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RESEARCH NOTE

FUNCTION OF THE CRYSTALLINE STYLE AND FIRST DETECTION OF LAMINARINASE ACTIVITY IN FRESHWATER MUSSELS OF THE GENUS *ANODONTA*

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Knowledge of the digestive physiology of molluscs is essential for understanding their ecological niches, as well as for their conservation and aquaculture. Freshwater mussels are primarily filter feeders and the complex mechanism of filtration has been studied intensively (Ward *et al*., 1998; Urrutia *et al*., 2001; Garrido *et al*., 2012). However, less is known concerning the physiological mechanisms following ingestion, in particular how food particles are processed in the digestive tract. In bivalves, energy is mostly stored in the form of glycogen, which accounts for 5–14% of the dry weight in *Anodonta cygnea* (Gäde & Wilps, 1975). For the digestion of various carbohydrates, the crystalline style, a transparent rod composed in part of glycoside hydrolases, is known to be a key component of digestion in the stomach of many snails and marine bivalves. Previous studies (e.g. Alyakrinskaya, 2001) have shown the ability of crystalline style to break down starch in *Pseudanodonta complanata* and cellulose in *Mytilus*. Moreover, it has been suggested that crystalline style material not only releases digestive enzymes, but also provides an optimal digestion milieu by buffering ambient pH. Its physiological characteristics, including pH and dissolution, have been described by Hameed (1985) and Warren (1987).

This study evaluates the ability of crystalline styles excised from *Anodonta anatina* to break down different carbohydrate sources and tests the buffering capacity of style material for providing optimal conditions for digestion.

To determine enzymatic activities of crystalline styles, 51 individuals of *A. anatina* were examined (23 for enzyme activity assays and 28 for buffering capacity assays). The mussels were purchased from Bavarian fish hatcheries, where they were raised as a byproduct in semi-natural carp ponds. They were brought to the Aquatic Systems Biology Unit of Technische Universität München in Freising, Germany. Mussels (length 10.9 ± 2.2 cm, mean $\pm$ SD) were kept in a water tank from 1 to 22 August 2012, with a water supply from the river Moosach (15.6 ± 2.5°C; 8.4 ± 5.3 mg/l O_2), to provide natural nutrition. The species was determined by morphometric (Killeen, Aldrige & Oliver, 2004) and—for a subset of eight individuals used in the laminarinase assay—by genetic (Zieritz *et al*., 2012) methods.

To test the pH buffering capacities, 28 crystalline styles were dissected from the posterior end of the stomach and transferred into seven media (H_2O·HCl or H_2O·NaOH), each with different pH (4.0, 5.0, 6.0, 7.0, 8.0, 10.0, 12.0). The volumes were adjusted according to the size of the crystalline styles, with weight-to-volume ratios of style material in media ranging from 0.04 to 0.42 g/ml. Before adding the style material and after its dissolution, pH was measured with a portable multiparameter instrument (WTW multi 340i).

The enzymatic activity of the crystalline style was investigated using 15 crystalline styles dissolved in deionized water. Cellulose (ARBOCEL®; final concentration 500 μg/ml) and commercially available starch (final concentration 500 μg/ml) were added separately to three samples, respectively. Laminarin (*Laminaria digitata*, Sigma-Aldrich; final concentration 2 mg/ml) was added to eight samples. Five controls contained water, style material or one of the substrates. After an incubation time of 24 h at 17°C, the samples were deep-frozen and stored at −20°C until further analysis to determine possible breakdown of substrates to glucose by UV-test (manufacturer's specifications, r-biopharm). The determination of glucose was based on two enzymatic reactions which eventually released NADPH in equivalent amounts as glucose. In this assay, D-glucose is phosphorylated to D-glucose-6-phosphate by the enzyme hexokinase under hydrolysis of ATP. D-glucose-6-phosphate is then oxidized by glucose-6-phosphate-dehydrogenase via reduction of NADP to NADPH, which is photometrically detectable at a wavelength of 340 nm.

The results of the UV-test confirmed the breakdown of starch and laminarin to glucose in all samples; however, glucose was not detected when cellulose was provided as substrate (Table 1). Within the control groups, glucose was absent. Laminarin is a complex glucan, built up of $\beta(1-3)$ and $\beta(1-6)$ linkages (Sena *et al*., 2011) and it is widely present in algae, protozoans and fungi. Most of the species farmed in current agricultural practice cannot use this source of glucose due to a lack of enzymatic capabilities (Piavaux, 1977). In contrast, laminarinase activity has been described for marine bivalves including Mytilidae, Pectinidae, Veneridae and Myidae (Sova, Elyakova & Vaskovsky, 1970) but,

Table 1. Experimental detection of activity and function of glucanases released by the crystalline style of *Anodonta anatina*; the table shows average percentages of initial substrate concentrations that have been converted to glucose during the enzyme activity assay and the standard deviation (n = sample size).

Crystalline style	Substrate							
	Starch		Cellulose		Laminarin		None	
No	0%	$n = 1$	0%	$n = 1$	0%	$n = 1$	0%	$n = 1$
Yes	35 ± 10%	$n = 3$	0%	$n = 3$	33 ± 20%	$n = 8$	0%	$n = 1$

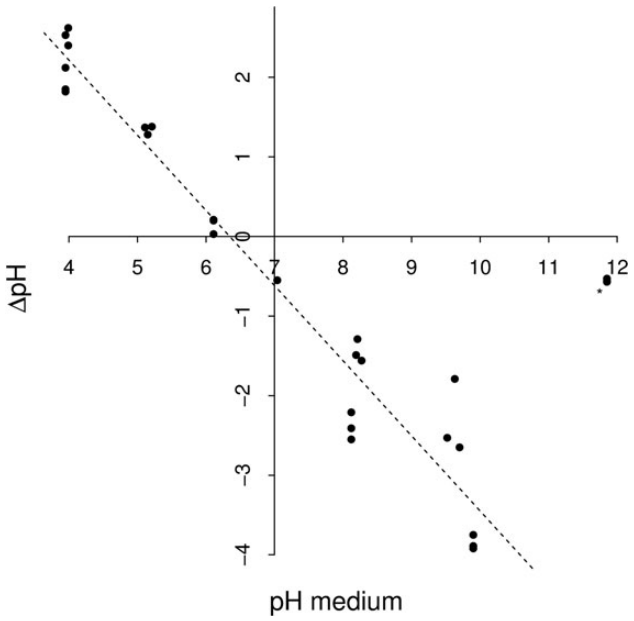

Figure 1. Influence of crystalline style material of *Anodonta anatina* on the pH of different media. The dotted line indicates a linear relation: $R^2 = 0.62$; *the three values at pH 12.0 were excluded from the linear model since they were found to be beyond the buffering capacity.

to our knowledge, the results of this study provide the first confirmation of this enzyme in freshwater mussels of the family Unionidae. Piavaux (1977) investigated the distribution of digestive laminarinases in animals and concluded that laminarinolytic activity is (1) common to different phyla and (2) strongly related to the presence of $\beta(1-3)$ glucans in the diet. As filter feeders, Unionidae feed on various food sources, i.e. phytoplankton, zooplankton, diatoms, bacteria, detritus or dissolved organic matter (Vaughn, Nichols & Spooner, 2008) and diatoms are an especially important source of $\beta(1-3)$ storage glucans (Chiovitti *et al.*, 2003).

The enzymatic activity of laminarinase and amylase requires pH values between 5.0 and 9.0, with optima in the slightly acidic to neutral range (pH 5.0–7.0) (Areekijseree *et al.*, 2004). The current results support Alyakrinskaya (2001) who suggested that crystalline style material releases digestive enzymes and also provides an optimal milieu by buffering ambient pH conditions. The dissolution of *A. anatina* crystalline styles in defined media with initial pH values from 4.0 to 10.0 changed pH to an average of 6.4 (SD 0.51). Figure 1 summarizes the change of pH in each medium. In media with pH 12.0 almost no change occurred (mean 11.3). However, the buffering action is linear from pH 4.0 to 10.0, i.e. within a pH range that is much wider than the pH values typically observed in freshwater mussel habitats. At low pH values, on the other hand, no threshold was detected at which regulation ceases. This may be due to the fact that no pH values below 4.0 were tested. Understanding the effect of pH on catalytic efficiency of laminarase is also important, but was beyond the scope of this study.

The range of pH regulation between <4.0 and 12.0 is quite large, compared with other biological buffer systems. Warren (1987) investigated style proteins of twelve bivalve species and concluded that the most abundent proteins are glycoproteins. Microscopic observations of the crystalline style in the current study revealed a two-layered structure, with a less-transparent tube within a transparent coating. This observation could reflect the two main functions of the style, which can be described as an integrated multi-component system based on several layers: the coating as a biological buffer and the inner for enzyme release. It remains unclear which components of the style contribute to this exceptional buffering ability, because most biological buffers show narrower optimal pH ranges. This indicates a buffer system possibly based on multiple components, for instance polypeptides and salts. To evaluate the full potential for digesting different types of food particles (e.g. phytoplankton species), further investigations on the enzymatic breakdown of polysaccharides by style material, as well as studies considering the particle-size selectivity during the filtering process, should be conducted. Such information could be useful to determine the nutritional requirements of freshwater mussels, which still remain rather vague.

In summary, this study provides the first evidence of laminarinase activity in freshwater mussels, along with the observation of pH buffering by crystalline styles to maintain the functioning of this enzyme. Information on the dietary requirements is required to improve existing efforts in conservation and breeding of endangered species of freshwater bivalves (Geist, 2010; Gum, Lange & Geist, 2011). At the same time, it underlines the great potential of bivalves for aquaculture, as they are able to digest plant material not utilizable by most of the other commonly farmed species.

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