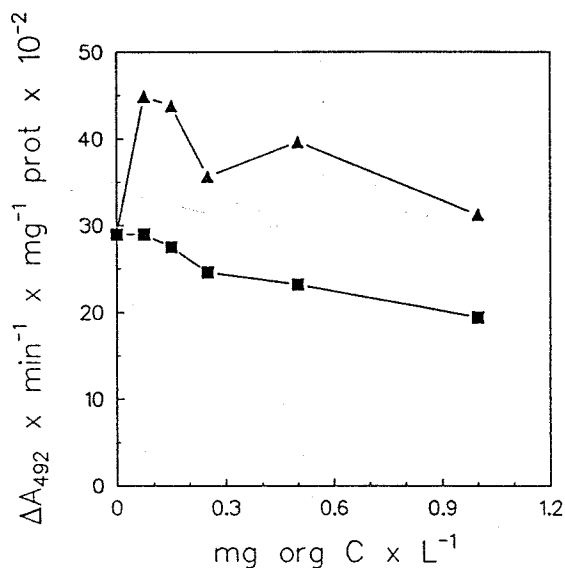


Fig. 1. Effect of LMW (▲▲) and HMW (■) humic substances on initial rate of ATP-dependent H^+ -transport of plasma membrane vesicles isolated from oat roots. Vesicles were incubated at 25°C for 10 min in the presence or absence of humic substances in the assay medium before starting the reaction by adding 100 mM KNO_3 . Data are means from an experiment run in triplicate representative of three independent preparations of plasma membrane enriched vesicles. S.D. did not exceed 5% of the means.

case an appropriate ionophore (e.g. gramicidin-D) is added when the proton accumulation has reached the steady state and the extent of the recovery of absorbance is determined. In the steady-state the proton influx is equal to passive proton efflux. Therefore this parameter gives also an indication on the ability of membrane vesicles to sustain proton gradients. In Table 3 data concerning the effect of LMW humic substances on H^+ transport, evaluated with the two parameters above described, are presented by comparing data from Figure 1 with those obtained calculating proton transport by means of ionophore reversible quench. Results show the absence of correlation between the two parameters. In fact, LMW humic substances progressively reduced the ionophore reversible quench, indicating that the stimulation of the initial rate of proton accumulation did not cause an enhancement of ΔpH generated at the steady state.

Effect of humic substances on passive proton permeability

Figure 2 shows the effects of LMW and HMW humic substances on passive proton permeability of plasma membrane vesicles measured as dissipation of an artificially imposed pH gradient. In this condition it is possible to measure the passive permeability of the vesicles to an outwardly directed proton flux. The presence of LMW humic substances at 0.075 mg org CL^{-1} or 0.5 mg org CL^{-1} concentration in the incubation medium enhanced both the rate of proton efflux

Table 3. Effect of LMW humic substances on initial rate of ATP-dependent H^+ -transport ($\Delta A_{492} \text{mg}^{-1} \text{prot min}^{-1}$) and ionophore reversible quench ($\Delta A_{492} \text{mg}^{-1} \text{prot}$) of A.O. measured after addition of 4 μM gramicidin-D at the steady-state of the H^+ -pumping reaction. Statistics as in Table 1

Humic Substances (mg org CL^{-1})	Initial rate	Ionophore reversible quench
0.000	0.29	0.88
0.075	0.45	0.76
0.250	0.36	0.68
0.500	0.40	0.52
1.000	0.31	0.52

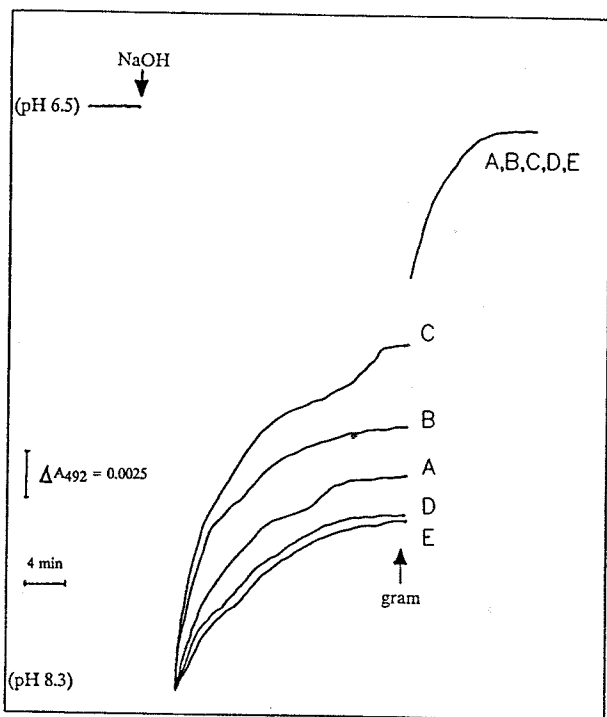


Fig. 2. Effect of LMW and HMW humic substances on the passive proton permeability of plasma membrane vesicles from oat roots. Vesicles were incubated for 10 min at 25°C in 1 mL of assay mixture in the presence or absence of humic molecules before adding 30 μ L of 1 N NaOH. At the time indicated by the arrow 4 μ M gramicidin-D was added. Trace A: control; trace B: 0.075 mg org CL⁻¹ LMW humic substances; trace C: 0.5 mg org CL⁻¹ LMW humic substances; trace D: 0.075 mg org CL⁻¹ HMW humic substances; trace E: 0.5 mg org CL⁻¹ HMW humic substances.

and the extent of recovery of absorbance evaluated 15 min after the imposition of the pH jump. These effects, which were highest when 0.5 mg org CL⁻¹ of LMW humic substances was applied, indicate that these compounds can induce an increase of passive permeability to protons of isolated plasma membrane vesicles. On the contrary, HMW humic substances induced a decrease of both parameters, suggesting a decrease of membrane proton permeability.

Effect of humic substances on anion transport

Figure 3 shows the effects of 0.5 mg org CL⁻¹ of LMW and HMW humic substances on anion dependent H⁺-transport. In this experiment we initiated proton transport with ATP-BTP in the absence of charge compensating monovalent anions. In this condition is generated a steady

state $\Delta \mu_{H^+}$ across the membrane which is dominated by $\Delta \psi$ (Lew and Spanswick, 1985). The subsequent addition of increasing concentrations of anions causes a considerable quench of A.O. absorbance. By following the anion stimulated A.O. quench is possible to obtain information on membrane permeability to different anions and on the kinetics of their transmembrane transport (Giannini and Briskin, 1987).

Results show that in the presence of Cl⁻ the stimulatory action of LMW humic substances was quite scarce and almost constant in the entire range of concentrations tested. HMW humic substances determined a slight inhibition of Cl⁻-dependent H⁺ transport without modifying the kinetic pattern. When the assay was run in the presence of NO₃⁻, LMW humic substances showed higher stimulatory capacity which appeared to increase with the increase of anion concentration. HMW humic substances were almost ineffective at low (non saturating) NO₃⁻ concentrations. When NO₃⁻ was present at concentrations higher than 30 mM these compounds showed an increasing inhibitory action.

Discussion

The results of this study show that transport properties of plasma membrane vesicles isolated from oat roots are affected by humic substances extracted from the soil. However, the effects on membrane activities and ion fluxes are dependent on the molecular size and concentration of humic fraction.

In the presence of 0.075 mg org CL⁻¹ of LMW humic substances the K⁺-stimulated component of plasma membrane ATPase activity was increased more than 5 times indicating that this humic fraction possesses a very high capacity of stimulation which is, at least in part, maintained also at concentrations above 0.5 mg org CL⁻¹. This stimulatory capacity seems to be a specific feature of the interaction between LMW humic substances and plasma membrane bound ATPase activity. In fact, when the interaction between LMW humic substances and tonoplast vesicles was considered (Pinton et al., 1992) it was shown that these compounds increased the activity of the tonoplast Cl⁻-stimu-

Fig. 3. proton or absorbance anion solution Figure

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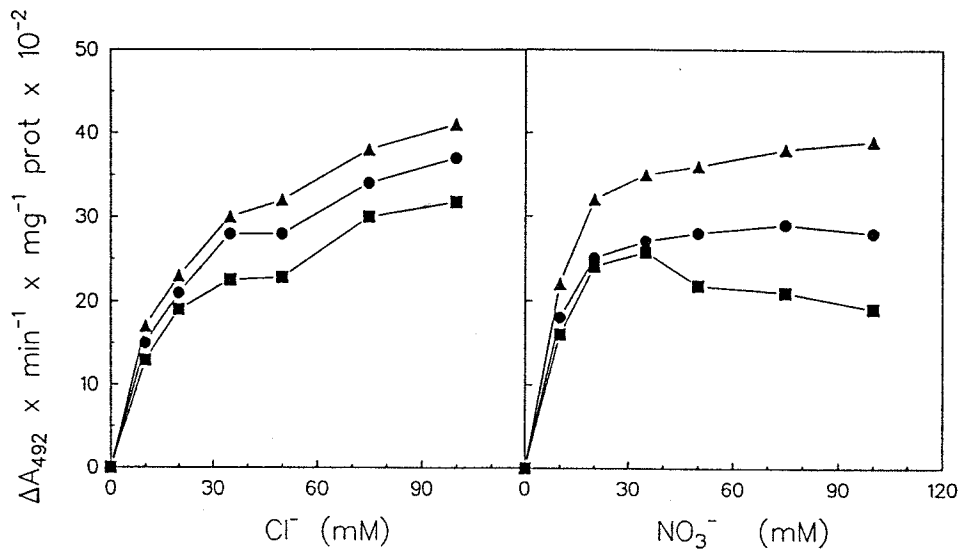


Fig. 3. Effect of LMW (▲▲) and HMW (■) humic substances ($0.5 \text{ mg org CL}^{-1}$) on the kinetics of anion-dependent proton transport in plasma membrane vesicles (●● control). Vesicles were incubated at 25°C for 10 min in the presence or absence of humic substances in the assay medium before starting the reaction by adding 5 mM ATP-BTP. The anion dependent A.O. absorbance quenching was followed after the addition of the desired amount of the K^+ -anion solution. The K^+ concentration was kept constant (100 mM) by addition of appropriate amount of K-IDA. Statistic as in Figure 1.

lated ATPase by only 30%. In addition, as demonstrated in this study, the stimulation exerted by HMW humic substances on plasma membrane K^+ -stimulated ATPase activity did not exceed the value of 30%. Nardi et al. (1991) reported that a humic fraction of molecular weight below 3.5 KDa increased of the KCl-stimulated ATPase activity of oat root microsomes by 87%, whereas humic components having molecular weight above 3.5 KDa stimulated the enzyme activity by only 16%.

The stimulation exerted by LMW humic substances was still evident when enzyme activity was measured in the presence of the detergent Brij-35. This can be interpreted as a direct activation of the phosphohydrolyzing activity of plasma membrane ATPase and not the consequence of an alteration of the transmembrane electrical potential generated by the activity of proton pump, as suggested as mechanism of interaction between LMW humic substances and tonoplast ATPase (Pinton et al., 1992).

Potassium ions can stimulate plasma membrane ATPase activity (Briskin, 1987). This action has been related to its ability to increase

the rate of hydrolysis of a phosphorylated intermediate formed during the catalytic cycle of the enzyme. However, at present it is not clear if K^+ is also directly transported by H^+ -ATPase and if the observed stimulatory effect is linked to the transport process (Briskin and Hanson, 1992). Our data show that the stimulatory effects of LMW humic substances on ATPase activity are evident only when K^+ is present in the assay mixture, suggesting a capacity of humic substances to increase somehow the efficiency of the interaction between K^+ and enzyme protein determining higher level of phosphohydrolytic activity.

The initial rate of ATP dependent H^+ accumulation in tightly sealed inside-out plasma membrane vesicles was stimulated by LMW humic substances. The pattern of stimulation of proton transport was similar to that reported for ATP hydrolysis, presenting two peaks, one at $0.075 \text{ mg org CL}^{-1}$ and one at $0.5 \text{ mg org CL}^{-1}$. However, the values of stimulation were lower (55% at $0.075 \text{ mg org CL}^{-1}$ and 51% at $0.5 \text{ mg org CL}^{-1}$) than those recorded for the hydrolytic activity. The complex pattern of stimulation of LMW humic substances might be due to the presence in the humic material of

different components, active at different concentrations.

Together with the stimulatory effect on the initial rate of inwardly directed H^+ -transport, LMW humic substances revealed a capacity to decrease the amplitude of ΔpH in the steady-state of the reaction (Table 3). In principle the steady-state ΔpH in isolated vesicles can be modified in several ways: H^+ -ATPase can be stimulated or inhibited; the permeability of the membrane can be altered; cotransport of permeable anions can be affected. Our data demonstrate that LMW humic substances stimulate ATP hydrolysis. On the other hand, as directly tested by evaluating the rate of dissipation of artificially imposed proton gradients, LMW humic substances can also induce an alteration of permeability of plasma membrane vesicles rendering them more leaky to protons. This latter effect could explain both the lower level of stimulation exerted by LMW humic substances on the initial rate of H^+ -accumulation in comparison with ATP hydrolysis and the lower ΔpH at the steady-state. Alteration of membrane permeability of potato cells caused by humic acids at the concentration of 40 mg L^{-1} has been described by Samson and Visser (1989) and interpreted as a surfactant-like behaviour of humic substances. Our data show that at concentration at least 100 times lower also a LMW humic fraction can modify the permeability of plasma membrane vesicles isolated from oat roots. However, LMW humic substances never induced a rapid and complete collapse of the artificially imposed pH gradients which were, in part, maintained up to 30 min even at the highest concentration tested. Therefore, LMW humic substances seem to possess both a weakly permeabilizing effect on membrane vesicles and a capacity to directly stimulate plasma membrane H^+ -ATPase.

The experiment run by starting the proton pumping reaction with the addition of NO_3^- and Cl^- at increasing concentrations, allowed us to get information on the permeability of plasma membrane vesicles to these anions and on the kinetic properties of their membrane transport (Giannini and Briskin, 1987). LMW humic substances differently affected the anion permeability of plasma membrane vesicles. In fact, when the H^+ -transport assay was run in the presence

of 100 mM anion, the anion dependent proton-transport was increased by about 50% for NO_3^- and 10% for Cl^- , while it was unaffected when the slowly permeant SO_4^{2-} ion was added (not shown). The kinetic of NO_3^- intravesicular accumulation was clearly modified by LMW humic substances. Therefore these results suggest that LMW humic substances might specifically influence the rate of NO_3^- transmembrane fluxes. However, our data do not allow to definitely assess which is the molecular site of action of these substances at plasma membrane level. The effects exerted by HMW humic substances on H^+ -ATPase activity and on the permeability of membrane vesicles proved to be different from those exerted by LMW humic substances. In fact these compounds seemed to reduce the permeability both to protons and NO_3^- ions.

The results presented here correlate well with previous reports indicating that low molecular size humic fractions are particularly active in stimulating ion uptake in root tissues (Dell'Agnoia and Nardi, 1987). Although care must be taken in exploiting 'in vivo' results with evidences obtained 'in vitro' with isolated system, it is worthy to note that the LMW humic fraction utilized in the present work has an average molecular size, as determined by HPLC, around 2.5 KDa (De Nobili M., pers. commun.). Therefore, a part of these substances seems to be small enough to penetrate cell wall pores (Carpita et al., 1979) and diffuse into the apoplast reaching and interacting with the plasma membrane. The demonstration that LMW humic substances can affect membrane activities relevant for cell growth and metabolism (Serrano, 1989) is consistent with the idea that components of humified soil organic matter may play a role in the complex interaction between plant and soil environment promoting plant growth and nutrition.

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