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The Ruminant Digestion Model Using Bacteria Already Employed Early in Evolution by Symbiotic Molluscs

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Abstract. The purification and some molecular properties of six lysozymes from the gills of different mytilids and vesicomyids are described: they belong to the previously described Invertebrate lysozyme family. The predominance of the bacterial nutrition in these organisms seems to necessitate the presence of a lysozyme as in the case of the ruminant digestion model.

Key words: Symbiotic molluscs — Ruminent digestion model — Bacteria

Introduction

Mammals have adapted to a wide range of both physical environments and of diets; some adapted to herbivorous diets containing a high amount of cellulose, which cannot be digested by mammals and thus remains inaccessible as a food source. Digestive (foregut) fermentation allows a symbiotic microbial population to ferment with the help of the plant material cellulases and to release useful compounds, more particularly short-chain fatty acids (acetate, proprionate, butyrate), which are absorbed through the stomach wall into the bloodstream (Dobson et al. 1984; Jollès and Jollès 1984; Stewart et al. 1987).

However, some of the carbon and most of the nitrogen and phosphorus in the ruminant diet end up as components of the rapidly growing population of bacteria in the foregut. To retrieve the nitrogen and phosphorus, which are essential for animal growth and milk production, the ruminant must digest some of the bacteria. This is no easy task because the bacteria are protected by an unusual chemical in their cell walls which resists attack by the enzymes in a conventional digestive tract.

Bacteria from the foregut pass into the true stomach where they encounter lysozyme (EC 3.2.1.17). Several lysozyme families have been described (Jollès and Jollès 1984) that, while differing in molecular weight, amino acid sequence, and molecular and kinetic properties, all have the same specificity for cleaving the cell walls of bacteria. Lysozyme is present in high concentration in ruminant stomachs but in low concentration in many nonruminant stomachs. Ruminants are enzymically equipped to digest bacteria, whereas many other mammals are not. Mammals of the latter type employ lysozyme mainly as a shield against bacterial invasion—for example, in tears and white blood cells. But in the true stomach of ruminants, lysozyme has evidently been recruited as a major digestive enzyme, a change from its normal function as an antibacterial defense enzyme (Dobson et al. 1984; Jollès et al. 1984).

Both regulatory and structural mutations made this recruitment of lysozyme possible. For example, the number of lysozyme molecules produced by the stomach lining increased 70-fold, and mutations in the structure of the protein fitted it better for function in the stomach fluid, which is very acidic and contains pepsin. Resistance to cleavage by pepsin is most likely the result of

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fewer pepsin-sensitive bonds and decreased electrostatic interactions with pepsin; when examining the distribution of charges on cow lysozyme, it is clear that all its faces bear more negative charges than the corresponding faces in hen and human lysozymes. Resistance to the acidic medium can be illustrated by the disappearance of the acid-sensitive Asp-Pro linkage usually encountered in lysozymes: Asp is replaced by Glu and Pro is deleted. In the ruminant lysozyme case, where a plausible link exists between molecular evolution and organismal evolution, regulatory change seems to have played a primary role and protein structural changes an important secondary role (Stewart et al. 1987; Jollès et al. 1984, 1989, 1990).

Bacteria have also been encountered as the chief source of food in the world of invertebrates, and in this case again, a lysozyme has been characterized.

That is the case for bivalve molluscs previously discovered as a main component of luxuriant communities (biomasses up to 100 kg/m^2) of giant organisms associated with deep hydrothermal vents on the Pacific Rise at the Galapagos rift (Lonsdale 1977). Further explorations demonstrated that the environments exploited by such populations are widespread on the ocean floor, including active deep hydrothermal vents on midocean ridges and cold seeps in subduction zones as well as hypersaline or oil seeps in passive margins and rich-sulfide littoral sediments (reviewed in: Grassle 1986; Southward 1987; Laubier 1989; Tunnicliffe 1991). It has been established that these communities are chemosynthetic based, living on bacterial primary production (Jannasch 1989).

The discovery of autotrophic pathways in the gill of the main species associated to the observation of endocellular bacteria led to the description of new symbiotic models between bacteria and these bivalves (reviewed in: Fiala-Médioni et al. 1990; Fisher 1990; Childress and Fisher 1992; Cavanaugh 1993). Vesicomyidae living either as epifaunal in the cracks of hydrothermal vents or half-burried in mud trenches are, along with Mytilidae, among the major organisms of these sites and represent ecological and biological models comparable to mollusc–algae symbioses in tropical waters.

An active lysosomic resorption of the bacteria was observed on transmission electronic microsis (TEM) (Fiala-Médioni 1984; Fiala-Médioni and Le Pennec 1987; Fiala-Médioni et al. 1990), and lysosomic enzymes as well as lysozyme were characterized in the gills of both *Bathymodiolus* and *Calyptogena* symbiotic models (Fiala-Médioni et al. 1994). So, as observed in ruminants, the digested bacteria appear to represent the main source of nutrients in these molluscs.

In the present paper we describe the purification and some molecular properties of a series of lysozymes isolated from the gills of mytilids (*Mytilus* and *Bathymodiolus*) and vesicomyids (*Calyptogena*). These all belong to the previously described invertebrate lysozyme family.

Materials and Methods

Materials. One species of Vesicomyidae (*Calyptogena magnifica*) was collected in an hydrothermal vent (2,250 m in depth) from the Galapagos Rise during the ''Galapagos'' Cruise (1985). Two other undetermined Vesicomyidae (*Calyptogena* KN5 and *Calyptogena* KN7) were obtained from Japan Trenches cold seep sites (depth: 1,999 m and 3,788 m, respectively) during the ''Kaiko-Nankai'' Cruise (1989). The Mytilidae (*Mytilus galloprovincialis*) were collected from the bay of Banyuls-sur-Mer (Golfe du Lion, southwestern France) in littoral waters and from the hydrothermal vents: in the West Pacific (Fiji Basin, depth: 1,970 m for *Bathymodiolus brevior*) during the ''Yokosuka'' Cruise (1991) and in the Atlantic (*Bathymodiolus* sp. LS, depth: 1,964 m) during the ''Lucky-Strike'' Cruise (1993).

The littoral mytilid was collected by scuba diving and all other species were collected using submersibles. The samples were dissected immediately on board ship after recovery and tissues were frozen in liquid nitrogen until analyses.

Determination of the Lytic Activity. Lytic activity was determined by observing spectrophotometrically at 540 nm the increase in transmittance which occurred during the lysis of a suspension of *Micrococcus luteus* according to the procedure of Jollès et al. (1965). However, the phosphate buffer (pH 6.2, *I*: 0.164) was employed after a fourfold dilution. Hen egg-white lysozyme was used as a standard.

Purification of the Lysozymes. The gills were ground in 0.1 M acetic acid. The suspension was centrifuged (12,000 rev/min, 50 min at 4°C). The clear supernatant was chromatographed on CM-52 cellulose (0.1 M ammonium acetate, pH 5.8, 4°C): the column was washed with the buffer until disappearance (at 280 nm) of all material not bound to the resin. The enzyme was eluted during a gradient elution (0.1 M ammonium acetate, pH 5.8, 0.1 $\rightarrow$ 0.8 M). The active fraction was dialyzed against distilled water at 4°C during 2 h (Spectrapor 1000). The purification was completed by SDS-PAGE electrophoresis through a 12.5% acryalmide gel.

N-terminal Sequence Determination. After SDS-PAGE, the lysozyme band was eluted during 18 h at 37°C by 0.1 M sodium acetate (pH 8.5, 0.1% SDS). The eluate was submitted to the ProSpin method (sample preparation cartridge; Applied Biosystems, Foster City, CA, USA) in order to submit the disk with the enzyme directly to sequence analysis. It was performed by Edman degradation using an automated gas-phase Sequencer (model 470 A, Applied Biosystems). The phenylthiohydantoin amino acid derivatives were automatically identified with an Applied Biosystems 120 A phenylthiohydantoin analyzer used on-line with the sequencer.

Results and Discussion

The lysozyme content of the Vesicomyidae and Mytilidae gills was slightly variable but generally had mean values between 0.1 and 0.3 mg lysozyme/g of gill (expressed as hen lysozyme).

Six different lysozymes were evidenced on six species and purified by chromatography on CM-52 cellulose. As an example, Fig. 1 shows the purification of the lyso-

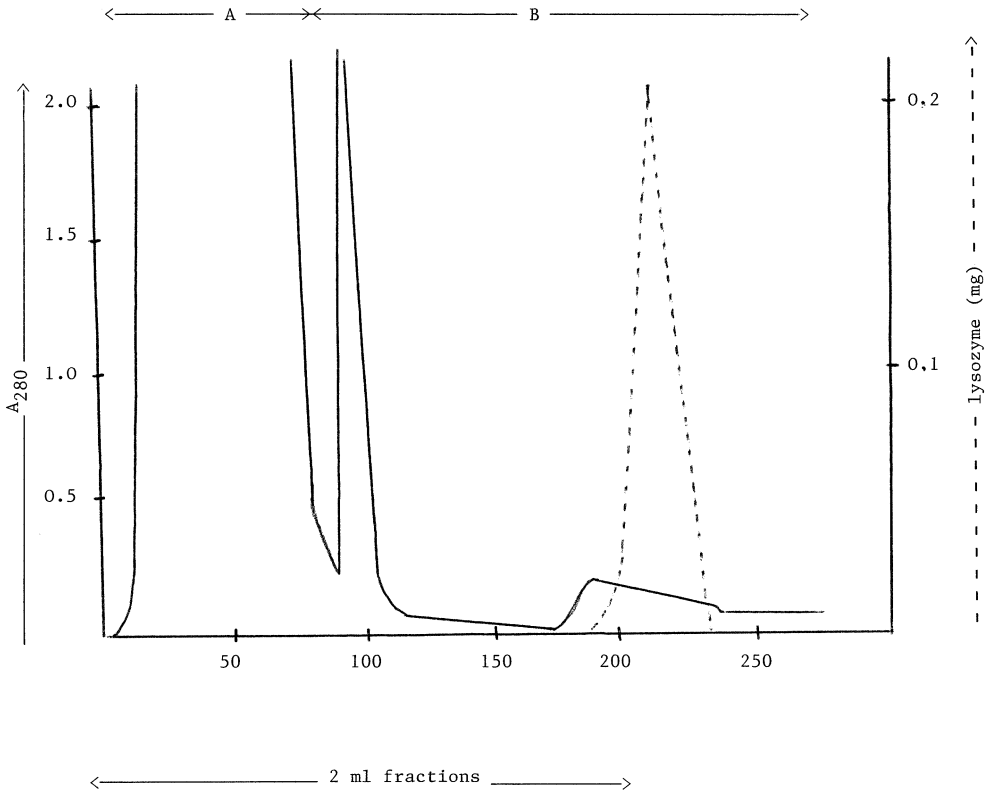

Fig. 1. Chromatography at 4°C on CM-52 cellulose (11 cm × 2 cm) of *Mytilus galloprovincialis* gills (45 g) ground in 0.1 M acetic acid (90 ml, clear supernatant). **A** 0.1 M ammonium acetate, pH 5.8; **B** gradient elution pH 5.8; 0.1 M → 0.8 M ammonium acetate; mixing chamber: 200 ml of solution A. Hen egg-white lysozyme was used as a standard.

zyme from *Mytilus galloprovincialis.* The enzyme was eluted with the ammonium acetate gradient between 0.45 and 0.5 M. This value is nearly the same for the *Calyptogena* KN5 and KN7 lysozymes and slightly different for the *Calyptogena magnifica* enzyme (0.28–0.35 M).

The purity of the lysozymes was ascertained by N-terminal sequence analysis and their molecular masses were determined by SDS-PAGE (15,500 ± 1,000).

Figure 2 reports the N-terminal sequences (between 16 and 24 amino acids) of the six lysozymes. The three Vesicomyidae lysozymes on one side and the three Mytilidae enzymes on the other side are quite homologous, with molecular differences more pronounced between the two families than among the species of a same family.

Using these data it can be asserted that all the lysozymes found are homologous to the previously described *Asterias rubens* lysozyme (Jollès and Jollès 1975), which was claimed to be the representative of a new lysozyme family—the invertebrate lysozymes—as its N-terminal sequence is not recognizably related to the other known N-terminal ones from the lysozyme families known thus far.

A characteristic common to all the symbiotic species is the simplification and reduction of the digestive tube. Different degrees can be found, and the vesicomyid clams appear more strictly dependent on their endocellular bacteria than the *Mytilidae Bathymodiolus* able to filter feed; yet this ability is reduced when compared with the littoral nonsymbiotic Mytilidae (Page et al. 1991; Pranal 1995).

The bivalve families considered the most ancient from a phylogenetic point of view comprise the majority if not the totality of the symbiotic species. Reid and Brand (1986) suggested that the acquisition of symbiotic bacteria by lucinarean bivalves in the Paleozoic sponsored their survival and subsequent diversification in the Mesozoic. Furthermore, fossil forms of the genera Bathymodiolus, Lucina, Thyasira, Solemya, and Achorax were observed in the fossil sources of the Pliocene and Miocene in different parts of the world (Niitsuma et al. 1989; Campbell 1992; Taviani 1994; Gaillard et al. 1992). More ancient fauna have ever been described at the end of the Jurassic (Gaillard and Rolin 1988) and the early Cretaceous, in which isotopically light carbonates appear to have formed from bacterial oxidation (Beauchamp et al. 1989).

The appearance of these symbiotic forms long before the emergence of mammals, in areas where the environment probably was very close to those containing symbiotic molluscs, could only materialize via the acquisition of a process of nutrition that allowed the host to recover the organic material elaborated by its bacterial symbionts. Thus, it seems justified to suggest that a ly-

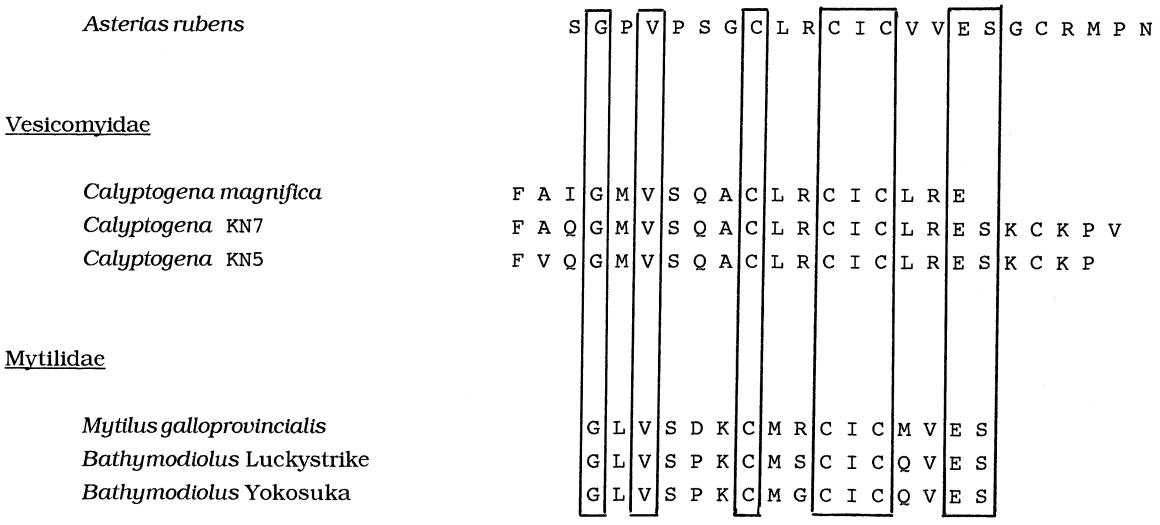

Fig. 2. N-terminal sequence of three Vesicomyidae and three Mytilidae lysozymes: comparison with the *Asterias rubens* lysozyme (Jollès and Jollès 1975). Identical amino acids in all the lysozymes are *boxed.* The *one-letter amino acid abbreviation* system was used.

sozyme with a digestive role allowed such a process to take place.

Insofar as the ''digestive'' mollusc lysozymes are structurally different from the ruminant enzymes, it seems to be a question of functional convergence which reappeared later in the course of the evolution.

The animals from very distant sites (Galapagos, Japan, Fiji, the Atlantic) all possess structurally related lysozymes which might derive from a common ancestor. In the case of the Mytilidae the lysozymes found in symbiotic as well as in nonsymbiotic species are quite similar.

Although lysozyme activity in the gills of the nonsymbiotic species *M. edulis* is much lower than in the symbiotic ones (Fiala-Médioni et al. 1994), a nonnegligible lysozyme activity is present in the digestive gland of *M. edulis*, where the absorption of the filtered bacteria associated with particles in the feeding current takes place (McHenery et al. 1986).

The lysozymes found in molluscs (present study) and in *Asterias rubens* (Jollès and Jollès 1975) belong to the same lysozyme family, invertebrate lysozymes, different from the other lysozyme families characterized so far (c-, g-, phage, bacteria, plant types) and not (yet) found in vertebrates (Jollès and Jollès 1984). But all lysozymes, whatever their origin, have the same specificity: they cleave the glycosidic bond between the C-1 of *N*-acetylmuramic acid and the C-4 of *N*-acetylglucosamine in the bacterial peptidoglycan, although the experimental conditions (pH, *I*) may vary.

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