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1 ***Post mortem* degradation of myosin heavy chain in intact fish muscle: Effects of pH and**
2 **enzyme inhibitors.**

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11 **Abstract**

12 Fish muscle is rapidly degraded during *post mortem* storage due to proteolytic enzymes acting
13 probably both on muscle cells and connective tissue. In this work we have developed a model
14 system which may be used to study the enzymatic degradation occurring in intact *post mortem*
15 fish muscle. Degradation of myosin heavy chain (MHC) was monitored in muscle with pH
16 adjusted to 6.05, 6.3 and 6.9 and in the presence of the enzyme inhibitors PMSF, EDTA,
17 Phenanthroline, Pepstatin A, Antipain, E-64 and the cysteine proteinase activator
18 Dithiothreithol (DTT). After storage, myofibrillar proteins were isolated and MHC-specific
19 antibodies used to study the degradation in the different samples. MHC from muscle with pH
20 6.05 and 6.3 was degraded while no severe degradation was observed at pH 6.9. Introduction
21 of enzyme inhibitors into the muscle tissue clearly showed that mainly cysteine and aspartic
22 proteinases are responsible for the *in situ* MHC-degradation. This is supported by the severe
23 breakdown of MHC in the muscle samples containing DTT.

24

25 Keywords: Atlantic cod, *post mortem* proteolytic degradation, myosin heavy chain, cathepsins.

26

1. Introduction

Fish muscle is rapidly degraded during *post mortem* storage due to proteolytic enzymes acting probably both on muscle cells and connective tissue (Cepeda, Chou, Bracho & Haard, 1990; Delbarre-Ladrat, Cheret, Taylor & Verrez-Bagnis, 2006). Unlike in mammalian meats where a certain amount of degradations usually are required for tenderization, the effects of the processes in fish are generally undesirable. These changes which are progressive during storage, may have significant impact on the quality and consumer acceptance of the fish fillets (Foegeding, Lanier & Hultin, 1996).

There are several proteolytic systems present in fish muscle tissue which may be involved in *post mortem* muscle degradation. These include the proteasome, an heat-stable alkaline proteinase, matrix metalloproteinases (MMPs), the calpains, and the lysosomal cathepsins (Delbarre-Ladrat et al., 2006). It has been suggested that cathepsins B, L and/or D could have major roles in degradations of proteins in *post mortem* fish muscle (Ladrat, Verrez-Bagnis, Nöel & Fleurence, 2003).

Three main strategies have traditionally been used when studying *post mortem* proteolytic degradation of fish muscle. One is to homogenize fresh muscle tissue and incubate extracted sarcoplasmic and/or myofibrillar proteins with selected purified enzymes and then follow the degradation of muscle proteins by methods such as SDS-PAGE and Western blotting with antibodies directed against specific muscle proteins (Aoki & Ueno, 1997; Delbarre-Ladrat, Verrez-Bagnis, Noel & Fleurence, 2004; Folco, Busconi, Martone, Trucco & Sanchez, 1984; Geesink, Morton, Kent & Bickerstaffe, 2000; Jiang, Lee & Chen, 1996; Ladrat et al., 2003; Ogata, Aranishi, Hara, Osatomi & Ishihara, 1998; Yamashita & Konagaya, 1991). As stated by Godiksen, Morzel, Hyldig, & Jessen, (2009) a major weakness with homogenizing muscle and extracting proteins is that the natural control mechanisms in the intact muscle tissue are lost. Sub-cellular compartments are disrupted, enzyme-inhibitor

52 complexes may be formed or dissociated and enzyme precursors may be activated. It is well
53 known that myofibrillar proteins (surimi) obtained after homogenization and washing of fish
54 muscle possess proteolytic enzyme activities (An, Weerasinghe, Seymour & Morrissey, 1994;
55 Cao, Jiang, Zhong, Zhang & Su, 2006; Hu, Morioka & Itoh, 2008; Wang, Martinez & Olsen,
56 2009). It is however an open question if these activities are native to the myofibrillar proteins
57 or if enzyme–myofibrillar complexes are formed during the preparation of the myofibrillar
58 fraction. A second strategy is to correlate the activities of the proteases present with specific
59 properties of the fish or muscle tissue (Bahuaud, Mørkøre, Østbye, Veiseth-Kent, Thomassen
60 & Ofstad, 2010; Cheret, Delbarre-Ladrat, de Lamballerie-Anton & Verrez-Bagnis, 2007;
61 Yamashita & Konagaya, 1992). Both when isolated protein fractions are incubated with a
62 specific protease and when the activity of a protease in a muscle is determined, the conditions
63 used are most often highly optimized and may therefore be quite different from the *in situ*
64 conditions in the *post mortem* muscle. For example when incubating with or analysing the
65 activity of the cysteine protease cathepsin B, both EDTA and a reductant such as
66 dithiothreitol, are usually included (Kirschke, Wood, Roisen & Bird, 1983). A third strategy is
67 to store intact muscle for a certain period under different conditions and then analyze the
68 degradation of proteins by electrophoretic techniques during the storage (Bonnal et al., 2001;
69 Jasra, Jasra & Talesara, 2001; Martinez, Friis & Careche, 2001; Papa, Alvarez, Verrez-Bagnis,
70 Fleurence & Benyamin, 1996; Tsuchiya, Kita & Seki, 1992). With this strategy it is not
71 possible to determine the protease or proteases that are responsible for the degradation.

72 To be able to identify the enzyme mechanisms responsible for the *post mortem*
73 proteolytic degradation in intact fish muscle we have adapted the system developed by Huang,
74 Huang, Xi, & Zhou (2009) for studies of poultry muscle degradation. The aims of our study
75 were to investigate how small changes in muscle pH and how the introduction of enzyme

76 inhibitors and activators into the intact muscle, affected the post mortem degradation of

77 myosin heavy chain (MHC) during storage of the muscle.

78

2. Material and Methods

2.1. Fish

The results presented are from an Atlantic cod (*Gadus morhua* L.) of approximately 5 kgs purchased from a local fish supplier in Tromsø. After slaughter the gutted fish had been stored in ice for 4 days before hand filleted (*post rigor*). The fillets were then frozen at -50°C prior to further use. Similar results were found using a cod of similar size obtained from the Kårvika Aquaculture Station, Tromsø, but these are not shown.

2.2. Adjusting the pH in intact fish muscle samples

Cubes, approximately 2 grams, of fish muscle were incubated at room temperature (RT) in 8 ml 200 mM phosphate buffers, pH 5.7, 6.3 and 7.0 with 0.02% NaN_3 included. After 0, 1, 5, 15, 30 and 60 min and after 1, 2, 3 and 5 days of incubation, muscle cubes were homogenised 1:1 (w/v) in 150 mM KCl for 30 seconds using a T25 Ultra Turrax (Ika Laboratory and Analytical Equipment, Staufen, Germany) with a S25N-10 G dispersing tool (Ika). The pH was measured in the solution immediately after homogenisation. To ensure that the pH measured in the samples was not due to buffer left on the surface of the muscle pieces, the cubes were carefully wiped with a tissue paper prior to homogenisation and pH measurement.

2.3. Storage of muscle in the presence of protease inhibitors and an activator

Storage of muscle was performed as described under section 2.2 at pH 5.7, 6.3 and 7.0. The inhibitors used were Phenylmethylsulfonyl Fluoride (PMSF) (Calbiochem, San Diego, CA, USA), EDTA (Calbiochem), Phenanthroline (Sigma-Aldrich Inc., St. Louis MO, USA), Pepstatin A (Calbiochem), Antipain (Sigma) and E-64 (Sigma). The concentration of the different inhibitors is indicated in the figure legends. Fish muscle cubes, 0.5 g, were

104 individually incubated with 2 mls of buffers containing the different inhibitors or activator in
105 glass containers with a plastic lid at room temperature. After the desired incubation times
106 indicated in the figures, myofibrils were isolated from the samples as described below. One
107 sample was incubated in the presence of the cysteine proteinase activator Dithiothreitol (DTT)
108 (Calbiochem).

109

110 *2.4 Isolation of myofibrils*

111 Isolation of myofibrils was done according to Cao et al. (2006) with some
112 modifications. 0.5 g of fish muscle were homogenized by Ultra Turrax (Ika) for 30 seconds in
113 2 mls of 50 mM phosphate buffer, pH 7.5, containing 0.02% NaN_3. The homogenate was then
114 centrifuged for 15 minutes at 4ºC and 4000g. The supernatant which contained sarcoplasmic
115 proteins was removed and the pellet resuspended in 0.8 ml of the same buffer. This washing
116 step was repeated three times. The third wash was performed with the same type of buffer
117 with pH of the of the incubation solution. After the last wash the pellet was dissolved in 50
118 mM phosphate buffer with pH of the incubation solution, containing 0.02% NaN_3 and 500
119 mM NaCl. Then the sample volume was doubled using 10% SDS to totally dissolve all
120 proteins.

121

122 2.5. SDS-PAGE and Western Blotting

123 SDS-PAGE was performed basically according to Laemmli (1970) using 4-10 %
124 NuPAGE® Novex® Bis-Tris polyacrylamide gels in an XCell *SureLock*™ Mini-Cell
125 (Invitrogen Life Technologies Corporation, Carlsbad, CA, USA). Samples mixed with sample
126 buffer (60 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, 0.1%
127 bromophenol blue) were heated at 95–100 ºC for 10 minutes prior to electrophoresis. The
128 staining of the gels was performed using SimplyBlue™ SafeStain (Invitrogen). The molecular

129 mass standard used was Seeblue Plus2 Pre-Stained Standard (Invitrogen). Approximately 1
130 µg of total protein was applied to each well on gels destined for Simplyblue staining and 0.5
131 µg was loaded on gels destined for western blotting (Fig. 2). Protein concentration in the
132 samples was determined using a BCA Protein Assay - Reducing Agent Compatible kit
133 (Thermo scientific, Rockford, IL, USA). In the western blots of isolated myofibrils from
134 samples incubated with enzyme inhibitors or activator an anti-actin antibody (A2066, Sigma)
135 was used in a 1:1000 dilution as a loading control (Fig. 3). Proteins separated by SDS-PAGE
136 were transferred to a PVDF-membrane, as described by Towbin, Staehelin, and Gordon (1979)
137 by using Power PAC 1000 electroblot apparatus (Bio-Rad). The transfer was performed using
138 30 V for 90 minutes at room temperature. Immediately after the blotting, the membrane was
139 washed 2x5 minutes at room temperature in TBS buffer (20 mM Tris-HCl, pH 7.5, containing
140 500 mM NaCl) and blocked with 25 ml TBS containing 0.05% Tween 20 and 5% fat-free
141 milk for 30 minutes (blocking buffer). The membrane was then incubated with primary
142 antibody in blocking buffer at 4 C° over night. The antisera dilutions used in the
143 immunoblotting experiments are 1:80000 for the anti-MHC antibody (anti-MHC) (Martinez
144 & Pettersen, 1992). The next day the membrane was washed 3 x 5 minutes in TBS containing
145 0.05% Tween (TTBS), followed by incubation for 1 hour at room temperature with a
146 secondary antibody conjugated to horseradish peroxidase (goat anti-rabbit IgG-HRP, Santa
147 Cruz Biotechnology Inc., Santa Cruz, CA, USA) at a dilution of 1:10,000 in TTBS. The
148 membrane was then washed 3 x 5 minutes in TTBS followed by 5 minutes in MilliQ water.
149 Subsequently, the membrane was incubated with 0.75 ml Supersignal West Pico
150 Chemiluminescent Substrate (Pierce) followed by exposure to film (Lumi-Film
151 Chemiluminescent Detection Film, Roche Diagnostics, Indianapolis, IN, USA) and
152 developing.
153

154 **3. Results**

155 *3.1 Adjustment of muscle pH*

156 Pieces of fish muscle were incubated in phosphate buffers with pH 5.7, 6.3 and 7.0 at
157 RT (20-22ºC) for up to 5 days, to adjust pH conditions in the muscle tissue during the storage.
158 Initial pH of the samples was 6.3. The results from the incubations for 1 hour are shown in
159 Fig. 1. Incubation for 1 minute did not alter the tissue pH for any buffer used, while after 5
160 minutes the pH was markedly changed in the samples with high- and low-pH buffer. Samples
161 kept in pH 7.0 had reached a tissue pH close to 6.6, while samples kept at 5.7 had a pH of 6.1.
162 The relatively fast change in pH of the muscle tissue continued during the first 15 minutes and
163 was then stabilized in the high-pH buffer at pH 6.9. The pH of the muscle samples incubated
164 in pH 5.7 obtained a stable pH of approximately 6.05 after 15 minutes. The samples kept in a
165 buffer with pH 6.3 showed no change in pH during the incubation period. Further incubation
166 of muscle samples for up to 5 days did not change the tissue pHs obtained after 1 hour (results
167 not shown).

168

169 *3.2. MHC-degradation in stored muscle*

170 Myofibrils were isolated from pieces of fish muscle stored under the three different
171 pH-conditions to determine if these adjustments had effect on the MHC-degradation. The
172 results in figure 2 clearly show a pH-dependent degradation of MHC in intact fish muscle.

173 Simplyblue staining of SDS-PAGE gels showed a decrease in intensity of the protein
174 band corresponding to MHC (220 kDa) for samples kept at pH 5.7 (Fig. 2A) for 5 days. In
175 addition protein bands at approximately 125 kDa appeared with increasing intensity when
176 analyzed after, 1, 3 and 5 days of incubation. The relative amount of the 220 kDa band and
177 the 125 kDa changed during the storage at pH 6.3 indicating similar, but less prominent

178 degradation. Little or no reduction in the 220 kDa band was seen in the samples kept at pH
179 7.0 and no appearances of bands at 125 kDa were detected.
180 The same samples were subjected to Western blotting with primary antibody directed
181 against MHC and corresponding results were found as in Fig. 2A. A marked decrease of
182 intact MHC (220 kDa) was detected in the samples kept at pH 5.7, together with an increase
183 of the 125 kDa band (Fig. 2B). The same relative changes in intensities of the 220 kDa and
184 125 kDa bands were also seen at pH 6.3. No degradation was observed during the incubation
185 period in samples kept at pH. 7.0.
186
187 *3.3. MHC-degradation in muscle samples stored in the presence of protease inhibitors*
188 Figure 3A shows a Western blot of extracted myofibrillar proteins from intact muscle
189 samples incubated for 120 hours in the pH 5.7 buffer (muscle pH 6.05) together with different
190 enzyme inhibitors and a cysteine proteinase activator (DTT). The results show that EDTA,
191 Pepstatin A, Antipain, E64 and DTT present in the buffer, all influenced the degradation of
192 MHC at pH 6.05. Pepstatin A, Antipain and E64 inhibited while EDTA and DTT accelerated
193 the MHC-degradation compared to the control sample (lane 2). Degradation products of MHC
194 (approximately 100-60 kDa) were present in high amounts in the control muscle sample (no
195 inhibitor) incubated for 120 hours (lane 2) and in the muscle sample incubated with PMSF
196 (lane 3). Less of these degradation products were observed in the muscles incubated with
197 Pepstatin A (lane 6), Antipain (lane 7) and E-64 (lane 8). The sample incubated with Pepstatin
198 A for 120 hours had about the same amount of these degradation products as the control at 0
199 hours (lane 1). The presence of Phenanthroline in the incubation buffer (lane 5) appeared to
200 slightly reduce the amount of intact MHC and the smaller degradation products
201 (approximately 55-60 kDa) compared to the control and PMSF-containing samples (lanes 2
202 and 3). The MHC in the muscle sample incubated with DDT appeared to be completely

203 degraded into two major bands of approximately 60 and 50 kDa (lane 9). The actin band
204 (loading control) was also degraded in this muscle sample. A similar 50 kDa band can also be
205 seen in the EDTA-containing sample (lane 4). In this sample, the high MW bands were just
206 faintly visible while the bands in the 60-100 kDa region are clearly visible.

207 Samples incubated at pH 6.3 (Fig. 3B) showed the same general pattern as samples in
208 pH 5.7 buffer (muscle pH 6.05), but the degradation appeared less pronounced. The band
209 corresponding to intact MHC was present with equal intensity in all samples except those
210 containing EDTA and DTT. In these two samples no intact MHC were discernable. The
211 samples with muscle pH 6.9 (Fig. 3C) showed no or very little degradation of MHC. A
212 possible exception is the muscle sample incubated with DTT (lane 9).

213

214 **4. Discussion**

215 Several reports have shown that a low ultimate muscle pH *post mortem* can lead to
216 negative effects on fish muscle quality. Among these are poor liquid holding capacity (Ang &
217 Haard, 1985; Love, 1979; Ofstad, Egelandsdal, Kidman, Myklebust, Olsen & Hermansson,
218 1996; Rustad, 1992), increased gaping (Love, 1980) and a more rapid degradation of the
219 muscle tissue (Ofstad et al., 1996). The reason for a low ultimate muscle pH is intensive
220 feeding leading to a high glycogen level in the muscle (Ang et al., 1985; Ofstad et al., 1996).
221 The ultimate muscle pH of farmed cod and wild cod is often around 6.3 and 6.8, respectively
222 and it is known that farmed cod suffers more from gaping and undergo more severe structural
223 alterations *post mortem* than wild cod (Ofstad et al., 1996). In this work we have developed a
224 simple system, which enables us to determine the effects of pH and enzyme inhibitors on *post*
225 *mortem* protein degradation in intact fish muscle. This makes it possible to identify the class
226 of enzymes responsible for the degradation. We have studied MHC breakdown *post rigor, but*
227 the technique could also be used in studies of other muscle proteins and probably also in the
228 *pre-* and *in rigor* phase *post mortem.*

229 The results show that it is possible to quickly adjust the pH-conditions inside the fish
230 muscle *post rigor*. The muscle pH of 6.3 at the start of the experiment suggests that the cod
231 used was of farmed origin. The pH of the muscles was stabilized at the required level after
232 only 15 minutes incubation. The samples kept in a low-pH buffer (pH 5.7) could not be
233 adjusted lower than approximately pH 6.05. This was probably due to limited buffering
234 capacity of the phosphate buffer in this pH range. A different buffer system could most likely
235 have solved this problem, however the values obtained are relevant for a *post rigor* fish
236 muscle. By altering the pH in the pieces of muscle, different types of enzymes can have
237 conditions suitable for their action, and their ability to degrade muscle structures can be
238 monitored. The increased degradation of MHC during the storage of the intact muscle at the

239 two lowest pHs indicates that lysosomal enzymes are responsible. Lysosomal cathepsins have
240 acidic pH-optimum while most other proteolytic enzymes in muscle tissue have optimum in
241 the more pH neutral range. Very little degradation of MHC appeared to have occurred during
242 the 120 hours of *post rigor* storage at the near neutral pH, indicating that enzymes with such a
243 pH optimum are not of major importance for this breakdown. We have previously shown that
244 degradation of MHC in isolated myofibrils were similarly dependent on pH (Wang et al.,
245 2009). Myosin has isoelectric point at pH 5.5 (Foegeding et al., 1996) and it is known that
246 proteins may be more susceptible to degradation at pH close to the proteins isoelectric point
247 (Dice & Goldberg, 1975). Consequently this may also contribute in the observed increased
248 degradation at the low pH.
249 When storing muscle pieces in the presence of specific protease inhibitors the results
250 support that mainly cathepsins are responsible for the MHC breakdown confirming more
251 directly the involvement of these enzymes in muscle degradation (Aoki et al., 1997; Bahuaud
252 et al., 2010; Cheret et al., 2007; Jiang et al., 1996; Ladrat et al., 2003; Ogata et al., 1998;
253 Yamashita et al., 1991). The results show that cysteine- and aspartic acid proteinase inhibitors
254 are more effective than serine- and metalloproteinase inhibitors in preventing breakdown of
255 MHC at the slightly acidic pHs of 6.05 and 6.3. No effects of the cysteine– and aspartic acid
256 inhibitors or the other inhibitors could be seen at the near neutral pH of 6.9. The results
257 therefore strongly indicate that cathepsin D and acid cysteine proteinases, possibly B and L,
258 are involved in MHC degradation in intact muscle *post mortem*. Schwartz & Bird (1977)
259 reported that cathepsins B and D isolated from rat liver could degrade myofibrillar proteins at
260 pH below 6. More recently, Ladrat et al. (2003) showed that isolated fish myofibrillar proteins
261 are degraded by cathepsins B, D and L *in vitro*.
262 The severe breakdown of MHC in muscle samples with Dithiothreitol (DTT) included
263 during the incubation confirms that cysteine proteinases are involved. DTT or other reducing

264 agents such as free cysteine, reduces oxidized cysteine or cystine in the active site thereby
265 activating such proteinases. This result suggests that the oxidation state of the muscle tissue
266 may be of importance for protein degradation and that the use of reducing agents when
267 determining cysteine proteinase activities may not reflect the true activities in the muscle. The
268 added DTT may also reduce cystine residues within the myofibrillar complex in the muscle
269 sample and thereby making the proteins more accessible to proteolytic attack. Also the
270 accelerated degradation of MHC when EDTA is used as inhibitor is an interesting observation,
271 suggesting that metal-dependent or activated proteinases such as calpains and matrix
272 metalloproteinases may not play a major role in this process. The mechanism behind the
273 increased degradation of MHC in the presence of EDTA is difficult to explain, but activation
274 of non-proteolytic enzymes by EDTA have previously been described (Kirkeby, 1976). Erdös
275 (1960) also saw an activating effect of EDTA and interpreted this effect as binding of an other
276 inhibitor present. Mort (1998) stated that in preparations of cathepsin B, EDTA together with
277 DTT in millimolar concentrations should be used to avoid active site cysteine oxidation and
278 formation of the dimeric amino acid cystine. The usage of metal chelators has been shown to
279 inhibit cysteine oxidation in cell cultures in vitro (Ishii & Bannai, 1985). EDTA may also
280 chelate divalent cations involved in stabilizing the myofibrillar protein structure and
281 consequently making the proteins more susceptible to proteolytic degradation. More work is
282 clearly required to elucidate the mechanisms behind the increased breakdown of MHC in
283 muscle when it is incubated with DTT and in particular EDTA. EDTA, and also DTT, is
284 commonly used in buffers for enzyme activity assays. Our findings indicate that addition of
285 such compounds might speed up the rates of enzymatic reactions. This implicate that the
286 measurements of enzymatic activity show total potential activity and not the amount of active
287 enzyme.

288 In this paper we have focused on degradation of myosin heavy chain in intact fish
289 muscle after the ultimate pH had been obtained (*post rigor*). The results showed increased
290 breakdown at pH values similar to what found in the flesh of intensive farmed fish and that
291 cathepsin D and cathepsin cysteine proteases probably are the main enzymes involved.
292 However, this increased breakdown of MHC may not be the direct cause of the more rapid
293 quality changes often seen in farmed fish. With the use of antibodies directed against other
294 contractile or structural proteins, the technique could probably be used to clarify which
295 enzymes are responsible for the quality related changes in muscle proteins occurring *post*
296 *mortem*.
297

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413

Figure legends

Figure 1. pH adjustment in muscle samples incubated at room temperature (20-22ºC) in 200 mM phosphate buffers. The buffer pHs are shown in the inserted box.

Figure 2. Coomassie stained SDS-PAGE gel (A) and Western blot (B) of myofibrils isolated from Atlantic cod muscle cubes incubated at three different pH-values without enzyme-inhibitors present. The samples were kept at room temperature (20-22ºC). Incubation time and pH-conditions are indicated in the top panel of the figure. Primary antibody in the western blot was directed against myosin heavy chain (1:80000).

Figure 3. Western blot of myofibrillar proteins isolated from Atlantic cod muscle cubes incubated at pH 5.7 (A), pH 6.3 (B) and 7.0 (C) in the presence of different enzyme inhibitors. The samples were kept at room temperature (20-22ºC) for 120 hours before isolation of the myofibrils. Primary antibodies were directed against actin (1:1000) and myosin heavy chain (1:80000). Type of inhibitor used and concentration is: Lane 1: No inhibitor 0h, lane 2: No inhibitor 120h, lane 3: 1 mM PMSF, lane 4: 5 mM EDTA, lane 5: 1 mM Phenanthroline, lane 6: 0.1 mM Pepstatin A, lane 7: 0.1 mM Antipain, lane 8: 0.1 mM E64 and lane 9: 5 mM DTT

Figure 1

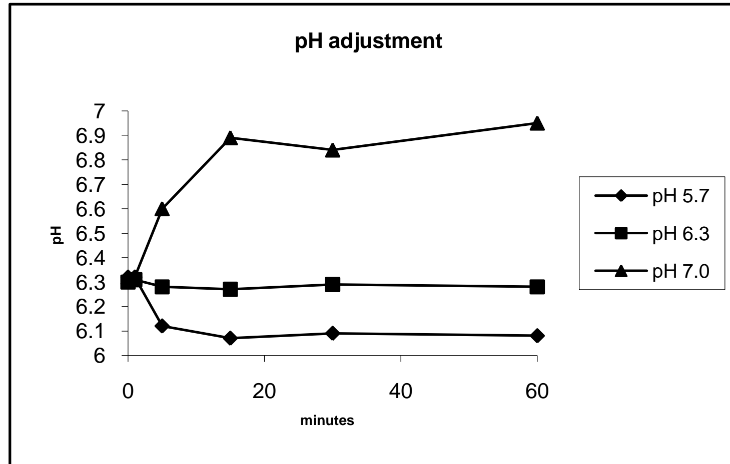
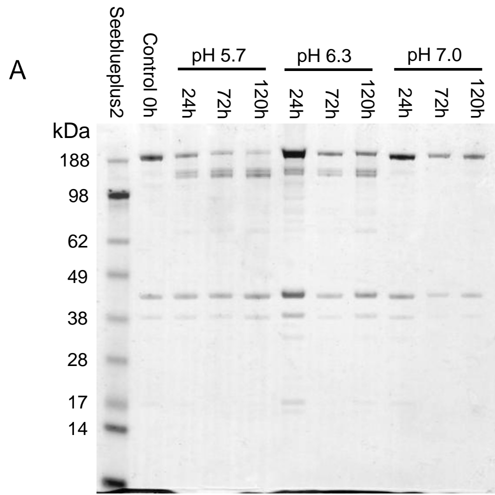


Figure 2



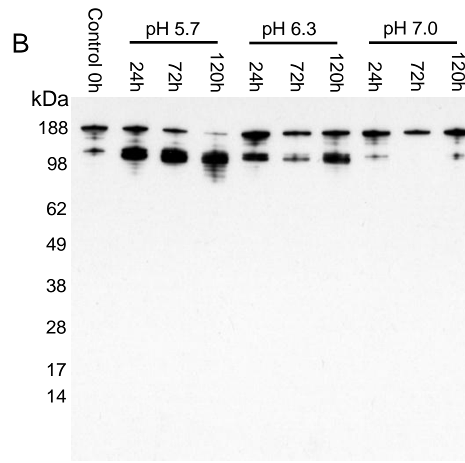


Figure 3