The role of bacteria in the digestion of seaweed by the abalone *Haliotis midae*

Jean H. Erasmus a, Peter A. Cook a, Vernon E. Coyne b,*

a Zoology Department, University of Cape Town, Private Bag, Rondebosch 7700, South Africa
b Microbiology Department, University of Cape Town, Private Bag, Rondebosch 7700, South Africa

Accepted 15 December 1996

Abstract

The polysaccharolytic enzymes of the abalone *Haliotis midae* and its resident gut bacteria were investigated. Bacteria isolated from the abalone gut were able to degrade the polysaccharides laminarin, carboxymethylcellulose (CMC), alginate, agarose and carrageenan. Detection of alginate lyase, CMCase, laminarinase, agarase and carrageenaase in the hepatopancreas, which was devoid of bacteria, indicated that *H. midae* produces a range of polysaccharases. The endogenous polysaccharases of abalone fed either *Ecklonia maxima* or *Gracilaria verrucosa* varied in response to diet. It is proposed that bacteria resident in the digestive system of *H. midae* assist in the digestion of alginate, laminarin, agarose, carrageenan and cellulose. © 1997 Elsevier Science B.V.

Keywords: *Haliotis midae*; Abalone; Gut bacteria; Polysaccharide digestion

1. Introduction

Recent research to improve the growth rate of cultured abalone has focused on feeding efficiency and artificial diets for enhancing growth rate (Britz, 1994). However, another potentially important aspect is the role of microorganisms resident in the gut. A number of studies have documented the interactions that occur between bacteria and their invertebrate hosts (reviewed by Harris, 1993). Vitalis et al. (1988) noted that it is necessary to assess the enzymatic activity of bacteria resident in species which feed on...
nutritionally poor seaweeds. This is because bacteria can enhance the digestion efficiency of the host by supplying polysaccharolytic enzymes, and consequently, improve the growth rate of the host (El-Shanshoury et al., 1994). Since many species of abalone feed largely on kelp, which is deficient in many essential nutrients (Simpson, 1994), it is possible that gut bacteria play an integral role in the digestion of complex algal polysaccharides. Although the digestive enzymes of abalone have been identified (Campbell, 1965; McLean, 1970; Knauer et al., 1996), no previous study has determined whether these are bacterial in origin.

Bacteria from the digestive tract of *Haliotis midae* were previously isolated, quantified and identified by Erasmus (1996). The present paper investigated whether these bacterial strains produce enzymes that assist in the breakdown of seaweeds. This hypothesis was tested with a range of polysaccharides from algal species commonly fed to farmed abalone. Thus, agarose and carrageenan (predominant in red seaweeds), alginate, carboxymethylcellulose (CMC) and laminarin (from kelp species) were investigated. The specificity of endogenous polysaccharases were also examined. The influence of bacterial enzymes on the digestion of seaweeds ingested by abalone is discussed.

2. Materials and methods

2.1. Bacterial strains and *H. midae*

Abalone were obtained from the Sea Fisheries Research Institute, Sea Point, Cape Town, South Africa. The animals were kept at 18°C (±0.5°C) and fed a diet of *Ecklonia maxima*. Abalone fed primarily on *Gracilaria verrucosa* were obtained from Marine Growers, Port Elizabeth. Bacterial strains were isolated during a previous study (Erasmus, 1996).

2.2. Production of gnotobiotic abalone

The minimal concentration of antibiotic required to kill abalone enteric bacteria was determined by inoculating abalone gut homogenates on bacterial culture media that were supplemented with antibiotics at various concentrations. The antibiotics screened were streptomycin, chloramphenicol, penicillin, kanamycin, tetracycline, ampicillin and cefotaxime.

Abalone were then treated with antibiotics to remove all microorganisms from the gut as follows. Abalone (3.5 cm shell length) were placed in sterile 1-l beakers containing 500 ml autoclaved sea water that was aerated by pumping sterile air through an autoclaved air stone placed at the bottom of the water column. The air entering the water was sterilised by filtration through a 0.2-μm acetate filter inserted in a plastic, autoclaved air line. The sea water was dosed with the following antibiotics (final concentration): ampicillin (600 μg/ml), chloramphenicol (250 μg/ml) and cefotaxime (250 μg/ml). The sea water and antibiotic cocktail was changed every 12 h over a 72 h period. Animals were fed autoclave-sterilised artificial food (Sea Plant Products, Hermanus, South Africa) and maintained in the dark to promote feeding.
2.3. Dissection of the digestive tract

The digestive tracts of normal and gnotobiotic abalone (n = 6 for each group) were dissected aseptically on ice. Each digestive tract was homogenised separately in 10 ml of 100 mM potassium phosphate buffer, pH 6.9, using autoclaved glass homogenisers. Samples of the gnotobiotic abalone digestive tract homogenates were tested for sterility by inoculating ten-fold dilutions on marine agar and incubating the cultures at 22°C for 2 days. The digestive tract homogenates from gnotobiotic and normal animals were clarified by centrifugation at 10,890 × g for 15 min and the supernatant retained for enzyme assays.

2.4. Dissection of the hepatopancreas

Polysaccharases produced by H. midae were identified by assaying hepatopancreas extracts using various polysaccharide substrates. The hepatopancreas was removed from animals fed primarily a diet of either E. maxima or G. verrucosa. The animals were dissected aseptically to avoid contamination with fluids containing bacteria from other regions of the digestive tract. The same region of the digestive gland was excised from each animal (Fig. 1), thus avoiding the possibility that different regions of the hepatopancreas may produce different enzymes.

Each hepatopancreas was homogenised in sterile 100 mM potassium phosphate buffer, pH 6.9, using an autoclaved glass homogeniser. Diluted samples of homogenised hepatopancreas were inoculated onto marine agar and incubated for 3 days at 22°C to determine whether bacteria were present in the hepatopancreas. As some strains of bacteria may not grow on synthetic bacterial culture media, 4',6-diamidino-2-phenylindole (DAPI) epifluorescence microscopy and scanning electron microscopy (SEM) was

![Fig. 1. Schematic representation of the abalone digestive tract showing the portion of the hepatopancreas excised for enzymatic analysis.](image-url)
performed on extracts of hepatopancreas to establish unequivocally that it is sterile. The preparation of samples for SEM was adapted from the protocol of Eisenman and Al ferment (1982). Dehydrated samples were coated with 60 μm gold palladium and observed with a Cambridge S200 SEM. DAPI epifluorescence microscopy was as previously described (Erasmus, 1996). The hepatopancreas homogenates were centrifuged at 1089 × g for 15 min to remove cell debris and the supernatant retained for polysaccharase assays.

2.5. Preparation of bacterial samples

Bacteria, isolated in a previous study (Erasmus, 1996), were grown in 5 ml defined culture media supplemented with either agarose, CMC, carrageenan, laminarin or alginate as a carbon source. The basal media consisted of (wt./vol): 0.1% yeast extract, 0.1% peptone and the appropriate polysaccharide substrate in filtered (Whatmans no. 1 filter paper) sea water. The final concentrations (wt./vol) of the polysaccharides added to the media were as follows: 1% alginate, 1% CMC, 0.2% laminarin, 0.1% agar and 0.1% carrageenan. Bacterial cultures were incubated at 22°C for between 12 and 16 h with agitation. Cells were harvested by centrifugation at 12096 × g and resuspended in either 5 ml 100 mM potassium phosphate buffer, pH 6.9 (Nelson–Somogyi assay), or 5 ml 20 mM Pipes buffer, pH 6.8 (ferricyanide reducing sugar assay). The cells were disrupted using a French press. The spent growth medium was retained in order to assay for the presence of extracellular bacterial polysaccharases.

2.6. Determination of polysaccharolytic activity

Polysaccharase activity was determined by assaying the amount of reducing sugar produced following hydrolysis of a particular polysaccharide substrate over a specific time period. The polysaccharide substrates were assayed at the following concentrations (wt./vol): 0.4% carrageenan, 1% CMC, 0.4% agarose, 0.4% laminarin and 1% alginate. Alginate lyase, laminarinase and CMCase were assayed according to the method of Nelson (1944) and Somogyi (1952), while agarase and carrageenase activities were determined with the ferricyanide reducing sugar assay of Gardner et al. (1987). Enzyme activities were expressed as mg-reducing sugar generated/mg protein/h, where the amount of reducing sugar liberated from each of the polysaccharide substrates was determined from standard curves constructed by plotting light absorbance (at the wavelength appropriate to the reducing sugar assay) against known concentrations of the appropriate sugar. The protein concentration of the sample was determined using the protocol of Bradford (1976). Since the hepatopancreas and digestive tract extracts contained large concentrations of protein, the activity of the enzymes assayed in these samples were expressed as mg-reducing sugar/ml/h (the bacterial culture supernatants contained negligible amounts of extraneous protein).

3. Results

All of the abalone enteric bacterial isolates secreted between 70–90% of their polysaccharases when grown in synthetic media supplemented with various polysaccha-
Table 1
Extracellular enzyme activity and substrate specificity of the polysaccharases of the enteric bacteria isolated from the digestive system of *H. midue*

<table>
<thead>
<tr>
<th>Gut region</th>
<th>Bacterial strain</th>
<th>Alginate&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Laminarin&lt;sup&gt;b&lt;/sup&gt;</th>
<th>CMC&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Agarose&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Carrageenan&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crop</td>
<td><em>Pseudomonas</em> C4</td>
<td>152.3 ± 4.7&lt;sup&gt;d&lt;/sup&gt;</td>
<td>11.7 ± 1.4</td>
<td>67.4 ± 11.9</td>
<td>0</td>
<td>24.3 ± 4.7</td>
</tr>
<tr>
<td></td>
<td><em>Flavobacteria</em> Z2</td>
<td>1.8 ± 1.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Alcaligenes</em> C3</td>
<td>5.9 ± 2.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total activity</td>
<td></td>
<td>152.3</td>
<td>17.6</td>
<td>69.2</td>
<td>0</td>
<td>24.3</td>
</tr>
<tr>
<td>Stomach</td>
<td><em>Vibrio</em> Y8</td>
<td></td>
<td>7.0 ± 1.3</td>
<td>13.8 ± 0.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Flavobacteria</em> Y2</td>
<td>4.1 ± 2.4</td>
<td>4.0 ± 1.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Pseudomonas</em> Y7</td>
<td>5.9 ± 3.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Pseudomonas</em> Y5</td>
<td>3.0 ± 1.8</td>
<td>2.9 ± 1.9</td>
<td>2.4 ± 0.4</td>
<td>29.2 ± 1.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Vibrio</em> S1</td>
<td>2.8 ± 2.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Flavobacteria</em> S2</td>
<td>5.6 ± 2.1</td>
<td>2.8 ± 1.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Vibrio</em> X6</td>
<td>8.8 ± 2.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total activity</td>
<td></td>
<td>21.5</td>
<td>8.5</td>
<td>9.9</td>
<td>9.4</td>
<td>43.0</td>
</tr>
<tr>
<td>Intestine</td>
<td><em>Vibrio</em> S2</td>
<td></td>
<td>2.6 ± 2.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Pseudomonas</em> S7</td>
<td></td>
<td>8.0 ± 2.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Moraxella</em> S3</td>
<td></td>
<td>6.3 ± 2.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Unidentified J2</td>
<td>4.9 ± 2.1</td>
<td></td>
<td>5.1 ± 1.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Vibrio</em> J4</td>
<td>6.1 ± 1.2</td>
<td>5.3 ± 0.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Vibrio</em> J3</td>
<td>2.8 ± 1.8</td>
<td>4.8 ± 1.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Pseudomonas</em> J3</td>
<td>3.9 ± 1.7</td>
<td>4.2 ± 1.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Alcaligenes</em> U6</td>
<td></td>
<td>3.1 ± 0.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Vibrio</em> J1</td>
<td>10.6 ± 0.5</td>
<td></td>
<td>8.8 ± 0.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Vibrio</em> R5</td>
<td>2.9 ± 1.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Bacillus</em> T4</td>
<td>1.8 ± 1.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Vibrio</em> J4</td>
<td></td>
<td>5.9 ± 1.0</td>
<td>24.8 ± 3.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Vibrio</em> J1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Alcaligenes</em> S4</td>
<td></td>
<td>98.9 ± 13.1</td>
<td>25.8 ± 1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Vibrio</em> U5</td>
<td>5.0 ± 1.0</td>
<td>31.1 ± 0.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Vibrio</em> U4</td>
<td>4.8 ± 1.2</td>
<td>26.6 ± 4.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Vibrio</em> S5</td>
<td>2.9 ± 0.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Flavobacteria</em> S6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>23.3 ± 3.9</td>
</tr>
<tr>
<td>Total activity</td>
<td></td>
<td>33.0</td>
<td>17.4</td>
<td>22.0</td>
<td>126.3</td>
<td>131.6</td>
</tr>
</tbody>
</table>

Each value = the average of 3 separate experiments.

*<sup>a</sup> mg galacturonic acid/mg protein/h.
*<sup>b</sup> mg glucose/mg protein/h.
*<sup>c</sup> mg galactose/mg protein/h.
*<sup>d</sup> Standard error.

rides which occur in macroalgae. In general, bacterial strains belonging to the genera *Pseudomonas* and *Vibrio* were the most effective at hydrolysing the polysaccharides tested (Table 1). In particular, *Pseudomonas* strain C4 from the crop of *H. midue* exhibited very high alginate lyase, laminarinase and CMCase activity. *Alcaligenes* strain S4 from the intestine produced the highest agarolytic activity, while the carrageenolytic activity of this strain was comparable to the other enteric bacteria. Although the number and diversity of bacteria is greater towards the latter region of the gut, the distribution of
extracellular bacterial polysaccharases did not follow the same trend. Bacterial alginate lyase and CMCase activity was highest in bacteria isolated from the crop of the abalone, while laminarinase activity was equivalent in bacteria colonising the crop and intestine (Table 1). Bacterial agarase and carrageenase enzyme activity was most pronounced in bacterial strains isolated from the intestine of *H. midae*.

Antibiotic susceptibility screening showed that streptomycin, kanamycin, tetracycline and penicillin had little effect on the abalone gut bacteria. Chloramphenicol and cefotaxime, at final concentrations of 20 μg/ml and 150 μg/ml respectively, inhibited the growth of abalone enteric bacteria on marine agar, but failed to reduce the numbers of gut bacteria when applied to live abalone. An antibiotic cocktail containing 600 μg/ml ampicillin, 250 μg/ml chloramphenicol and 250 μg/ml cefotaxime was found to render 3.5 cm abalone gnotobiotic without any toxic side effects.

Reducing sugar assays conducted on the digestive tracts of gnotobiotic *H. midae* previously fed *E. maxima* demonstrated the presence of enzymes which hydrolysed CMC, alginate and laminarin (Fig. 2). Similarly, CMCase, alginate lyase and laminarinase was detected in the hepatopancreas of *H. midae* fed *E. maxima* (Fig. 3). High carrageenase and agarase activity, and decreased levels of CMCase, alginate lyase and laminarinase activity, occurred in the hepatopancreas of animals fed primarily *G. verrucosa* (Fig. 3). Agarolytic and carrageenolytic enzyme activity was not detected in the gut of gnotobiotic *H. midae* (Fig. 2) or the hepatopancreas of animals fed *E.*
**Fig. 3.** Substrate specificity of *H. midae* hepatopancreatic enzymes. Each bar represents the average of results obtained from 8 different animals. Vertical lines indicate standard error.

Maxima (Fig. 3). DAPI epifluorescence microscopy, SEM and culture on laboratory growth media failed to detect bacteria in the hepatopancreas.

**4. Discussion**

The gut bacteria of *H. midae* were able to hydrolyse agar, carrageenan, CMC, laminarin and alginic acid. In fact, most of the bacterial strains isolated from the gut were able to utilise two or three polysaccharides. The observation that approximately 70–90% of the bacterial polysaccharolytic activity was extracellular suggests that bacterial enzymes are secreted into the lumen of the gut. The polysaccharase activity of bacteria isolated from the crop was higher than that in stomach or intestinal isolates with respect to two of the five polysaccharides tested. The alginate lyase activity produced by *Pseudomonas* strain C4 from the crop was 15 times greater than that of other alginolytic bacteria. Similarly, the CMCase activity expressed by strain C4 was three times greater than the other cellulolytic bacteria. This is surprising as the number of bacteria colonising the crop (8.9 × 10^8 cells/g tissue) was an order of magnitude lower than in the stomach (3.0 × 10^9 cells/g tissue) and intestine (4.4 × 10^9 cells/g tissue) (Erasmus, 1996).

In contrast to the location of the alginolytic and cellulolytic abalone gut bacteria, agarolytic and carrageenolytic activity was predominantly associated with intestinal
bacterial strains. Although the difference in pH of the crop and stomach to that of the intestine (pH 5.6 and 6.5, respectively) may account for this, the reason for the intestinal location of the majority of the agarolytic and carrageenolytic bacteria remains to be determined.

Assays of the hepatopancreatic enzymes of *H. midae* showed that the abalone produces its own cellulase, alginate lyase, laminarinase, agarase and carrageenase. Confirmation that these enzymes were produced by the abalone themselves was provided by the finding that the hepatopancreas was sterile. The detection of an abalone cellulase is of interest because the ability to degrade cellulose is not a common attribute of eukaryotes. Although a number of studies concerning the digestive enzymes of abalone have concluded that these animals synthesise their own cellulase (Elyakova et al., 1981; Boyen et al., 1990; Gomez-Pincheiti and Garcia-Reina, 1993) and alginate lyase (Ostgaard and Larsen, 1993; Ostgaard et al., 1994), none of these studies have considered the possibility that the cellulolytic and alginolytic enzyme activity may be bacterial in origin. The present study is the first to address this possibility.

Enzyme assays performed on the abalone hepatopancreas indicated that the endogenous enzymes varied with diet. Alginate lyase, CMCase and laminarinase activity was greater in abalone fed with kelp than in those fed primarily on *G. verrucosa*. The latter animals had very high agarase and carrageenase activity, while no agarase or carrageenase activity was detected in animals fed with *E. maxima*. Since the cell wall of *E. maxima* is composed predominantly of alginate, kelp-fed animals appear to adjust their polysaccharide-digesting enzymes to utilise this substrate. Similarly, Harris et al. (1986) reported a difference in the enzyme activity of adult *Calanus helgolandicus* fed diets containing different amounts of starch. They suggested that there may be a compensatory mechanism between digestive enzymes and the substrate ingested. Similar reasoning could be applied to the present study. Although the cell wall of *G. verrucosa* consists of agar, both agarase and carrageenase activity increased in animals fed with *G. verrucosa*. Carrageenan and agar galactans occur in the cell walls of certain Rhodophyceae. The former polymer consists of alternating α-1,3- and β-1,4-linked β-D-galactopyranose units, while the latter polymer is comprised of alternating α-1,3-linked β-D-galactose and β-1,4-linked anhydro-L-galactose units (Craigie and Leigh, 1978; McCandless, 1985). Thus, carrageenan and agar are closely related polymers (Barbeyron et al., 1994). Indeed, the amino acid sequence of the κ-carrageenase of the marine bacterium *Alteromonas carrageenovora* has been shown to be similar to the β-agarase of *Streptomyces coelicolor* (Barbeyron et al., 1994). Since β-agarases and κ-carrageenases are β-1,4-endoglucanases, it is possible that the carrageenase produced by *H. midae* is induced by the presence of agar.

Although Knauer et al. (1996) found that juvenile *H. midae* fed diatoms and artificial food exhibited different protease and amylase activities, it is not possible to discern whether this variation involved endogenous or exogenous (bacterial) enzymes. Since bacteria were not detected in the hepatopancreas of *H. midae*, our data showed unequivocally that abalone themselves produce different enzymes with respect to diet, and thus, support the study of Knauer et al. (1996).

Comparison of the levels of polysaccharolytic activity in gnotobiotic and untreated abalone indicates that bacteria play a role in the digestion of alginate, CMC, laminarin
and agarose. The very low levels of agarase and carrageenase enzymes in the gnotobiotic abalone was due to their previous diet of *E. maxima*, which is devoid of agar.

In conclusion, this study has shown that abalone themselves synthesise agarase, carrageenase, alginate lyase, CMCase and laminarinase enzymes. Expression of these endogcnous polysaccharases is regulated by diet. In addition, bacteria isolated from the gut of *H. midae* can hydrolyse carrageenan, laminarin, alginate, CMC and agarose, all of which occur in algae ingested by abalone. Thus, bacteria resident in the abalone digestive tract may assist in the digestion of complex polysaccharides. Future studies will address the effect of bacterial polysaccharases on the growth rate of *H. midae*.

**Acknowledgements**

This work was supported by a Foundation of Research Development research grant (VEC) and a postgraduate student bursary (JHE). The authors express their thanks to Mr. Nick Loubser of Irvin and Johnson, Danger Point and Mr. Connie Muller of Marine Growers, Port Elizabeth for providing the abalone. In addition, the authors express their gratitude to Sea Plant Products, Hermanus for supplying the artificial food used in this study.

**References**


