Chapter 1

Curcumin and its derivatives prevent iron-induced lipid peroxidation in a teleost *Anabas testudineus* (Bloch)

Introduction

Aquaculture is the sector expected to meet the challenges of current and future food requirements of the world. Success of aquaculture depends on developing and adopting technologies which allow efficient and environmentally sustainable production. The increased rate of utilization of antibiotics and other chemicals have now become a risk to humans because of chemical residues in food and of antibiotic resistance being passed on to human pathogens. The advent of antibiotics led to a decline in their usage. As a result, scientists have intensified efforts in exploiting plants as potential natural alternatives for enhancing livestock productivity. The plant kingdom might provide a useful source of new medicines, pharmaceutical entities and bioactive compounds for enhancing animal production and health, food safety and quality, while conserving environment.

Curcumin, the active constituent of *Curcuma longa*, is one of the best-studied natural compounds. A wide spectrum of pharmacological activities has been attributed to curcumin based on studies conducted in higher mammals or mammalian cell-lines. It appears that no studies are available on the effect of curcuminoinds in lower vertebrates except a recent study on the effect of turmeric
tuber powder on the in vitro inhibition of fish lipid peroxidation (D’Souza & Prabhu, 2006). In vitro experiments were conducted as a preliminary step in order to examine whether curcumin is efficient in a lower vertebrate like fish. The aim was to promote curcumin supplemented food in aquaculture to improve the quality and quantity of food after generating in vivo data. The present study evaluates the role of natural curcumin which contains three curcumins, the separated curcumin I, curcumin II, curcumin III and curcumin-boron-oxalic acid complex on lipid peroxidation in a fish model – Anabas testudineus (Bloch).

Experimental material

CUR Mix, CURI, CURII, CURIII and CUR BOx were prepared as described in the materials and methods section.

Experimental design

Acclimated adult A. testudineus weighing 40 ± 5 g were fasted overnight prior to sacrifice. Fish were killed by decapitation, and liver tissue was dissected out. About 100 mg of the tissue were cut in to very small pieces in 1 ml tissue culture medium (TCM), which consisted of RPMI-1640 supplemented with 10% of 200 mM L-glutamine solution and 0.1% of 50 mg/ml gentamycine sulphate solution. Cell suspension (CS) was prepared with a slight modification (Sunny et al., 2002; Sreejith & Oommen, 2006) of a reported procedure (Slater & Schreck, 1997) and was placed on ice until the tissue fragments settled down. Cell viability was checked using trypan blue and was found to be 90-95%. The CS were
transferred to a multi-well culture plate for the *in vitro* experiments. Six sets of experiments were conducted to study the effect of five curcuminoids CURI, CURII, CURIII, CURMix and CURBox on lipid peroxidation. Dimethyl sulphoxide (DMSO) was used to dissolve curcumin based on previous studies conducted in other animal models. First, stock solutions of 0.1M of the five curcuminoids were prepared and diluted to get final concentrations, $10^{-2}$, $10^{-3}$, $10^{-4}$ M in the TCM. For each compound, three different concentrations were tested ($10^{-2}$M, $10^{-3}$M & $10^{-4}$M) at two incubation periods (30 min and 60 min) in hepatocytes derived from 6 different fish in each set. For each concentration, 30 and 60 min experiments were done on the same day using hepatocytes derived from different animals.

Set 1: $10^{-2}$M CUR (x) + control; 6 CS each (30 min)
Set 2: $10^{-2}$M CUR (x) + control; 6 CS each (60 min)
Set 3: $10^{-3}$M CUR (x) + control; 6 CS each (30 min)
Set 4: $10^{-3}$M CUR (x) + control; 6 CS each (60 min)
Set 5: $10^{-4}$M CUR (x) + control; 6 CS each (30 min)
Set 6: $10^{-4}$M CUR (x) + control; 6 CS each (60 min)

Where x is I, II, III, Mix and Box

Lipid peroxidation was induced in both the tests and controls by adding 100μl 15mM ferrous sulphate. Total volume of the system was 1.1ml. To this, various curcuminoids (0.1ml) were added to the hepatocytes after being gently
stirred using a Pasteur pipette and the same volume of DMSO to corresponding controls. After incubation, the excess TCM was removed with the help of a pipette. Cells were washed and homogenized (MICCRA D-8 homogenizer) in respective buffers (sucrose, for enzyme analysis and Tris – HCl buffer for estimation of lipid peroxidation products). Homogenate was centrifuged at 5,000rpm for 10min, the supernatant was collected and used for biochemical analysis.

Biochemical analyses

Lipid peroxidation in the liver was determined by measuring the peroxidation products, TBARS content, CD, the activities of antioxidant enzymes, SOD, CAT and protein were measured according to the methods described in materials and methods section. Absorbance was measured using a UV-visible spectrophotometer (UV-1601, Shimadzu, Japan).

Statistical analyses

The data were statistically analyzed by one way analysis of variance (ANOVA), using the SPSS set up. The results were expressed as mean ± S.E. of 6 fish. The significant difference among means was determined by Duncan's multiple range test at the level, p<0.05.
Results

Results of the present study revealed that all the curcuminoids prevented lipid peroxidation in fish hepatocytes. The CURMix and CURIII were more potent compared to others.

Set 1:- SOD activity increased in the CURII and CURIII groups [Fig. 1.1(a)]. CAT activity enhanced in CURII, CURIII and CURMix but unaffected in the CURI treated group [Fig. 1.2(a)]. TBARS decreased in all treated groups with a lower value in the CURII treated group. CD also decreased in all groups with the lower value in the CURIII group (Table 1.1).

Set 2:- SOD increased in CURIII group [Fig. 1.1(a)]. CAT activity suppressed in all the groups [Fig. 1.2(a)]. TBARS decreased invariably in all groups. CD also decreased with the least value in the CURmix group (Table 1.1).

Set 3:- SOD was higher in the CURBOx group [Fig. 1.1(b)]. CAT had no difference [Fig. 1.2(b)]. TBARS decreased in all groups. Maximum effect was shown by the CURI and CURII groups. CD also lowered with the least value in the CURMix treated group (Table 1.1).

Set 4:- SOD activity decreased in the CURI and CURIII groups with an increase in the CURMix and CURBOx groups [Fig. 1.1(b)]. CAT increased in CURII and CURIII groups [Fig. 1.2(b)]. TBARS and CD decreased in all treatment groups (Table 1.1).
Set 5:-SOD activity was enhanced in the CURBOx group [Fig. 1.1(c)]. CAT activity increased in the CUR I and CUR Box group [Fig. 1.2(c)]. TBARS and CD decreased in all treatment groups (Table 1.1).

Set 6:-SOD activity increased in all treated groups [Fig. 1.1(c)]. CAT increased only in the CURBOx group [Fig. 1.2(c)]. TBARS and CD decreased in all groups (Table 1.1).

Discussion

Curcuminoids represent a class of valuable phytonutrients with unique bioprotective properties. The antioxidant property of curcumin explains many of its wide ranging pharmacological activities. Curcumin is known to inhibit lipid peroxidation in rat liver microsomes, erythrocyte membranes, and brain homogenates (Pulla Reddy & Lokesh, 1994). It has been suggested that curcumin exerts its action by maintaining the activities of antioxidant enzymes like SOD, CAT and GPx (Pulla Reddy & Lokesh, 1992). Administration of curcumin and curcumin analogs decreased lipid peroxidation by improving the antioxidant status and thereby preventing the damage to liver and subsequent leakage of enzymes glutamyl transferase and alkaline phosphatase (Rukkumani et al., 2004). Curcumin is also known to protect hemoglobin from oxidation (Unnikrishnan & Rao, 1995). In vitro, curcumin can significantly inhibit the generation of ROS such as superoxide anions, hydrogen peroxide and the nitrite radical generation by activated macrophages which play an important role in inflammation (Joe &
Lokesh, 1994). Oral administration of 30 mg/ kg body weight of curcumin in rats for 10 days reduces the Fe- induced hepatic damage by lowering lipid peroxidation (Pulla Reddy & Lokesh, 1996). The protection of mice from radiation by dietary curcumin also has been attributed to its antioxidant property (Inano et al., 2000).

The time and dose dependent effect of various curcuminoids on iron-induced lipid peroxidation in fish liver, *in vitro* was investigated. Initiation of lipid peroxidation by ferrous sulphate takes place either through ferryl-perferryl complex (Gutteridge, 1985) or through hydroxyl radicals by Fenton reaction (Halliwell, 1978). The elevated TBARS and CD levels in the controls of all groups in the present study clearly indicate increased lipid peroxidation and oxidative stress. There was a significant reduction of TBARS and CD by various curcuminoids - treated groups in all sets invariably, assuring their protective role in *A. testudineus*.

In the present study, SOD activity increased in CURII and CURIII treated group of set 1, CURIII of set 2, CURBOx of set 3, CURMix and CURBOx of set 4, CURBOx group in set 5 and all groups in set 6, but decreased in CURI and CURIII groups of set 4. In rest of the groups, it was unaffected, indicating that curcumins do not always depend on the enzymatic pathway to mediate their antioxidant effect. Catalase increased in the CURII, CURIII and CURMix groups of set 1, CURII and CURIII of set 4, CURBOx of set 5 and CURBOx of set 6, but lowered in CURI of set 1, all treated groups of set 2 and in the rest, it
remained unchanged. The decreased activity of the enzyme may be due to the feed back inhibition or the oxidative inactivation of the enzyme proteins due to excess ROS generation. The inhibition of CAT activity is suggestive of enhanced generation of superoxide anion by FeSO₄ since superoxide anion is a powerful inhibitor of CAT (Ashakumary & Vijayammal, 1996). Studies have shown that curcumin prevented oxidative damage during indomethacin - induced gastric lesion, not only by blocking inactivation of gastric peroxides, but also by direct scavenging of H₂O₂ and hydroxyl radical (Halliwell & Gutteridge, 1990; Bandyopadhyay et al., 1999). Since there was a reduction in TBARS and CD in all treated groups of the six sets, either both SOD and CAT had done their action very early and exhausted at the time of measurement or curcumin might have used some other mechanism along with enzymic pathway to reduce the peroxidation products. The antioxidant property of curcumin is attributed to their unique conjugated structure, which includes two methoxylated phenols and an enol form of beta diketone, the structure shows typical radical - trapping ability as a chain breaking antioxidant (Sreejayan & Rao, 1994; Masuda et al., 2001). This mechanism may include one or more of the following interactions; scavenging or neutralizing of free radicals (Soudamini et al., 1992), interacting with oxidative cascade and preventing its outcome (Unnikrishnan & Rao, 1992), oxygen quenching and making it less available for oxidative reactions (Soudamini et al., 1992), inhibition of oxidative enzymes like cytochrome p450, chelating and
disarming oxidative property of metal ions such as iron (Sreejayan & Rao, 1994), inhibits peroxidation of membrane lipids and maintaining cell membrane integrity and function (Balasubramanyam et al., 2003; Rukkumani et al., 2003). Interestingly, curcumin not only exhibits antioxidant and free radical scavenging properties, but also enhances the activities of other antioxidants such as SOD, CAT, GPx (Pulla Reddy & Lokesh, 1994). Another mechanism by which curcumin protects oxidative stress in endothelial cells is by induction of heme oxygenase-1 (Motterlini et al., 2000).

A time and dose-dependent effect of curcumin on fish lipid peroxidation is confirmed, as has been reported by Ambegaokar et al. (2003), Swarnakar et al. (2005) and Ahuja et al. (2006), in other living systems. The present results suggest that the curcuminoids involved prevent lipid peroxidation in fish as well. They directly scavenge free radicals, stimulate antioxidant enzymes, or employ some other pathways to protect tissues from oxidative stress. However, the actual mechanism remains to be elucidated. Curcumin is used as a food preservative due to its ability to protect the integrity of biomolecules, to prevent rancidity of meat and to provide edible meat containing less oxidized fat. AL-Sulthan (2003) reported that turmeric as a feed additive enhanced the overall performance in broiler chicken farming. The in vitro results are to be confirmed and strengthened in in vivo conditions in this teleost model. The next chapter deals with the in vivo studies regarding the protective effect of curcumin.
Fig. 1.1 Effect of curcuminoids; control, CURI, CURII, CURIII, CURMix and CURBOx on SOD activity in the cell suspension of hepatocytes of *A. testudineus* at 30 and 60 min (a) 10^{-2} M (b) 10^{-3} M (c) 10^{-4} M. Values are mean ± S.E. of 6 fish. Groups with different letter headings (a, b, c & d) are significantly different (P<0.05; One-Way ANOVA; Duncan's test)

Fig. 1.2 Effect of curcuminoids; control, CURI, CURII, CURIII, CURMix and CURBOx on CAT activity in the cell suspension of hepatocytes of *A. testudineus* at 30 and 60 min (a) 10^{-2} M (b) 10^{-3} M (c) 10^{-4} M. Values are mean ± S.E. of 6 fish. Groups with different letter headings (a, b, c, d & e) are significantly different (P<0.05; One-Way ANOVA; Duncan's test)
Table 1.1 Effect of curcuminoids on the lipid peroxidation products, Thiobarbituric acid reactive substances [μmol MDA g⁻¹ tissue] and Conjugated Dienes (μmol g⁻¹ tissue) at different concentrations (10⁻², 10⁻³, 10⁻⁴ M) and time periods.

<table>
<thead>
<tr>
<th>Group</th>
<th>Parameters</th>
<th>Control</th>
<th>CUR I</th>
<th>CUR II</th>
<th>CUR III</th>
<th>CUR Mix</th>
<th>CUR Box</th>
</tr>
</thead>
<tbody>
<tr>
<td>Set A 10⁻² M 30 min</td>
<td>TBARS</td>
<td>7.80 ± 0.72 a</td>
<td>1.31 ± 0.26 bc</td>
<td>0.53 ± 0.1 bc</td>
<td>0.99 ± 0.10 b</td>
<td>0.99 ± 0.10 bc</td>
<td>1.70 ± 0.30 b</td>
</tr>
<tr>
<td></td>
<td>CD</td>
<td>30.3 ± 1.70 a</td>
<td>14.3 ± 2.90 b</td>
<td>12.5 ± 1.80 bc</td>
<td>7.78 ± 0.60 c</td>
<td>15.3 ± 0.10 b</td>
<td>10.3 ± 1.20 bc</td>
</tr>
<tr>
<td>Set B 10⁻³ M 60 min</td>
<td>TBARS</td>
<td>7.80 ± 0.72 a</td>
<td>0.46 ± 0.03 b</td>
<td>0.42 ± 0.02 b</td>
<td>0.41 ± 0.02 b</td>
<td>0.45 ± 0.04 b</td>
<td>0.36 ± 0.10 b</td>
</tr>
<tr>
<td></td>
<td>CD</td>
<td>28.6 ± 1.50 a</td>
<td>14.7 ± 0.80 bc</td>
<td>15.4 ± 1.10 b</td>
<td>22.2 ± 1.70 a</td>
<td>7.80 ± 1.90 d</td>
<td>10.5 ± 1.30 bc</td>
</tr>
<tr>
<td>Set C 10⁻⁴ M 30 min</td>
<td>TBARS</td>
<td>7.40 ± 0.30 a</td>
<td>0.38 ± 0.04 b</td>
<td>0.45 ± 0.10 a</td>
<td>1.13 ± 0.30 c</td>
<td>1.59 ± 0.11 d</td>
<td>2.90 ± 0.20 c</td>
</tr>
<tr>
<td></td>
<td>CD</td>
<td>22.8 ± 1.70 a</td>
<td>7.90 ± 0.70bd</td>
<td>13.4 ± 2.20 a</td>
<td>17.4 ± 3.10 a</td>
<td>5.60 ± 1.50 d</td>
<td>18.5 ± 1.80 “c”</td>
</tr>
<tr>
<td>Set D 10⁻⁵ M 60 min</td>
<td>TBARS</td>
<td>7.40 ± 0.50 a</td>
<td>2.10 ± 0.33 b</td>
<td>2.30 ± 0.18 b</td>
<td>1.50 ± 0.20 c</td>
<td>4.40 ± 0.50 c</td>
<td>5.80 ± 0.50 d</td>
</tr>
<tr>
<td></td>
<td>CD</td>
<td>31.2 ± 1.60 a</td>
<td>16.4 ± 1.70 bc</td>
<td>13.2 ± 1.30 c</td>
<td>16.0 ± 2.50 bc</td>
<td>21.8 ± 2.90 bd</td>
<td>23.9 ± 1.40 bd</td>
</tr>
<tr>
<td>Set E 10⁻⁶ M 30 min</td>
<td>TBARS</td>
<td>8.40 ± 0.50 a</td>
<td>2.30 ± 0.20 b</td>
<td>3.40 ± 0.20 b</td>
<td>3.30 ± 0.11 b</td>
<td>3.17 ± 0.30 b</td>
<td>6.00 ± 0.60 c</td>
</tr>
<tr>
<td></td>
<td>CD</td>
<td>30.3 ± 1.80 a</td>
<td>25.2 ± 1.20 b</td>
<td>23.6 ± 0.80 b</td>
<td>27.8 ± 0.70 a</td>
<td>23.7 ± 0.60 b</td>
<td>27.7 ± 3.60 bh</td>
</tr>
<tr>
<td>Set F 10⁻⁷ M 60 min</td>
<td>TBARS</td>
<td>8.40 ± 0.10 a</td>
<td>2.70 ± 0.20 bc</td>
<td>3.00 ± 0.40 ce</td>
<td>3.40 ± 0.50 b</td>
<td>2.10 ± 0.30 c</td>
<td>2.70 ± 0.40 ce</td>
</tr>
<tr>
<td></td>
<td>CD</td>
<td>30.0 ± 1.60 a</td>
<td>10.5 ± 0.90 b</td>
<td>18.0 ± 0.90 b</td>
<td>23.2 ± 1.50 d</td>
<td>21.3 ± 1.30 bd</td>
<td>18.0 ± 2.50 b</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± S.E. of 6 fish. The significant difference between groups was analyzed by One way ANOVA. Mean values of different letter headings (a, b, c, d & e) are significantly different (p<0.05) as determined by One-way ANOVA using Duncan’s Multiple range test, in SPSS set up.