Fluorescence tagging of phosphatase and chitinase activity on different structures of *Utricularia traps*

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Introduction

The genus *Utricularia* (Lentibulariaceae) comprises about 220 species and represents the largest genus of carnivorous plants (Taylor 1989; Guisande *et al.* 2007). The trap of *Utricularia* is a hollow utricle usually 1-4 mm long, with walls mostly two cells thick, and filled with water. It is the most sophisticated trap within carnivorous plants (Juniper *et al*. 1989), with a variety of glands and trichomes both on the inner and outer surfaces (see Figure 1). After the prey irritates trigger hairs situated close to the trap door, it is sucked in as a result of the underpressure maintained inside the utricle. After firing, the trap restores the underpressure by rapidly removing approximately 40% of the water from the lumen. This process lasts about 30 min and the trap is ready to fire again (Sydenham & Findlay 1975).

Little is known about the mechanisms of digestion in *Utricularia* traps although protease, acid phosphatase, and esterase were detected cytochemically in the quadrifid glands (for review see Juniper *et al.* 1989). Recently, Sirová *et al.* (2003) investigated fluorometrically the activities of five extracellular enzymes directly in the trap fluid from four aquatic *Utricularia* species. Phosphatase always exhibited the highest activity, while the activities of the other enzymes were usually lower by one or two orders of magnitude and could have entered the trap from the ambient water. Furthermore, the trap enzyme activities are independent of prey digestion and the enzyme production is probably constitutive, *i.e.*, not induced by prey, unlike Droseraceae traps (*cf.* Juniper *et al.* 1989). Phosphatases represent a broad group of enzymes catalyzing the hydrolysis of phosphate esters. Acid phosphatases are common plant enzymes of low substrate specificity that appear to be important in the production, transport, and recycling of phosphate (Duff *et al*. 1994).

The importance of phosphatase activity in trap digestion also was supported by Płachno *et al.* (2006), who proved the presence of phosphatases using the ELF (enzyme-labeled fluorescence) method both inside the terminal gland cells (arms) and on the surface of quadrifid glands in traps of all 26 *Utricularia* species investigated. Although trap hydrolytic enzyme secretion is accepted as one of several principal criteria for plant's carnivorous syndrome (Juniper *et al*. 1989), the ELF method still remains problematic and partly unreliable as to the exact interpretation of data (*cf.* Sirová *et al.* 2003; Płachno *et al.* 2006). As each plant cell contains phosphatases in its cytoplasm or organelles, it may not be clear (*e.g.*, due to endocytosis of the ELF substrate) that the positive ELF reaction visualizes *strictly* the externally released or bound enzyme. Furthermore,

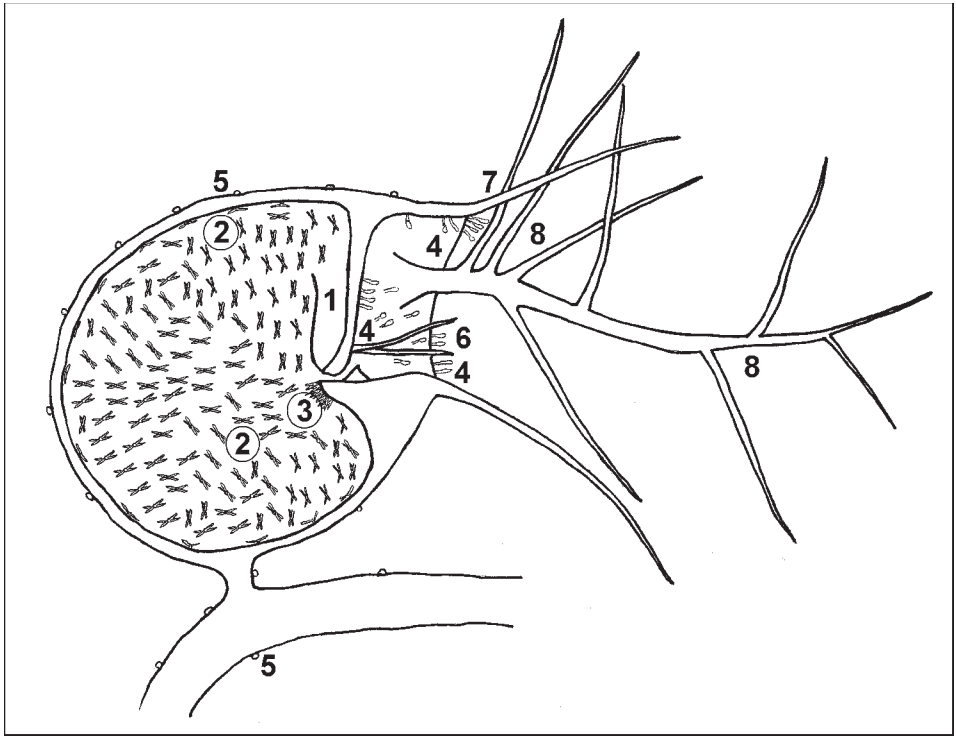

Figure 1: Schematic longitudinal section through a trap of *Utricularia* with glands and other structures (modified from Juniper *et al.* 1989). 1: trap door, 2: quadrifid glands, 3: bifid glands, 4: stalked mucilage glands, 5: spherical sessile glands, 6: trigger hairs, 7: rostrum, 8: antennae.

Płachno *et al.* (2006) used 12.5 times more concentrated substrate than the former authors did, which could be the reason for the difference in the extent of positive ELF results between the two studies.

Utricles are also inhabited by diverse communities of microorganisms (*e.g.*, Richards 2001). Considering that aquatic *Utricularia* species frequently grow in P-limiting conditions, that trap phosphatase activity is invariably high (Sirová *et al*. 2003), and that the affinity of phosphate uptake of some microorganisms (bacteria) is at least one order of magnitude greater than that of phytoplankton (Currie & Kalff 1984), it is highly probable that this microbial community enhances P acquisition for the trap under nutrient-poor growth conditions. Moreover, it has been found recently (Sirová *et al.* 2003, unpubl.; Płachno *et al*. 2006; Płachno & Wołowski 2008) that bacteria, cyanobacteria, and algae in the utricles and relative *Genlisea* traps also show extracellular phosphatase activity.

The aim of our study was to detect extracellular phosphatase and chitinase activity in traps of nine *Utricularia* species and distinguish it within the trap structures (*e.g.*, glands), commensal organisms, and periphytic organisms attached externally on the traps. Fluorescence tagging was used to visualize both enzymes. In four aquatic *Utricularia* species, the enzyme activity was investigated in intact traps after two days of prey capture. The greatest attention has been focused on aquatic *Utricularia* species as they have large traps.

Table 1: Summarization of phosphatase and chitinase activity found on different trap structures (for explanation see Figure 1), commensal (C, in trap fluid) or epiphytic (E) microorganisms. The numbers indicate structures, on which any (*weak*, medium, **high**) activity was detected; n.d.--not determined. Dotted line separates aquatic and terrestrial species; note that traps of the former were tagged as intact, but the latter as halved.

Species	Traps without prey		Traps with prey after 2 d	
	phosphatases	chitinases	phosphatases	chitinases
U. vulgaris	2, *3*, 5, *E*, *C*	E	2, **4**, 5, E	*8*, *E*
U. australis	2, 3, **4**, *C*	*8*, *E*	*3*, 4, 5, E	*8*, *E*
U. floridana	**2**, **4**, 7, *C*	**8**, *E*	**4**, **5**, 6, 8, E	8
U. intermedia	**4**, **5**, *C*	*8*, C, *E*	**4**, **5**, 8, E	*5*, 8, *E*
U. stygia	1, **4**	*8*	n.d.	n.d.
U. bremii	*2*, 6	*6*, *8*, *E*	n.d.	n.d.
U. sandersonii	1, *2*, **4**, **5**	**4**, **5**, 8	n.d.	n.d.
U. livida	2, 4, 8	4, 5, 8	n.d.	n.d.
U. reniformis	4, **5**	*2*, E	n.d.	n.d.

Materials and Methods

All plant species were obtained from the collection of the Institute of Botany at Třeboň, Czech Republic. Aquatic species, *Utricularia vulgaris* L. (from Czech Rep.), *U. australis* R.Br. (Czech Rep.), *U. intermedia* Hayne (Czech Rep.), *U. stygia* Thor (=*U. ochroleuca s.l.*; Czech Rep.), and *U. bremii* Heer *ex* Kölliker (Lake Oniega, NE Russia) were grown in plastic containers outdoors, *U. floridana* Nash. (N Florida, USA) in a greenhouse aquarium, and terrestrial species, *U. sandersonii* Oliver (South Africa), *U. livida* E.Mey. (South Africa), and *U. reniformis* A.St.-Hil. (Brazil) in a greenhouse terrestrial culture.

Both enzymes, phosphatases (phosphomonoesterases) and chitinases (β-N-acetylhexosa-minidases), were fluorescently tagged in intact traps without prey in six aquatic species (*U. vulgaris*, *U. australis*, *U. intermedia*, *U. floridana*, *U. stygia*, and *U. bremii*). Several leaves with intact traps cut from 11th and 12th adult leaf whorls (intermediate age, see Sirová *et al.* 2003) on shoots of *U. vulgaris* and *U. australis*, from younger parts of green photosynthetic shoots of *U. bremii*, or pale carnivorous shoots of *U. intermedia*, *U. stygia*, and *U. floridana* were used. The cuttings were thoroughly rinsed. Four excised intact traps without prey were transferred to the solution of 0.1 mM Trisma buffer (pH 7.5; *cf.* Štrojsová & Vrba 2006), supplemented with a substrate, and filled twice, successively after two firings after 1 h each by means of mechanical irritation. ELF 97 phosphate and ELF 97N-acetylglucosaminide were applied (at 20 μM final concentrations) for tagging phosphatase and chitinase sites, respectively. The filled traps were then thoroughly rinsed with distilled water and were transferred to the solution of 0.5 mM KCl + 0.02 mM $CaCl_2$. They were kept at 23-25°C in very weak daylight for 2-3 h until the microscopical evaluation (see below). The trap fluid from other 6-10 traps was collected by a glass pipette (for details, see Sirová *et al.* 2003), allowed to desiccate on a cover slip, and microscopically inspected for enzyme tagging of commensals, such as protists and bacteria.

Parallel leaf or shoot cuttings of these species (except for *U. stygia* and *U. bremii*) were placed into plastic jars (0.2 l) with culture water, fine zooplankton (*Chydorus* sp., Cladocera, size 0.5-0.6

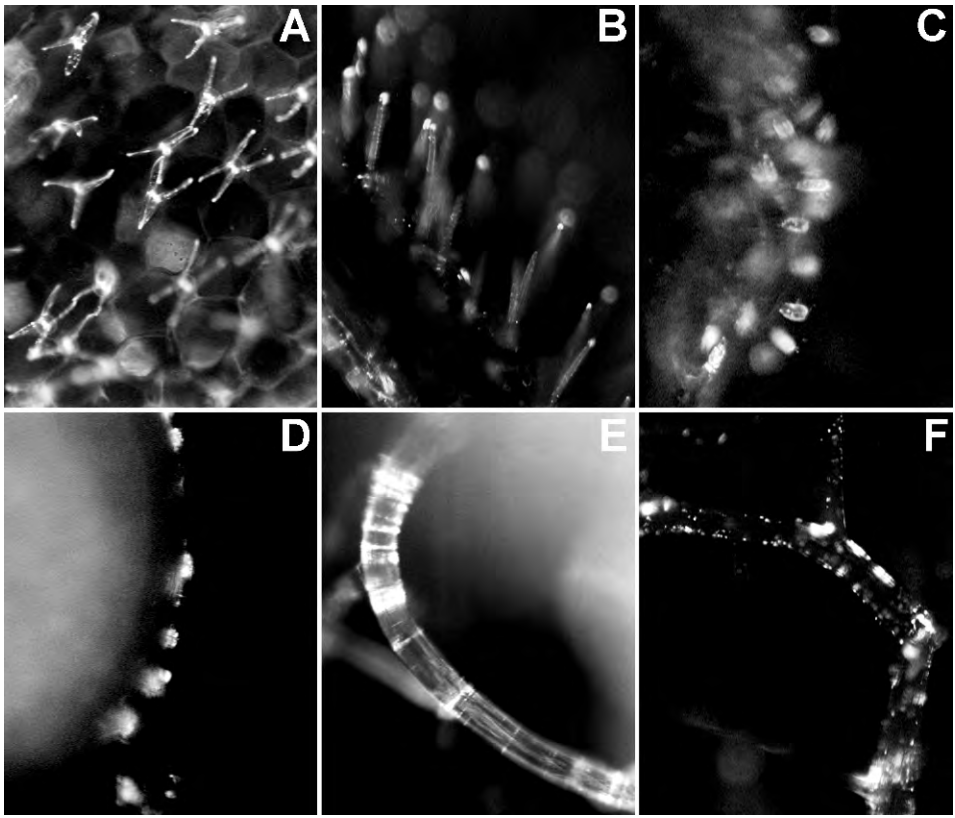

Figure 2: Examples of fluorescently tagged phosphatases (Ph) or chitinases (Ch) on different trap structures of *Utricularia* species. A: quadrifid glands (Ph, U. *floridana* without prey); B: bifid glands (Ph, *U. vulgaris* without prey); C: stalked mucilage glands (Ph, *U. intermedia* with prey); D: spherical sessile glands (Ph, *U. sandersonii*); E: antennae (Ph, *U. floridana* without prey); F: antennae (Ch, *U. floridana* with prey). Note distinct pattern of the enzyme tagging in E and F (dots likely tag enzymatic activity of epiphytic microorganisms, *e.g.*, suspect bacteria).

mm) was added, and carefully removed after 4-10 h. By this time, most of the traps contained prey. The cuttings were thoroughly rinsed and exposed in the filtered (mesh size 44 µm) culture water at 20-26°C (68-79°F) in dim daylight for 2 d. Afterwards, four excised intact traps with prey were filled twice by the solutions and further treated as described above for the empty traps. The traps of all aquatic species used were 1.5-3.5 mm large.

Before microscopic evaluation, the traps were halved lengthwise by a razor blade and thoroughly rinsed with distilled water. They were inspected with an epifluorescence microscope (Olympus BH-60, magnification 40-100×) and the images in green fluorescence were recorded with a monochromatic camera (for more details, see Štrojsová & Vrba 2006). Eight halves of four traps, either with or without prey, were inspected for each enzyme in all plant species. The attention was paid not only to trap structures but also to various attached trap organisms (periphyton).

Four traps without prey of three other terrestrial species (*U. sandersonii*, *U. livida*, and *U. reniformis*) were lengthwise halved by a razor blade, thoroughly rinsed with distilled water, put into the

same solution of either substrate as described above for 2-3 h, and were observed in the same way. The traps of the terrestrial species were only about 1 mm large.

Results and Discussion

Both phosphatase and chitinase activities were detected in all nine *Utricularia* species under study (Table 1). The brightest fluorescence, tagging the highest enzymatic activities, was frequently observed in stalked mucilage glands around the trap doors (see Figure 2B) and in quadrifid glands inside the traps (see Figure 2A). Other highly active structures of *Utricularia* included spherical sessile glands distributed on both trap surfaces and stalks, particularly in terrestrial species (see Figure 2D). Bifid glands in *U. vulgaris* and *U. australis* were moderately tagged for phosphatases only (see Figure 2C). Surprisingly, both substrates also tagged certain activity on various trichomes and, particularly, on the trap antennae (Table 1). Some enzymatic activity tagging the plant surfaces (*i.e.*, on traps, trichomes, stalks, etc.) was likely due to epiphytic microorganisms (probably bacteria, with characteristic single-dot tagging, *cf.* Figure 2F), some tagging pattern of antennae or rostrum was very distinct (*cf.* Figure 2E) and its epiphytic origin was unlikely. While chitinases were rarely tagged on any glands (indeed 3 of 4 such species were terrestrial), they were tagged on the antennae of all six aquatic species (*cf.* Table 1, Figure 2F). There was little difference in the phosphatase tagging of *U. vulgaris* and *U. australis* between the traps without prey and those with prey captured two days prior to labeling, whereas more outer trap structures were tagged in the traps with prey compared to those without prey of other two species (*U. floridana* and *U. intermedia*).

Commensal microorganisms, in particular algae (detected by chlorophyll *a* autofluorescence), were found in many trap fluid samples. Algal cells, however, were rarely tagged with either substrate used. Abundant *Euglena* spp. (rarely tagged for phosphatase) and other algal species occurred in the *U. vulgaris* traps (*cf.* Sirová *et al.* 2003). Another algal species (apparent "monoculture") was abundant in the *U. floridana* traps, but it was not tagged at all. One large cell of heterotrophic Eukaryote (suspect ciliate) was tagged for phosphatase in the trap fluid sample of *U. australis* and likely some suspect bacterial cell (*i.e.*, single dots of ELF alcohol fluorescence) were tagged for chitinase in the trap fluid of *U. intermedia* (*cf.* C in Table 1).

Overall, we can conclude that chitinases were almost untagged on inner trap structures and therefore could hardly participate in chitin decomposition of prey carcasses in *Utricularia* traps. On the contrary, phosphatases were much more regularly distributed on trap structures both inside and outside the traps as well as on microorganisms characterized as commensal or epiphytic. Moreover, the pattern of phosphatase tagging inside the traps with or without captured prey is largely very similar within each species suggesting that the enzyme production is constitutive, independent of prey, and supports our previous results (Sirová *et al.* 2003, unpubl.; Płachno *et al.* 2006) on the key role of phosphatases in prey digestion in *Utricularia* traps. Nevertheless, the present results together with those of Sirová *et al.* (2003) on fluorescence tagging of phosphatase activity in *Utricularia* traps are in a certain methodological controversy with the results of Płachno *et al.* (2006). The latter authors, however, applied far more concentrated ELF 97 phosphate (250 µM *vs.* 20 µM final concentrations) for tagging halved traps, as opposed to this study and Sirová *et al.* (2003) tagging intact traps. Therefore, it is obvious that the consistently very high intensity of phosphatase tagging of internal glands in halved traps in their results (Płachno *et al.* 2006; Płachno & Wołowski 2008) could be due to some penetration of the substrate into the gland cells. Nedoma *et al.* (2007) have recently proved that the 20 µM concentration of ELF 97

phosphate is adequate for tagging extracellular enzymes in plankton; also pH<8 (*i.e.*, buffering) may be important (*cf.* Štrojsová & Vrba 2006). Thus, one should be careful by applying ELF 97 substrates for tagging extracellular enzyme activities. Experimental conditions, such as substrate concentration, intactness of structures, and time of tagging, should be considered and chosen carefully.

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