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Characterisation of cell types in abalone (*Haliotis* spp.) tissues using immunohistochemical techniques

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Abstract

The increasing popularity of abalone as a seafood delicacy has led to the rapid development of abalone aquaculture worldwide. The commercial and economic importance of this industry has resulted in an increasing interest in the biology of this particular shellfish genus. In this study we focus on the identification of structural, functional and proliferative proteins in two species of abalone shellfish, *Haliotis discus hannai* and *Haliotis tuberculata*. Monoclonal and polyclonal antibodies that react with proteins in vertebrate and invertebrate tissues were selected and applied to abalone tissues. Cross sections of whole animals were analysed using avidin–biotin immunoperoxidase staining protocols. In total, twenty-four antibodies were tested on shellfish tissues. Six antibodies out of twenty-four detected antigens in *Haliotis* spp. Cytokeratins, proliferating cell nuclear antigen, neuron specific enolase, laminin and vimentin were detected in abalone tissues. Positive immunohistochemical results were confirmed using western blot. The expression of these proteins aids in the characterisation of cell types present in abalone tissues, which contributes to a better understanding of the fundamental biology of this shellfish genus.

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Keywords: Abalone; Cell markers; *Haliotis tuberculata*; *Haliotis discus hannai*; Immunohistochemistry

1. Introduction

Abalone are marine gastropods consisting of 56 described species found in both temperate and tropical waters of both hemispheres (Bevelander, 1988; Geiger, 2000). Abalone aquaculture has become an economically important activity worldwide owing to a significant increase in consumer demand for this shellfish product. World cultured abalone production has soared phenomenally in recent times with over fifteen species currently in commercial cultivation (Gordon and Cook,

2004). Abalone shellfish are not native to Ireland but they were introduced in 1970. Land-based farms were set up along the Western seaboard for the commercial cultivation of two particular species of abalone, *Haliotis tuberculata* and *Haliotis discus hannai*. The histology of prosobranch and abalone shellfish has been studied (Bevelander, 1988; Voltzow, 1994) but there is a scarcity of information on the cellular and molecular constituents of shellfish in general.

Immunohistochemistry is a technique that demonstrates phenotypic antigen expression and was developed in the early 20th century. It allows for the demonstration of antigens in tissue sections by the use of specific immunological (antibody–antigen) interactions culminating in the attachment of a visible marker

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(usually an enzyme) to the antigen (Avrameas and Uriel, 1966; Nakane and Pierce, 1966). Cell markers are proteins characteristic of certain cell types. They may arise as surface cell markers that are molecules or proteins characteristic of the plasma membrane of a cell or as intracellular proteins that occur within the cell. Cell types and tissues express cell specific markers, which can be identified through immunohistochemistry (Miller, 2002).

The characterisation of cell types in higher vertebrates has been researched extensively but the information available for invertebrates remains scarce (Bacetti et al., 1984; Lyons-Alcantara et al., 1999). The human antibody repertoire can collectively target thousands of antigens but there is currently a limited range of antibodies that react with invertebrate antigens (Vullings et al., 1989; Lyons-Alcantara et al., 1999, 2002; Panasophonkul et al., 2004). Owing to this factor immunohistochemical techniques have rarely been employed in the study of shellfish but there are a few studies that have utilised immunolocalisation methods. In marine research the use of monoclonal antibodies and immunohistochemical techniques has been concentrated in the study of marine immunology and infectious disease (Yoshino and Granath, 1983; Mialhe et al., 1988; Noël et al., 1994; Coll and Dominguez-Juncal, 1995; Roch, 1999; Xue and Renault, 2001). More recently immunohistochemical methods, using rabbit and mouse polyclonal antibodies, were employed to locate serotonergic and FMRF-amidergic

neurons within the cerebral, pleuropedal and visceral ganglia of *Haliotis asinina* (Panasophonkul et al., 2004).

Immunohistochemistry has also been used in the study of cytokeratins in fish and invertebrates (Markl and Franke, 1988; Markl et al., 1989; Bunton, 1993; Diogo et al., 1994). Cells of the digestive gland of the Norwegian prawn, *Nephrops norvegicus*, were characterised using immunohistochemistry (Lyons-Alcantara et al., 1999) and the localisation of the nuclear protein PCNA within the nuclei of both fish and prawns was also established using this technique (Suzuka et al., 1989; Ortego et al., 1994).

This research concentrates on the evaluation of antigen expression and protein typing in shellfish tissues. It has been suggested that all vertebrate and invertebrate intermediate filament proteins share a common antigenic determinant (Pruss et al., 1981). Homologues of vertebrate type I, II and III intermediate filament proteins were identified in the invertebrate *Branchiostoma lanceolata* (Karabinos et al., 1998). It is evident that some proteins are highly conserved in evolution due to the cross reaction of antibodies to human antigens with epitopes in shellfish tissues. This study focuses on the immunohistochemical evaluation of structural and functional proteins in shellfish tissues that have not been previously characterised. A wide range of antibodies to fish and human antigens are investigated to determine their cross reactivity with similar proteins present in abalone tissues using

t1.1 Table 1
t1.2 Specifications and controls for antibodies that bind with antigens in human/mammalian species

t1.3	Antibody*	Source of immunogen	Clone	Optimal dilution	Antigen retrieval	Positive control
t1.4	CD† 20	Human	L26	1:1200	Microwave	Tonsil
t1.5	CD† 34	Human	QBEnd 10	1:50	Microwave	Tonsil
t1.6	CD† 68	Human	KP1	1:50	Microwave	Tonsil
t1.7	Chromogranin A	Human	DAK-AE	1:500	Microwave	Ileum
t1.8	Cytokeratin AE1/3	Human	AE1/3	1:800	Protease	Pancreas
t1.9	Cytokeratin 8	Human	4.1.18	1:50	Microwave and protease	Pancreas
t1.10	Ki67	Human	MIB-1	1:25	Microwave	Tonsil
t1.11	Cytokeratin MNF 116	Human	MNF 116	1:120	Protease	Ileum
t1.12	Collagen IV	Human	CIV22	1:450	Microwave	Kidney
t1.13	Human Desmin	Human	D 33	1:50	Microwave	Intestine
t1.14	34βE12	Human	34 βE12	1:20	Protease	Pancreas
t1.15	LP 34	Human	LP34	1:50	Protease	Skin
t1.16	Muscle Specific Actin (MSA)	Human	HHF 35	1:100	Microwave	Pancreas
t1.17	Neurofilament	Porcine	2F 11	1:50	Microwave	Cerebellum
t1.18	Neuron Specific Enolase (NSE)	Human	BB/NC/VI-H14	1:600	Microwave	Intestine
t1.19	S100	Bovine	S100	1:400	Microwave	Skin/Sal gland
t1.20	Synaptophysin	Bovine	SY38	1:20	Microwave	Pancreas
t1.21	Vimentin	Bovine	VIM 3B4	1:50	Microwave	Tonsil
t1.22	Von Willebrand Factor	Human	F8/86	1:75	Microwave	Tonsil

t1.23 *All antibodies used were supplied by DakoCytomation™, Galway, Ireland.

t1.24 †CD — Cluster of Differentiation.

t2.1 Table 2
t2.2 Specifications and controls for antibodies that bind with antigens in both vertebrate and invertebrate species

t2.3	Antibody	Source of immunogen	Clone	Supplier	Optimal dilution	Antigen retrieval	Positive control
t2.4	Cytokeratin AE1	Human	AE1	Serotec	1:100	Protease	Pancreas
t2.5	PCNA	Rat	PC10	Serotec	Neat	Microwave	Tonsil
t2.6	Vimentin	Porcine	V9	Serotec	1:80	Microwave	Smooth muscle
t2.7	Neurofilament	Rat	RmdO-20	Zymed	1:50	Microwave	Cerebellum
t2.8	Laminin	Mouse	Polyclonal	Abcam	1:100	Protease	Liver

105 immunohistochemistry. Where positive immunohisto-
106 chemical results are observed, western blot analysis is
107 employed to confirm the presence of these specific
108 proteins in abalone tissues.

109 2. Materials and methods

110 2.1. Specimen collection and processing

111 *H. tuberculata* and *H. discus hannai* specimens were
112 obtained from the Boet Mór shellfish farm in Clifden,
113 Co. Galway, Ireland. The animals ranged from one to
114 three years old.

115 Animals were anaesthetised in a solution of alcohol
116 and seawater (1:1) for 30 min and transferred to 100%
117 absolute alcohol for 30 min. The shells were removed
118 and the animals were placed in Davidson's fixative for
119 24 h. An incision was made lengthwise down the foot
120 muscle of each mollusc to allow penetration of the
121 fixative.

122 Individual organs were dissected from shellfish 5 cm
123 in length and animals less than 2 cm in length were
124 cross-sectioned longitudinally.

125 2.2. Paraffin sections

126 All animals were processed through the following
127 solutions: 10% formalin, spirit (95% ETOH), absolute
128 alcohol ($\times 5$), xylene ($\times 3$) and paraffin wax ($\times 2$). Tissues
129 were embedded in paraffin wax and 5 μm sections were
130 cut using a microtome. Sections were set onto adhesive
131 APES (3-aminopropyltriethoxysilane) coated slides and

incubated at 56 °C for 2–3 h. The following antigen
retrieval methods were used on some tissues to enhance
immunostaining: (a). microwaving: dewaxed and rehy-
drated tissue sections were placed in 500 ml citrate buffer
(2.1 g citric acid in 1 l of distilled water at pH 6 using 2 M
NaOH) microwaved (800 W) for 18 min and incubat-
ed in buffer for a further 20 min and (b). proteolytic
digestion: dewaxed and rehydrated tissue sections were
incubated in 0.1% protease (*Streptomyces griseus*, Type
24, Sigma) in PBS for 10 min at 37 °C.

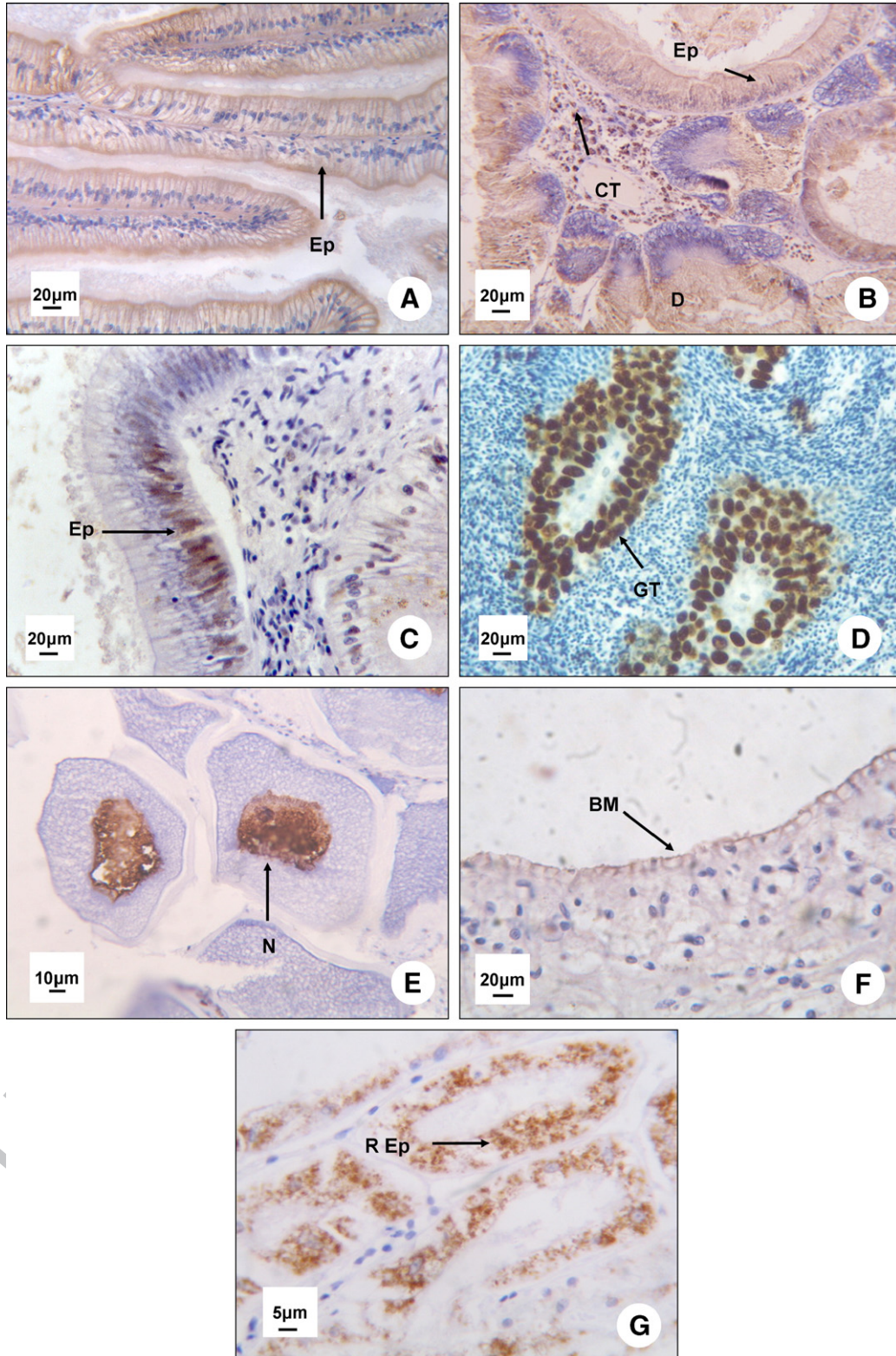
142 2.3. Immunostaining protocol

143 Shellfish and human tissues were stained using the
144 Vectastain® Elite Avidin–Biotin Complex kits. After
145 antigen retrieval the slides were treated with 3%
146 hydrogen peroxide in methanol for 10 min. Following
147 rinsing in water, sections were incubated in PBS for
148 5 min. Sections were covered with normal horse serum
149 (1:100 universal kit; 1:67 mouse kit) for 5 min. They
150 were then drained and the primary antibody diluted in
151 PBS was applied to sections for 60 min (Tables 1 and 2).
152 The slides were rinsed in PBS ($\times 3$) and incubated in
153 biotinylated secondary antibody (1:25 universal kit;
154 1:50 mouse kit) for 15 min. Slides were washed in 3
155 changes of PBS buffer and incubated for 15 min in
156 avidin–biotin peroxidase complex reagent (1:25 with both
157 kits). Sections were rinsed in buffer and the chromogen,
158 diaminobenzidine-peroxidase (0.06 mg ml⁻¹ with 0.03%
159 hydrogen peroxide) was applied for 5 min to produce a
160 brown reaction product. Slides were counterstained in
161 Mayer's Haematoxylin for 1 min and dehydrated

t3.1 Table 3
t3.2 Specifications and controls for antibodies used in western blotting

t3.3	Antibody	Source of immunogen	Clone	Supplier	Optimal dilution	Positive cell line control	Molecular weight (kDa)
t3.4	Cytokeratin AE1	Human	AE1	Serotec	1:100	HeLa	40; 48; 50; 56
t3.5	Cytokeratin MNF	Human	MNF 116	DAKO	1:120	HeLa	45–56.5
t3.6	PCNA	Rat	PC10	Serotec	Neat	HeLa	36
t3.7	Vimentin	Porcine	V9	Serotec	1:80	CHO ^a	57

t3.8 ^a CHO — Chinese Hamster Ovary cell line.



162 through graded alcohols, cleared in xylene and mounted
163 in Distrene Plasticiser Xylene (DPX). Human tissues
164 were used as positive controls as listed in Tables 1 and 2.
165 Human tissues used as positive controls were supplied
166 by other DIT ethically approved projects. For negative
167 controls, all reagents were applied to abalone tissues
168 except the primary antibody.

169 2.4. Protein extraction

170 Human cell lines were grown in culture and protein
171 was extracted from fully confluent cells which served
172 as positive controls in western blotting experiments
173 (Table 3). Protein was extracted from cell lines using
174 NP40 Triple detergent lysis buffer (0.1% SDS; 150 mM
175 NaCl; 50 mM Tris–Cl (pH 8.0); 1% Nonidet P-40 (NP-
176 40)). 3 ml of NP40 lysis buffer was added to a flask of
177 confluent cells. The lysis buffer containing the cells was
178 centrifuged at 13,000 rpm for 20 min at 4 °C and the
179 supernatant was stored at –20 °C. For protein extraction
180 from abalone tissues, approximately 200 mg of fresh
181 frozen tissue was macerated and suspended in 2 ml SDS
182 lysis buffer (2% SDS; 50 mM Tris–HCl (pH 7.2); 1 mM
183 β -mercaptoethanol) and boiled for 3 min (Cummins and
184 Hanna, 2004). The sample was homogenised and boiled
185 for 5 min. Samples were centrifuged at 13,000 rpm for
186 10 min and the supernatant was collected for storage at
187 –20 °C. Soluble protein concentrations (μ g/ml) of
188 human cell line extracts and abalone tissue extracts were
189 analysed using the Bradford protein assay.

190 2.5. Western blot analysis

191 For western blot analysis proteins separated by SDS-
192 PAGE analysis were transferred to a PVDF (polyvinyl-
193 idene difluoride) membrane using a semi-dry western
194 blotter (Apollo™ Instrumentation). The blotter was run at
195 2 mA per cm² of gel for 2 h after which the membrane was
196 removed and washed briefly with TBS (Tris Buffered
197 Saline) buffer. The membrane was incubated in 5% BSA
198 blocking solution overnight at 4 °C on a shaker at a low
199 setting. After blocking the membrane was washed briefly
200 in TBS and the primary antibody was added to the
201 membrane at the appropriate dilution in 5% BSA blocking
202 solution (Table 3). The membrane was incubated in

primary antibody at room temperature for 2 h on a shaker. 203
The membrane was washed in 5% BSA blocking solution 204
(\times 3) for 15 min. The biotinylated secondary antibody 205
(diluted in 5% BSA blocking solution) was added and the 206
membrane was incubated for 1 h at RT. The membrane 207
was washed in 5% BSA blocking solution (\times 3) for 208
15 min. The ABC reagent (diluted in 5% BSA blocking 209
solution) was added and the membrane was incubated for 210
1 h at RT. The membrane was washed in 5% BSA 211
blocking solution (\times 3) for 15 min. The membrane was 212
finally incubated in DAB (0.06 mg ml⁻¹ in PBS with 213
0.03% hydrogen peroxide) to produce a brown reaction 214
product for the protein of interest. The membrane was 215
washed in 5% BSA blocking solution and allowed to air 216
dry prior to storage in the dark. 217

218 3. Results

219 3.1. Application of antibodies that bind with human/ 220 mammalian antigens to abalone tissues

221 Twenty antibodies that bind to mammalian antigens 221
were applied to abalone tissues (Table 1). Cross sections 222
of whole animals were used in preliminary trials and any 223
positive staining was subsequently verified by staining 224
individual shellfish organs. Two out of the twenty anti- 225
bodies tested reacted with antigens in abalone tissues: 226
cytokeratin MNF 116 and NSE (Neuron Specific Enolase) 227
(Fig. 1A and B). All human positive control tissues were 228
positive for each antibody tested and negative controls 229
were negative (Table 1). A brown reaction product 230
indicates a positive reaction. 231

232 Cytokeratin MNF expression was concentrated in the 232
epithelial cells of the intestine of abalone. The surfaces 233
of the epithelial cells were labelled for this antibody 234
(Fig. 1A). Other epithelial cells of the digestive tract also 235
stained positively for MNF but a more intense intestinal 236
epithelial expression was observed. 237

238 NSE produced a pattern of positive staining in the 238
epithelia of the digestive system. This antibody is 239
directed against a neural protein. Antigens within the 240
epithelial cells of the digestive tract and in the duct cells 241
of the hepatopancreas stained positively with this 242
antibody. NSE positive cells were also observed within 243
the connective tissue of the digestive tract (Fig. 1B). 244

Fig. 1. A. Epithelial cells (Ep) of the intestine of *Haliotis discus hannai* positive for cytokeratin MNF (brown). B. Duct cells (D), epithelial cells (Ep) and connective tissue (CT) of the digestive gland of *Haliotis discus hannai* positive for NSE (brown). C. Epithelial cells (Ep) of the intestine of *Haliotis discus hannai* positive for cytokeratin AE1 (brown). D. Nuclei in the male gonadal tubules (GT) of *Haliotis discus hannai* positive for PCNA (brown). E. Nuclei (N) of the female eggs of *Haliotis discus hannai* positive for PCNA (brown). F. Basement membrane (BM) of the foot of *Haliotis discus hannai* positive for laminin (brown). G. Right renal epithelial cells (REp) of *Haliotis tuberculata* positive for vimentin (brown).

245 3.2. Application of antibodies that bind with other
246 vertebrate/invertebrate antigens to abalone tissues

247 Five antibodies that bind to both vertebrate and
248 invertebrate antigens were tested on cross sections of
249 abalone tissues (Table 2). A positive result was then
250 verified by staining sections from individual organs
251 such as the digestive gland, epipodium, foot, gonad and
252 kidney. All human positive control tissues were positive
253 for each antibody tested and negative controls were
254 uniformly negative (Table 2). Cytokeratin AE1 was
255 expressed in some epithelial cells of the digestive
256 system of abalone tissues but not in all epithelial cells.

257 The positive reaction observed was not uniform rather
258 scattered throughout the cells of the intestine (Fig. 1C).
259 This cytokeratin was also expressed in the pedal epi-
260 thelia of some abalone.

261 In abalone tissues both the male and female gonads
262 expressed PCNA. PCNA positive cells were concen-
263 trated in the gonadal tubules of the reproductive organs
264 of the male abalone. The cells of the gonad were
265 strongly positive for the presence of this protein (Fig.
266 1D). Epithelial cells of the digestive system were also
267 positive when stained with this antibody. The nuclei of
268 large but immature ova in the female gonad were also
269 PCNA positive (Fig. 1E).

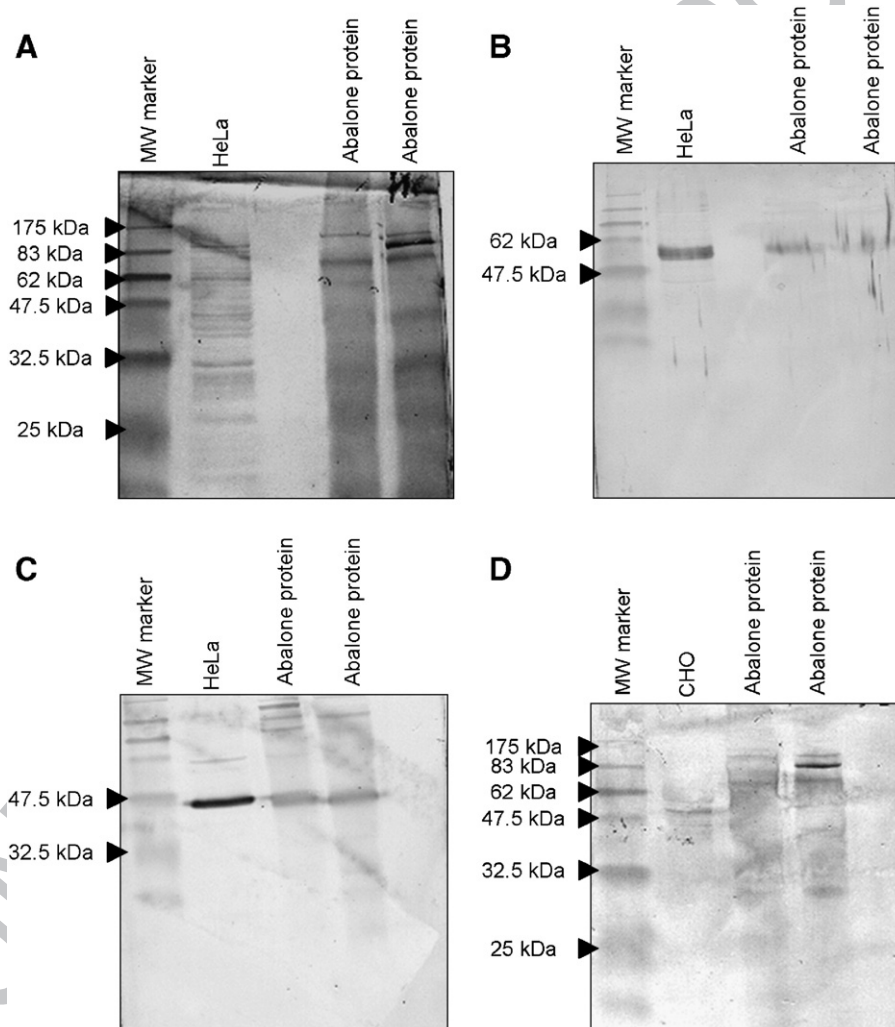


Fig. 2. A. Western blot of HeLa cells (11.3 μ g) and abalone (*H. discus hannai* and *H. tuberculata*) tissue extracts (6.3 μ g; 7 μ g) using cytokeratin MNF 116 (DAKO). B. Western blot of HeLa cells (11.3 μ g) and abalone (*H. discus hannai* and *H. tuberculata*) tissue extracts (6.3 μ g; 7 μ g) using cytokeratin AE1 (Serotec). C. Western blot analysis of HeLa cells (11.3 μ g) and abalone (*H. discus hannai* and *H. tuberculata*) tissue extracts (6.3 μ g; 7 μ g) using PCNA (Serotec). D. Western blot of CHO cells (8.1 μ g) and abalone (*H. discus hannai* and *H. tuberculata*) tissue extracts (6.3 μ g; 7 μ g) using vimentin V9 (Serotec).

270 The antibody laminin was expressed in the basement
271 membrane below the pedal epithelial layer of the
272 abalone foot (Fig. 1F).

273 Another clone of vimentin was used in this section of
274 the study. A granular staining pattern was expressed in
275 the cytoplasm of the right renal organ of abalone using
276 this antibody (Fig. 1G).

277 3.3. Western blotting results

278 Proteins identified in abalone tissues by immunohisto-
279 chemistry were further investigated and identified by
280 ABC/DAB detection in western blotting using SDS-
281 PAGE and transfer to PVDF membranes. Western blot
282 analysis confirmed the presence of cytokeratin MNF,
283 cytokeratin AE1, PCNA and vimentin in abalone
284 tissues.

285 Western blot analysis confirmed the presence of
286 cytokeratins in abalone using MNF and AE1 antibodies.
287 The broad spectrum antibody cytokeratin MNF which
288 detects a wide range of proteins, revealed proteins with
289 molecular weights of between 25 and 175 kDa in the
290 HeLa positive control (Fig. 2A). With abalone samples,
291 3 strong bands with molecular weights of 100, 83 and
292 70 kDa approximately were identified and weaker bands
293 were observed in the 32.5–47.5 kDa range (Fig. 2A). A
294 very strong band at 83 kDa in the second sample can be
295 observed in Fig. 2A. The lower molecular weight bands
296 correspond to keratins 5, 6, 8, 17 and 19 while the high
297 molecular weight proteins identified do not correspond
298 to known cytokeratins.

299 The antibody cytokeratin AE1 identifies proteins
300 with molecular weights of 40–56.5 kDa. A single band
301 with a molecular weight of 56.5 kDa approximately was
302 identified in both the positive HeLa control cells and
303 abalone samples, which corresponds to cytokeratin 10
304 (Fig. 2B).

305 Western blot analysis with PCNA identified clear
306 bands with a molecular weight of approximately 36 kDa
307 in the HeLa positive control and whole protein extracted
308 from abalone tissues (Fig. 2C). In Fig. 2C specific
309 bands between 83 and 175 kDa can be observed de-
310 spite a lower concentration of protein than the positive
311 control.

312 Stronger bands observed with HeLa positive controls
313 compared with abalone protein in western blots with
314 antibodies AE1 and PCNA, could be indicative of
315 higher concentrations of extracted protein.

316 Protein bands were detected in abalone tissues with
317 the vimentin antibody using western blotting (Fig. 2D).
318 A band was observed in the CHO positive control which
319 corresponds to the 57 kDa band expected with vimentin.

A very strong band and two weaker bands were
identified with molecular weights of between 83 and
175 kDa in the second abalone sample while the
stronger band was not observed in the first sample. The
strong band observed in Fig. 2D is very specific despite
lower concentrations of abalone compared to the HeLa
positive control.

4. Discussion

Immunohistochemistry is a highly valued technique
that allows for the study of functional and structural
entities within tissues. It allows for the analysis of
protein distribution within tissues and has the potential
for application in marine research. However, the lack of
specific antibodies to shellfish antigens remains a major
limitation.

Previous studies have used immunohistochemistry to
study the hemocytes/immune cells of molluscs (Yoshino
and Granath, 1983; Ottaviani, 1989; Noël et al., 1994;
Xue and Renault, 2001). These studies focused on the
definition of molluscan hemocyte type and the deter-
mination of hemocyte distribution within tissues using
antibodies produced specifically to detect these cells.
Few if any shellfish studies are available that have
aimed to characterise cells other than immune cells.

This study demonstrated cytokeratin expression in
abalone tissues using broad spectrum antibodies MNF
116 and cytokeratin AE1. Keratins are intermediate
filaments of epithelial cells and they have a structural
role in tissues. The paper of Diogo et al. (1994) was the
first study to characterise invertebrate cytokeratins.
Since then cytokeratins have been characterised in the
prawn and shrimp (Lyons-Alcantara et al., 1999, 2002).
Cytokeratin MNF recognises proteins of molecular
weight 45–56.5 kD and cytokeratin AE1 recognises
proteins with a molecular weight of 56.5, 50, 48 and
40 kD. The epitopes that are thus being targeted by these
two antibodies are in the same molecular weight range.
Both antibodies react positively to normal human
epithelia and their neoplasms showing a broad range
of reactivity (Leong et al., 2002). Four cytokeratins were
tested, and two were expressed in abalone tissues.
Cytokeratin AE1 reacted positively with cytoplasmic
proteins in the intestinal epithelia of abalone and
cytokeratin MNF was present on the surface of the
intestinal epithelia. These results were reinforced by
western blot analysis and while AE1 produced a specific
band of 56.5 kDa in abalone samples, western blotting
with cytokeratin MNF revealed many protein bands in
abalone tissues indicating numerous cytokeratins, some
of which were very distinct.

370 PCNA is a multifunctional cell marker representing a
 371 component of DNA polymerase- δ . It is a proliferating
 372 cell marker producing a staining pattern that is generally
 373 confined to the nuclei of actively proliferating tissues
 374 and cells. This cell marker has been detected in
 375 mammals, prawns, fish and some higher plants (Suzuka
 376 et al., 1989; Ortego et al., 1994; Lyons-Alcantara et al.,
 377 1999). In this study PCNA was detected in abalone
 378 tissues. Antigens in the nuclei of the reproductive organs
 379 of both male and female abalone reacted positively with
 380 this antibody. Cells of the gonad are constantly
 381 undergoing proliferation giving rise to new cells thus
 382 explaining the presence of PCNA in these areas. Not all
 383 abalone proliferating cells expressed this antigen,
 384 mainly epithelial proliferating cells. PCNA positivity
 385 was also located in the nuclei of epithelial cells of the
 386 hepatopancreas, however the degree of positivity within
 387 epithelial tissues was scattered. Western blot analysis
 388 also confirmed the presence of PCNA in abalone tissues
 389 and additional molecular weight bands, higher than the
 390 36 kDa specific band were observed.

391 Vimentin, a protein expressed in virtually all
 392 mesenchymal cells was also present in abalone tissues.
 393 Vimentin has been shown to produce a cytoplasmic
 394 pattern of staining in the invertebrate *Drosophila*
 395 *melanogaster* (Walter and Biessmann, 1984) and has
 396 been recognised as a cytoskeletal constituent of many
 397 fish tissues (Nelson and Traub, 1982; Gyoeva et al.,
 398 1987) and in invertebrate tissues (Karabinos et al.,
 399 1998). Markl et al. (1989) used gel electrophoresis,
 400 immunostaining and immunoblot assays to identify
 401 vimentin in rainbow trout tissues. They concluded that
 402 the identified cytoskeletal protein is homologous to
 403 mammalian vimentin but it only makes up a very small
 404 component of the cytoskeleton. In this study the V9
 405 clone (57 kDa) detected vimentin in the kidney of
 406 abalone and a granular cytoplasmic staining pattern was
 407 observed which was confirmed by western blotting with
 408 bands in the 57 kDa range but more definite bands were
 409 observed at 83 kDa.

410 Three different antibodies were employed to detect
 411 elements of the nervous system of abalone, NSE,
 412 neurofilament and S100. Neurofilament and S100
 413 were not detected while NSE, an isoenzyme of enolase
 414 present in neurons and neuroendocrine cells (Leong et
 415 al., 2002) was demonstrated in the epithelial cells of the
 416 digestive gland and in the connective tissue of the
 417 digestive tract. The prosobranch body is entirely
 418 innervated and NSE could be cross-reacting with an
 419 enolase enzyme released by the intestinal ganglia.
 420 Western blot analysis did not confirm the presence of
 421 NSE in abalone tissues. Nerve cells have recently been

characterised in *H. asinina* Linnaeus using antibodies to
 serotonin and FMRF-amide neurotransmitters (Panaso-
 phonkul et al., 2004).

Another antibody that cross-reacted with antigens in
 abalone tissues was laminin. Its presence indicates the
 highly conserved nature of this structural protein, being
 present in many species from invertebrates to verte-
 brates (Sarras et al., 1994; Zhang et al., 1994; Yurchenco
 and Waddsworth, 2004). Laminin is an embryonically
 expressed protein that is essential for basement
 membrane assembly and is one of the most ancient
 proteins within extracellular matrices (Cooper and
 McQueen, 1983; McCarthy et al., 1987; Montell and
 Goodman, 1989; Yurchenco and Waddsworth, 2004).
 Previous studies on invertebrate laminins have
 employed immunofluorescent and immunohistochemi-
 cal techniques to identify these proteins (Sarras et al.,
 1993, 1994). Laminin was observed in the basement
 membrane of the abalone foot. Western blot analysis
 was not performed with laminin since only a small
 number of basement membranes expressed this protein
 with immunostaining.

The use of mammalian and veterinary antibodies in
 this study gives an indication of the structural,
 functional and proliferative framework that makes up
 the tissues of abalone, which will be helpful in studying
 the effects of pathogens on this shellfish through
 comparisons of both healthy and diseased organisms.
 The key findings of this study are that PCNA,
 cytokeratins, NSE and vimentin are important biomar-
 kers of proliferation and differentiation in abalone
 species. Immunohistochemistry acts as a helpful adjunct
 to histology, histochemistry and other studies of cellular
 function. However further progress in this area is
 dependent upon antibodies specific to shellfish antigens
 being developed.

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