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The kinetics of transport of oleanolic acid monoglycosides into vacuoles isolated from *Calendula officinalis* leaf protoplasts

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Two series of oleanolic acid glycosides, i.e. glucosides (derivatives of 3-*O*-monoglucoside) and glucuronides (derivatives of 3-*O*-monoglucuronide) have been found in leaves of *Calendula officinalis* [1, 2]. Our previous studies [3, 4] on intracellular distribution of these compounds pointed to the vacuole as practically the only cellular compartment containing all glycosides of both series. We demonstrated effective transport of 3-*O*-monoglucoside (I) and 3-*O*-monoglucuronide (F) of [3-^{3}H]oleanolic acid into vacuoles isolated from *C. officinalis* leaf protoplasts [5]. Moreover, it was shown that the two monoglycosides significantly differed in the dependence of their transport on the pH value and the presence of ATP [6]. These results suggested the existence of different mechanisms for translocation of oleanolic acid monoglucoside I and monoglucuronide F across the tonoplast.

The aim of the present studies was to examine the kinetics of transport of the two monoglycosides (Fig. 1) into isolated vacuoles as well as to check whether this transport is influenced by various inhibitors. Isolated vacuoles were obtained from *C. officinalis* leaf protoplasts [4, 6]. We used radioactive monoglycosides obtained by chemical synthesis 3-*O*-monoglucoside of [3-^{3}H]oleanolic acid and 3-*O*-monoglucuronide of [3-^{3}H]oleanolic acid, of the same specific activity (3.82 mCi/mmol). The incubation of isolated vacuoles with radioactive compounds was carried out for 2 h at illumination of 3000 lux in phosphate buffer, pH 6.0 or 7.0 for monoglucoside I and monoglucuronide F, respectively. The monoglycosides nonabsorbed to the vacuoles were washed off by centrifugation in the mannitol-sucrose-Ficoll gradient [4]; the purified vacuoles were extracted with ether and n-butanol. The extracts were separated by t.l.c. [7], and the radioactivity of the individual compounds was estimated.

The kinetics of transport of the two monoglycosides into isolated vacuoles is presented in Figs. 2 and 3. Vacuoles (4×10^5/ml) were incubated with radioactive monoglucoside or monoglucuronide supplied in the concentration range from 1.2 to 41.2 μM. Other conditions of the incubation were as described above.

It was found that the rate of transport of the two monoglycosides was dependent on their concentration and exhibited a saturation phase. The transport rate of monoglucoside I was stimulated by 1.5 mM ATP (Fig. 2), which however, had no effect on the transport of

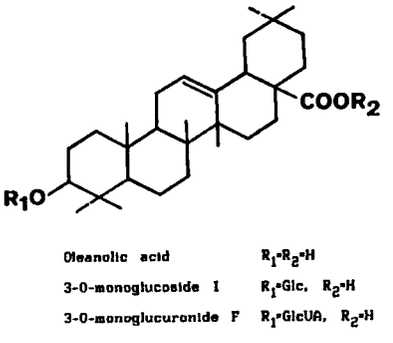

Fig. 1. The structure of oleanolic acid and its monoglycosides I and F

[1]Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; PCMBS, *p*-chloromercuribenzenesulfonic acid

monoglucuronide F (Fig. 3). The shape of the monoglucoside I kinetics plot was practically identical in the presence and the absence of ATP. The K_m value calculated according to Michaelis-Menten was 10 μM both in the absence (Fig. 2, inset A) and in the presence of 1.5 mM ATP (Fig. 2, inset B). For monoglucuronide F concentrations from 0.1 to 13.2 μM the transport rate followed not a linear but a sigmoid pattern (Fig. 3). Therefore, two apparent K_m

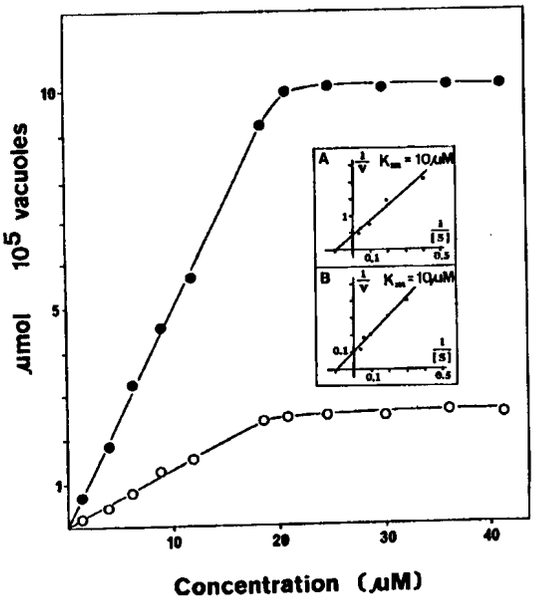

Fig. 2. Effect of monoglucoside I concentration on its transport into isolated vacuoles in the absence (○) and in the presence of 1.5 mM ATP (●).
For K_m determination the concentration of substrates ranged from 1.2 to 41.2 μM. K_m in the absence (inset A) and in the presence of 1.5 mM ATP (inset B)

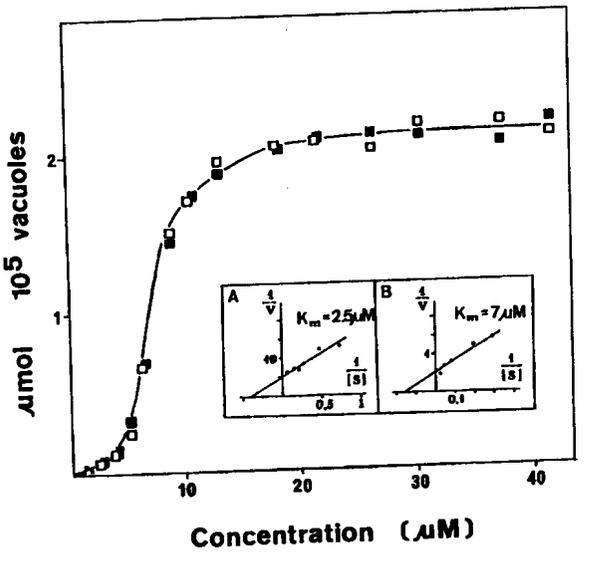

Fig. 3. Effect of monoglucuronide F concentration on its transport into isolated vacuoles in the absence (□) and in the presence of 1.5 mM ATP (■).
For K_m determinations the concentration of substrates ranged from 0.1 to 1.1 μM. K_m determinations for the steeper and less steep slopes (inset A and B, respectively) of the concentration-dependence curves

Table 1
The effect of various inhibitors on the transport of oleanolic acid monoglycosides (I and F) into isolated vacuoles of C. officinalis leaf protoplasts

Inhibitor		Inhibition (%)			
		−ATP		+ATP	
		I	F	I	F
DCCD	20 μM	23	0	85	0
	50 μM	60	0	89	0
	100 μM	79	1	95	2
CCCP	50 μM	28	1	63	2
	100 μM	57	1	88	2
DIDS	50 μM	55	0	87	1
	100 μM	78	2	93	2
KNO_3	30 mM	19	0	56	0
	50 mM	45	0	73	0
	100 mM	79	1	94	2
Na_3VO_4	0.1 mM	0	0	1	0
	50 mM	1	0	2	1

Table 2

The effect of the protein-modifying agent (PCMBS) on the transport of oleanolic acid glycosides (I and F) and the Neutral Red into isolated vacuoles from C. officinalis leaf

Inhibitor		Inhibition (%)					
		−ATP			+ATP		
		I	F	Neutral Red	I	F	Neutral Red
PCMBS	0.1 mM	88	86	0	92	87	1
	1.0 mM	96	98	1	98	97	0

values were calculated. For the lower monoglucuronide F concentration range (0.1-2.4 μM) the K_m value was 2.5 μM (Fig. 3, inset A) and for the higher concentration, approx. 7 μM (Fig. 3, inset B).

The results concerning the influence of various inhibitors, i.e. uncoupling agents, protonophores and membrane ATPase inhibitors, on the transport of the two oleanolic acid monoglycosides into isolated vacuoles are presented in Table 1. Vacuoles were preincubated with the inhibitor tested for 20 min in the presence or absence of 1.5 mM ATP, prior to the addition of radioactive monoglycoside (2×10^5 d.p.m. per 4×10^5 vacuoles). Other conditions of the incubation and the remaining steps of the procedure were unchanged.

It was found that DCCD[1], the proton-channel blocker of both tonoplast and plasma-membrane ATPase; DIDS, the anion-channel blocker, which is also a rather specific inhibitor of tonoplast ATPase; as well as CCCP, the protonophore acting as the proton-gradient dissipator and uncoupler, significantly reduced the transport of monoglucoside I. In the absence of ATP the transport of I was inhibited by approx. 80% by DCCD and DIDS, and 57% by CCCP. In the presence of ATP all those inhibitors reduced the transport of monoglucoside I by approx. 90%. The transport was not inhibited by vanadate, although this could be expected since this inhibitor is specific for the plasma-membrane ATPase; whereas the presence of nitrate, which in relatively high concentrations inhibits tonoplast ATPase, strongly reduced the transport of monoglucoside I. On the contrary, none of the inhibitors tested had any effect on the transport of monoglucuronide F.

The effect of PCMBS, the protein-modifying agent, on the transport of both monoglycosides as well as Neutral Red into isolated vacuoles is shown in Table 2. It was found that PCMBS inhibited the transport of both monoglycosides by approx. 90% in the presence and absence of ATP, whereas accumulation of Neutral Red was not affected.

Inhibition of monoglucoside I transport by nitrate, CCCP, DCCD, DIDS and PCMBS, and the saturation phase in the transport kinetics point to the presence of a carrier-mediated and ATP-dependent mechanism. On the other hand, the saturation kinetics and sensitivity to protein-modifying agent PCMBS of the transport of monoglucuronide F would suggest a passive, carrier-mediated process. Thus, the presented results support the conception that the two oleanolic acid monoglycosides differ in the mechanism of their transport to vacuoles isolated from *C. officinalis* leaf protoplasts.

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