Chapter 9

EFFECT OF TREATMENTS ON LYSOSOMAL STABILITY

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9.1. Introduction

The problem that could arise out of stored flesh foods are the tenderisation, change in colour and texture, production of undesirable flavours and spoilage by microorganisms.

The spoilage of flesh foods stored at refrigerated temperatures and freezing temperatures has been attributed to intracellular autolysis. The autolytic enzymes comprise proteases, including lysosomal hydrolases. These enzymes are known to tenderise the meats, and the hydrolytic products released may also influence the acceptability of the stored flesh foods. The release of lysosomal enzymes occurs at a faster rate when the samples are stored at ambient temperature. Storage in freezer and subsequent thawing facilitates the release of hydrolases. Hence it is very important to consider the role of lysosomes in the preservation of fish and fishery products that are stored either in iced storage or in freezer.

In this chapter studies are undertaken regarding the stability of lysosomes of two species of squid, loligo and needle squid under various processing variables, using the enzyme acid phosphatase as an indicator. Assessment of the total lysosomal enzyme activity of the squid muscle was done using mantle homogenate in 0.25M sucrose containing EDTA in the presence of a non-ionic detergent like Triton X-100. Latent and free activity of the enzyme was determined in a homogenate in a manner wherein the integrity of lysosomal particles was maintained as much as possible.

9.2. Review of Literature

Various factors are capable of altering the structure of the lysosomal membrane and releasing the hydrolases. The occurrence, distribution and properties of enzymes can influence the stability of food. For instance, modifications in refrigerated storage for enhancing the shelf life (2000) recommend the use of endogenous active enzymes in muscle tissues and deterioration of muscle tissue during extract from skeletal muscles. The activated muscle proteins are involved in drastic changes in disorganization of the basis of disorganization and need for evaluation of muscle tissue cannot be evaluated).

The lysosomal enzymes were studied under non-ionic detergent and thawing treatments. Bombay duck muscle proteins (WDW) with different classes of enzymes were used for evaluating the muscle tissue integrity.
properties of lysosomes were reviewed by Ninjoor, et al.,(1969). The storage stability of cattle spleen lysosomal enzymes was investigated using refrigerated storage and frozen storage by Melendo et al., (2001). Shann (2000) recommended that the lysosomal cathepsins and calpains, and their endogenous inhibitors were considered to be involved in meat tenderisation and deterioration of fish protein gel. Characterisation of crude lysosomal extract from bovine spleen for its use in processing muscle foods, was studied by Melendo et al., (1999) and (1998). Lysosomal cathepsins are calcium-activated muscle proteinases, involved in the post-mortem rheological changes in tissues of fish and marine invertebrates (Kolodziejska and Sikorski, 1996). Assessment of lysosomal nature of hydrolytic enzymes of skeletal muscles is rendered difficult for different variety of fish, owing to drastic homogenisation procedures required to obtain a uniform homogenate and need for prolong incubation periods. Such treatments cause disorganization of intact lysosomes and hence the latency of these enzymes cannot be established (Duve, 1959; Weinstok and Iodice, 1969).

The lysosomal enzymes in drip and the muscle of *Harpodon nehereus* were studied during repeated freezing and thawing in the presence of non-ionic detergent before and after irradiation. Irradiation and freezing and thawing treatments bring about increased exudation of soluble proteins of Bombay duck into the drip, possibly by the denaturation of myofibrillar proteins (Warrier et al., 1972). This treatment may also enrich the solubility of enzymes in the drip. Hari Kumar et al., (1974) have developed a method for evaluation of latency of lysosomal enzymes from chicken skeletal muscle tissue. Several mechanisms have been put forward to explain the increase in acid phosphatase activity. A direct correlation was noticed
between the duration of exposure to mercury and activity of acid phosphatase in catfish (Rema and Philip, 1999).

Lesly et al., (1996) investigated the role of myosin in the endosomal traffic and lysosomal system. The biosynthetic pathway for lysosomal hydrolysis has been extensively characterised by Cardelli (1993). The transport of materials to and from lysosome requires the proper movement and fusion of membrane vesicle.

Since cathepsin D is believed to constitute the major proteolytic activity in lysosomes (Barrett, 1972), lysosomes may also be involved in the protein degradation of squid mantle muscle. The activity at pH 3.0 is mainly contributed by carboxyl proteinases such as pepsin and cathepsin D. Migita et al., (1958) has observed that the squid mantle muscle actomyosin was much less stable than that of carp and rabbit. Sakai et al., (1986) have purified and characterised the acid cysteine proteinase from squid mantle. In studies on the proteinases of the squid mantle muscle, authors have found that there is high autoproteolytic activity in squid muscle. The activity was observed in both acidic and alkaline pH ranges with a maximum pH 3.0 (Sakai and Matsumotto, 1981). Sub cellular distribution study revealed that the acid proteinase activity is localised in lysosomes. These studies indicated the presence of a cathepsin D like proteinase and a cysteine preproteinase in squid mantle muscle, the former being the major enzyme in lysosomes. When the extraction was carried out in the presence of Dithiotritol (DTT), the acid proteinase activity increased, indicating that the squid mantle muscle contains a considerable amount of cysteine proteinase, thus refuting the presence of cathepsin B, H and L in the squid mantle muscle. The cathepsin D-like proteinase and cysteine proteinase were

9.3. Material

The animal samples were obtained from P. Warrier, et al.

9.3.1. Reagents

- 0.25M sucrose solution
- 0.25M sodium acetate buffer
- Citrate buffer
- Substrates: Acid urea, myosin and insulin
- Standard PH 3.0

9.3.2. Preparation

- Total A

Fresh animals were minced as explained above and homogenised with a 0.25M sucrose solution at 10000G speed for 20
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separated by Diethylaminoethane (DEAE)-Sephadex A-50 column chromatography.

9.3. Materials and Methods

The analyses of lysosomal enzymes were done as per the method of Warrier, et al., (1972).

9.3.1. Reagents

- 0.25M sucrose containing 1mM EDTA (8.5575g of sucrose and 40mg of EDTA in 100ml of distilled water).
- 0.25M sucrose containing 1mM EDTA with 0.1% Triton X-100.
- Citrate buffer (100mM, pH 4.8) 4.1g of citric acid and 11.25g of sodium citrate. Dissolved and made up to 500ml with distilled water and stored at 4°C.
- Substrate- \( p \)-nitro phenyl phosphate (400mg dissolved in 100ml of distilled water).
- 0.1N NaOH
- Standard \( p \)-nitro phenol -1mM, 0.13911 g/1000ml.

9.3.2. Preparation of tissue homogenate

➢ Total Activity

Fresh and treated samples of both species of squid, loligo and needle, as explained in 8.3 were taken for this study. The sample tissues were finely minced with scissors and 10% (w/v) tissue suspension was prepared in 0.25M sucrose containing 1mM EDTA using a homogeniser set at minimum speed for 20 sec. The homogenates were centrifuged at 4°C and collected.
the supernatant. The residue was washed twice in the same media and washings were combined with the supernatant. A similar homogenate was also prepared by thorough homogenisation of the sample (10% w/v) in the above solution with 0.1% Triton X-100 and taken as total activity.

For each assay, samples were taken in triplicate.

> **Free Activity**

The free activity was determined from 10% w/v tissue cut into small pieces using scissors and suspended in cold 0.25 M sucrose solution containing 1 mM. EDTA. The suspensions were passed through a single layer of surgical gauze and the residue obtained were washed 2 or 3 times with the same media and the washings were added together.

> **Drip**

The frozen samples were thawed and the exudate or drip was collected.

> **Bound Activity**

Bound Activity (Latent activity) was determined by subtracting free activity from total activity.

\[
\% \text{ Bound Activity} = \frac{\text{Total activity} - \text{Free activity}}{\text{Total Activity}} \times 100
\]

9.3.3. Assay of Acid phosphatase

0.5ml of Buffer was taken in a test tube, added 0.5ml of substrate and 0.5ml of the enzyme extract. Incubated the test tubes at 37°C for 30 minutes and stopped the reaction by adding 4ml of 0.1N NaOH. To the control extract was added after adding the NaOH. The optical density was read at 405nm along, the activity was estimated by plotting a standard curve.

The lysozyme activity in phosphatase was measured as U per ml (EU) as 1 U per minute produced 1 micro mole of p-nitrophenol.

9.3.4. Protease

Proteases and free were present in the medium.

The protease activity was estimated.

9.4. Results

![Figure 9.1 Effect of pH on acid phosphatase activity](image)
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405nm along with standards and blank. The protein in the extracts was estimated by Lowry, et al., (1951).

The lysosomal enzyme activity was expressed in terms of acid phosphatase activity as micrograms of p-nitro phenol liberated per minute per ml (EU) and specific activity as micrograms of p – nitro phenol liberated per minute per mg protein.

9.3.4. Protease activity

Protease (cathepsin-D like) activity in all the above extracts- total, latent and free were assayed according to the method described in 8.3. The pH of the medium was 3.0. Here also enzyme assays were conducted in triplicate. The protease activity was expressed as μg of tyrosine per ml per minute.

9.4. Results

Figure 9.1 Effect of treatments on lysosomal
on acid phosphatase in needle squid

Figure 9.2 Effect of treatments on lysosomal on acid phosphatase in loligo

![Graph showing enzyme activity for different treatments](image-url)
Profiles of lysosomal activity of both the species studied under various treatments are given in Fig. 9.1 and 9.2. The activity was observed to be higher in needle squid when compared with loligo. As in the case of proteolytic enzymes, lysosomal enzymes also showed a very low activity in samples treated with acetic acid alone. Untreated sample showed maximum activity followed by lime juice. Sample treated with acetic acid citric acid mixture and acetic acid STPP mixture exhibited almost similar activity in both the species. The samples treated with ascorbic acid showed a better result when compared with the above treatments.

Figure 9.3 Lysosomal acid phosphatase in drip of both species of squid after various treatments.

The activity in samples treated with high activity. In the drip also, was less when samples treated enzyme activity showed favourable phosphatase activity when compared to the untreated species.

<table>
<thead>
<tr>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>6</td>
</tr>
</tbody>
</table>

The best activity was 60%-80% (Table 9.3). Compared to sample 5 and treated sample, the activity was less.
in samples treated with acetic acid STPP mixture, but the drip showed a high activity. Acetic acid alone treated sample showed minimum activity in the drip also. The quantity of drip in samples treated with acetic acid alone was less when compared with other treatment systems, but more than the samples treated with acetic acid STPP mixture. The quantity of drip and the enzyme activity was maximum in the untreated samples. Ascorbic acid showed favourable results in the drip also. In all the treated samples, acid phosphatase activity was slightly higher in needle squid than loligo, while the untreated samples showed significant difference between the two species.

Table 9.1. Profile of percentage of bound activity (latent activity) of lysosomal acid phosphatase and protease in the squid mantle tissue

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Protease</th>
<th>Acid phosphatase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Loligo</td>
<td>Needle</td>
</tr>
<tr>
<td>1</td>
<td>66.72</td>
<td>61.49</td>
</tr>
<tr>
<td>2</td>
<td>79.83</td>
<td>75.25</td>
</tr>
<tr>
<td>3</td>
<td>67.61</td>
<td>65.19</td>
</tr>
<tr>
<td>4</td>
<td>62.3</td>
<td>72.02</td>
</tr>
<tr>
<td>5</td>
<td>80.75</td>
<td>79.85</td>
</tr>
<tr>
<td>6</td>
<td>67.81</td>
<td>63.61</td>
</tr>
</tbody>
</table>

The bound activity of cathepsins in loligo and needle ranged from 60%-80% (Table 9.1). In needle squid the latent protease activity was less compared to loligo in all treatments except sample 4. Among treatments sample 5 and 2 showed the highest values. In needle, the sample 4 (STPP treated samples) resulted in higher retention of lysosomal bound enzyme activity compared to loligo.
The latent activity in terms of acid phosphatase showed higher values in needle compared to loligo in all the treated samples, while in untreated samples needle showed a lower value.

**Table 9.2 Lysosomal Protease activity in the mantle tissue and the drip of both species of squid after treatments.**

*(EU microgram of tyrosine / ml / minute)*

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Loligo (EU)</th>
<th>Needle (EU)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>1</td>
<td>63.04</td>
<td>90.76</td>
</tr>
<tr>
<td>2</td>
<td>5.76</td>
<td>9.86</td>
</tr>
<tr>
<td>3</td>
<td>15.59</td>
<td>27.36</td>
</tr>
<tr>
<td>5</td>
<td>14.63</td>
<td>18.03</td>
</tr>
<tr>
<td>6</td>
<td>19.89</td>
<td>30.49</td>
</tr>
</tbody>
</table>

A, A' - without Triton X100, B, B' – with Triton X100, C, C' - Free activity, D, D' - Drip

Table 9.2 shows the lysosomal protease activity in the mantle tissue and the drip in both species of squid. In this also, a same trend was obtained as in the case of protease activity, which showed a minimum activity in sample 2 and maximum activity in sample 1. Sample 2 and 5 showed significantly lower activity than the other treatments.

**9.5. Discussions**

Sakai et al. (1983) have separated a cysteine proteinase and cathepsin D like proteinase from squid mantle muscle by using a buffer containing Triton X-100, acid treatment, ammonium sulphate fractionation and column chromatography. Their findings were confirmed to be trypsin-like. Urea, pH 3.0 and the addition of Triton X-100 to cathepsin D inhibited the trypsin-like activity. From the study, 3.0 of pH, which was taken as the appropriate

D like protease activity in mantle muscle, could be a major protease present and involved in the degradation process.

When mantle muscle was homogenized with 20 seconds of sonication and sucrose containing homogenisation, the trypsin-like activity was still present. The homogenisation process, Triton X100, and pH 3.0 were used to separate the protease activities, as mentioned by Sakai et al. (1983).
higher values were in untreated

**Results**

<table>
<thead>
<tr>
<th>C'</th>
<th>D'</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.609</td>
<td>5.35</td>
</tr>
<tr>
<td>17</td>
<td>0.81</td>
</tr>
<tr>
<td>186</td>
<td>3.78</td>
</tr>
<tr>
<td>40</td>
<td>3.86</td>
</tr>
<tr>
<td>17</td>
<td>2.33</td>
</tr>
<tr>
<td>203</td>
<td>2.97</td>
</tr>
</tbody>
</table>

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Chromatography. The latter proteinase has been recently purified and confirmed to be cathepsin D. The optimum temperature was at 45°C and pH 3.0 and the activity was almost zero at pH 5.0 (Sakai, 1986). Most of the cathepsin D activity was removed by pepstatin and leupeptin, which strongly inhibited the squid cysteine proteinase activity (Umezawa and Aoyagi, 1977). From the study, it was clear that cathepsin D is present in the squid meat, which was taken for the present study. Thus the result indicated that cathepsin D like proteinase contributed mainly to the protein degradation in squid mantle muscle at this pH. Since cathepsin D is believed to constitute the major proteolytic activity in lysosomes (Barrett, 1972), lysosomes may be involved in the protein degradation of squid mantle muscle.

When 10% homogenate of the mantle tissue prepared in 0.25M sucrose containing 1mM EDTA using a homogeniser at minimum speed for 20 seconds were employed for assessing the free and bound activity of Cathepsins (EC 3.4.4.2) and acid phosphatase (EC 3.1.3.2), it was observed that these enzymes were mostly in free form in muscle homogenate. The tissue was subjected to homogenisation with Triton X100 to solublize the lysosomal membrane lipoproteins and to liberate all enzyme into solutions. Treatment such as blenderisation, repeated freeze-thaw cycles, addition of Triton X100 etc. bring about almost complete release of the enzymes.

Homogenisation technique due to limitations could give high free activities, as evidenced by the high activity in the homogenate prepared without Triton X100, in control as well as test samples. Therefore using homogenisation technique to monitor endogenous free activity will be misleading. A method reported by Hari kumar et al., (1974), using tissue slices was used to study the *invivo* changes occurring in the lysosome due to various
treatments. The difference in the total and free activities is computed as bound or latent activity thus accounting for 60-80% for cathepsins and 82-95% in acid phosphatase. The results on acid phosphotase activity (both total and free) show significant variation in the release or solubilisation of the enzymes under various treatments in both the species of squid. The homogenisation with and without triton X 100 showed almost similar values confirmed the high solubility of squid proteins including enzymes into the extracting medium. Irradiation or freezing of muscle of Harpodon neheraus (Bombay duck) enhanced the levels of lysosomal activities (Warrier, et al., 1972).

9.6. Conclusion

The presence of cathepsin D like proteases of lysosomal origin is involved in the protein degradation in squid mantle muscle. Treatment is inevitable in the case of squid in order to maintain the storage quality. The treatment with acetic acid, acetic acid STPP mixture reduced the quantity of drip, while acetic acid STPP mixture treated sample showed a higher lysosomal enzyme activity in the drip than the acetic acid treated sample. In the present study, the level of lysosomal enzyme released into the medium was reduced by various treatments. The reduced amount of lysosomal enzymes in the drip of acetic acid treated sample and their low activity in the drip confirm the ability of acetic acid to maintain the inter-protein network and retain proteins including water soluble enzymes in the gel matrix to the maximum. The difference in the rates of release of enzymes could be due to the difference in the binding of these enzymes to the lipoprotein membrane matrix. This is very well supported by the increased bound activity in the respective samples.
9.7. References


Umezawa, H and Aoyagi, T., (1977) Activities of proteinase inhibitors of microbial origin. In Proteinases in Mammalian cells and Tissues
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* Not referred in original