CHANGES IN IN-VITRO AND IN-VIVO DIGESTIBILITY OF MACKEREL UPON CURING AND DRYING

By

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ABSTRACTS

Indian mackerel (Rasrrelliger kanagurta) was cured in saturated brine and dried at 50°C. The dried fish was desalted (rehydrated) and cooked in steam, as were the fresh fish. Digestibility studies were carried out on these two samples using pepsin, trypsin, chymotrypsin, bromelin, alcalase and neutrase for in vitro studies, and male albino rat for in vivo studies. Digestibility was found to suffer as a result of the curing and drying process for all enzymes with the degree of decrease in the following order: Trypsin > pepsin > neutrase > bromelin > alcalase > chymotrypsin. In animals, decrease in the digestibility of the dried fish protein was highly significant and the utilization and retention of the proteins were also significantly affected. But the difference in PER between the fresh and dried proteins was not significant. Amino nitrogen in the enzymatic digests determined by the trinitrobenzene sulphonic acid reaction was found to be highly correlated to the total nitrogen in digest determined by the Kjeldahl procedure.

INTRODUCTION

Curing and drying of fish is still a major method of preservation of fish catches in the developing countries. This is particularly true of the lower priced fish and those that are landed in quantities too large to be preserved by refrigeration or freezing. Because of their relatively low cost, dried fish products form an important source of animal protein for the economically poorer sections of the society.

Curing and drying bring about a number of changes in the fish muscle proteins like the formation of covalent disulphide bonds, hydrophobic bonds and covalent bonds between oxidized lipids and amino acids (Opstvedt, 1991). The effect of these changes on the digestibility of proteins in dried fish and the amenability of the proteins to hydrolysis by various proteases are aspects that demand detailed study. Such studies can also throw more light on the amino acid residues that are affected during the drying process. The differences in digestion of dried fish proteins by different proteases and the correlation between in vitro and in vivo protein digestion as followed by animal feeding studies are aspects which have not received proper attention.

This study is aimed (i) at finding out the difference in in vivo digestion by various proteases with a view to understand the nature of changes that occur during curing and drying. As most foods are cooked before being eaten, fresh and dried fish used in the study were cooked, to make it nutritionally more relevant.
MATERIALS AND METHODS

Indian mackerel, *(Rastrelliger kanagurta)* was procured in fresh condition (iced immediately after catch) from the local fish market during September 1993. The enzymes pepsin and trypsin used in this study were purchased from Sisco Research Laboratories Pvt.ltd, India. Chymotrypsin was from Sigma Chemical Company, U.S.A. Alcalase and Neutrase were a kind gift from M/s Novo-Nordisk a/s of Denmark. Male rats of Wistar strain used in the study were bred in the Institute’s animal house. All the chemicals used were of AR or GR grade.

Fish were eviscerated and washed thoroughly. Half of the lot was cooked by steaming in trays over boiling water in a covered vessel 20 min, followed by cooling and picking of the cooked meat. The remaining fish were split open from the ventral side for drying, washed, and cured by immersing in saturated brine for 24 h and dried in a cross-flow drier at 50°C for 48 h. After ambient storage for a week, the dried fish were desalted and rehydrated by soaking in ten volumes (w/v) of tap water overnight and drained. Rehydrated meat was cooked as was the fresh meat. The cooked meats were thoroughly mixed and held frozen at -20°C till use. The activity of the enzymes were assayed by the following methods: pepsin as per Ryle (1970), chymotrypsin and trypsin as per Laskowski (1955), Bromelin as per Murachi (1970) and alcalase as per Novo Industries method (1978). Neutrase was assayed as for alcalase but at pH 6.5 and in the presence of 0.02 M Ca$^{2+}$. Activity was determined as the Folin positive material in the trichloroacetic acid (TCA) filtrates using tyrosine as a standard and expressed as μM Tyr released per ml or g of enzyme per min (unit).

For *in vitro* digestibility studies, /g of the sample was homogenized with 20 ml buffer in a Polytron homogenizer at 10,000 rpm for 1 min. The following buffers were used: trypsin, chymotrypsin and alcalase: 0.3 M, pH 8.0 phosphate buffer, bromelin: 0.3M, pH 5.5 citrate phosphate buffer and neutrase: 0.3 M, pH 6.5 phosphate buffer. The probe was washed twice with further aliquot of buffer and the volume was made up to 45 ml with buffer. For pepsin the buffer was replaced with distilled water and after making up to 40 ml, 5 ml of 3 N HCL as added slowly with stirring. In the case of bromelin, 4 ml of 0.0625 M - mercaptoethanol was also added and the volume adjusted, such that the final reaction volume after addition of enzyme was 50 ml. After 10 min of equilibration at 37°C in a water bath, 5 ml of the enzyme solution containing 0.05 units enzyme/mg substrate protein (TN x 6.25) was added, mixed and incubated for 1 h. The reaction was terminated with 5 ml of 55% trichloroacetic acid (TCA). For controls, the order of addition of enzyme and TCA were reversed. After 30 min, the reaction mixture was filtered through Whatman no.1 paper. Total nitrogen was determined in a suitable aliquot of filtrate, cooked meats and enzyme solutions by the microkjeldahl procedure (AOAC, 1990). Amino nitrogen in the digest was determined by the trinitro benzenesulphonic acid (TNBS) reaction (Adler-Nissen, 1970), but the developed colour was read at 450 instead of 340 nm. Proximate composition of meats and salt were analyzed as per AOAC (1990).

Digestibility, protein efficiency ratio (PER), net protein utilization (NPU) and biological value (BV) of the protein samples were determined by standard methods.
RESULTS AND DISCUSSION

The proximate composition of the fresh mackerel and during subsequent stages of processing are presented in Table 1. It can be seen that full rehydration was not achieved even after the dried mackerel was rehydrated overnight and cooked, which is a common problem in all dried foods. The lower moisture content of the rehydrated product however, probably explains its higher protein and fat content. After desalting (rehydration) and cooking, the salt content became relatively low, but was still slightly higher than the fresh cooked mackerel.

Table 1

<table>
<thead>
<tr>
<th>Processing stage</th>
<th>Moisture</th>
<th>Fat</th>
<th>Protein</th>
<th>Salt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh mackerel</td>
<td>72.57</td>
<td>4.86</td>
<td>23.21</td>
<td>0.20</td>
</tr>
<tr>
<td>Fresh mackerel after cooking and picking of meat</td>
<td>69.38</td>
<td>3.37</td>
<td>23.89</td>
<td>0.16</td>
</tr>
<tr>
<td>Cured and dried mackerel</td>
<td>20.13</td>
<td>10.16</td>
<td>35.81</td>
<td>31.53</td>
</tr>
<tr>
<td>Rehydrated, cooked and picked meat</td>
<td>58.48</td>
<td>8.3</td>
<td>28.01</td>
<td>2.89</td>
</tr>
</tbody>
</table>

Results of the feeding trials using albino rats are presented in Table 2. It was seen that there was a highly significant decrease in digestibility upon drying and significant decreases in NPU and BV as well. Although the rats fed with dried mackerel showed a higher PER, it was not statistically significant.

Table 2

<table>
<thead>
<tr>
<th>Nutritional parameter</th>
<th>Fresh</th>
<th>Cured</th>
<th>M.S.S.</th>
<th>D.F.</th>
<th>F value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digestibility, %</td>
<td>89.64</td>
<td>85.38</td>
<td>45.37</td>
<td>1.8</td>
<td>29.75**</td>
</tr>
<tr>
<td>Protein efficiency rate</td>
<td>3.04</td>
<td>3.31</td>
<td>0.18</td>
<td>1.8</td>
<td>4.42</td>
</tr>
<tr>
<td>Net protein utilization</td>
<td>80.40</td>
<td>74.99</td>
<td>72.00</td>
<td>1.6</td>
<td>8.33*</td>
</tr>
<tr>
<td>Biological value</td>
<td>88.98</td>
<td>85.93</td>
<td>24.96</td>
<td>1.6</td>
<td>6.62*</td>
</tr>
</tbody>
</table>

1 Fresh cooked mackerel
2 Cured, dried rehydrated and cooked mackerel.
3 Mean sum of squares* significant at 5% level **significant at 1% level.
The amino nitrogen in the digest can be determined by the TNBS reaction, which however measures only the terminal amino N portion of the total nitrogen in the digest. Since the amino N is more quickly and accurately determined than total N by the Kjeldahl procedure, an attempt was made to find out if a significant correlation existed between total N and amino N in a random sample of 67 digests. The results of the regression of TNBS N on Kjeldahl N are shown in Figure 1. It was seen that the correlation between the two was highly significant and had a low error of estimation.

![Figure 1](image)

Figure 1  Regression of α amino nitrogen determined by the TNBS reaction on Kjeldahl nitrogen in the enzyme digests.

Hence, total N in the digests was estimated using the regression equation, from TNBS amino N values and the results expressed as % digestibility (net increase in the TCA soluble N/total N fraction) which are presented in Figure 2. In the fresh cooked muscle the digestibility increased with the enzymes used in the following order: alcase < neutrase < trypsin < chymotrypin < pepsin < bromelin. This order was changed slightly after the fish was cured, dried, rehydrated and cooked, but bromelin still showed the maximum digestibility (trypsin < neutrase < alcalase < pepsin < chymotrypsin < bromelin). It can be seen that there were substantial decreases in enzymatic digestibilities of all enzymes after the fish had been dried and cured. Digestibility was affected in the following order: trypsin > pepsin > neutrase > bromelin > alcalase > chymotrypsin.

The decrease in in vitro enzyme digestibility upon drying fish has been noticed earlier by Sheikh and Shah (1974) who reported that digestibility with pepsin, trypsin and pancreatic extract decreases with increase in drying temperature. But Shinno et al. (1965) did not find any such decrease in pepsin digestibility although they found reduction in total tryptophan content upon drying. In contrast, an increase in trypsin digestibility for yellow corvina and anchovy upon salting, was noticed by Lee and Ryu (1987) who, however, did not find appreciable difference for other fishes like Atlantic pollack and flounder. Perhaps, apart from the temperature of drying, salting and the level of salting can also affect the in vitro
Digestibility. These differences in digestibility could also be a result of differences in assay conditions like enzyme: protein ratio, activity variations in enzyme preparations, incubation time etc.

Figure 2 Changes in the digestibility of mackerel upon curing and drying with different proteolytic enzymes. Digestibility is expressed as the net increase in the ratio of TCA soluble nitrogen/total nitrogen, between experimental and control samples.

Feeding trials, as seen in this experiment, support the data of in vitro digestibility. It was seen that apart from decrease in digestibility of dried proteins, their utilization and retention by the animals were also affected significantly. Reports of previous studies, however, are conflicting as some workers found a decrease in true digestibility upon drying (Mustafa, 1996, Yanes et al., 1970), while others found only marginal decreases (Njaa et al., 1968, Herborg et al., 1974) or that heating did not affect the utilization of absorbed protein (Opstvedt et al., 1987).

Drying of fish proteins leads to the formation of different inter and intranolecular bonds. Disulphide bonds resulting from oxidation of -SH groups have been reported to reduce digestibility (Opstvedt et al., 1984). Isopeptide bonds between free -NH2 and -COOH groups, which get exposed on chain unfolding during drying, or between these groups and lipid oxidation products, are other possible bonds formed. Such covalent bonds are resistant to the action of proteinases which can explain the general decrease in digestibility upon drying.

The differing decrease in digestibility of dried fish with various proteinases can be partly attributed to the different substrate specificities of these enzymes. Thus, basic amino acids appear to be the worst affected by the drying process as digestibility by trypsin which cleaves at Lys and Arg residues (Fruton, 1975) at alkaline pH was affected the most. Yanes
et al. (1970) have reported a 20% reduction in FDNB reactive lysine when the fish was dried at 170°C in comparison to freeze dried samples.

Pepsin and neutrase digestibilities which were the next most affected, indicate the involvement of aromatic and hydrophobic amino acids in changes during drying, as these two enzymes cleave at such amino acid residues at acid and neutral pH (Fruton, 1975) respectively. A previous study did not find any decrease in pepsin digestibility in spite of a decrease in tryptophan content on drying (Shinno et al., 1965). The unfolding of polypeptide chains if any, at the low pH values encountered during pepsin digestion does not appear to have helped in exposing more susceptible sites for digestion by this enzyme.

Although the sites of enzyme attach for chymotrypsin and alcalase are similar to those of pepsin, albeit at alkaline pH (Fruton, 1975), digestibility by these enzymes was comparatively less affected. While the decrease in digestibility by alcalase and chymotrypsin with dried fish indicates that aromatic and hydrophobic amino acids are involved, as in the case of pepsin, the relatively lesser amount of decrease points to the more successful exposure of susceptible residues at alkaline pH. Since the electrostatic repulsion of polypeptide chains at acid and alkaline pH are a result of the protonation of NH2 groups and deprotonation of -COOH groups respectively, the protonation process appears to be more affected by the drying process. The highly reduced trypsin digestibility, which cleaves Lys and Arg residues responsible for protonation at acid pH, supports this hypothesis. However, reaction of amino acid side chains between themselves or with lipid oxidation products during drying, could also be another reason for the decreased digestibility.

Digestibility with bromelin, which has a broad substrate specificity, was highest with both fresh and dried fish, but was only slightly more affected than chymotrypsin and alcalase by the drying process, as it has a marginal preference for cleaving at Lys and Arg residues (Fruton, 1975).

The study was able to show that both in vitro and in vivo digestibilities are affected by the curing and drying of fish. In vitro digestibility with various proteases is not affected equally, reflecting different substrate specificities, or the formation of bonds other than the normal peptide bond.

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