Süleyman Demirel Üniversitesi
Fen Bilimleri Enstitüsü Dergisi

Suleyman Demirel University
Journal of Natural and Applied Science

17(1), 5-14, 2013

Characterization of Brain and Liver Aromatase of Gilthead Seabream (*Sparus aurata* L., 1758) using Dibenzylfluorescein

Demet HANÇER AYDEMİR*[1], Alaattin ŞEN[2]

[1]Süleyman Demirel Üniversitesi, Isparta Sağlık Hizmetleri Meslek Yüksekokulu, Tıbbi Hizmetler ve Teknikler Bölümü, 32200, Isparta

[2]Pamukkale Üniversitesi, Fen Edebiyat Fakültesi, Biyoloji Bölümü, 20070, Denizli

(Alınış Tarihi: 10.12.2012, Kabul Tarihi: 01.04.2013)

Key Words
Aromatase
Gilthead seabream
Brain
Liver
Dibenzylfluorescein.

Abstract: Aromatase catalyzes the conversion of androgens into estrogens. Data on native aromatase enzyme kinetics and thus actual catalytic activity are scarce in fish, impeding comparison of aromatase activity (AA) from different organs within and between species. In the present study, fluorescence aromatase assay was optimized to measure AA in the gilthead seabream (*Sparus aurata* L., 1758) using dibenzylfluorescein (DBF) as a fluorometric substrate in brain and liver microsomes. Brain and liver AA have showed linearity until 20 and 40 µg protein concentration throughout 30 minutes, respectively. In brain and liver optimum pH of the enzyme was found to be 6.50 and 8.25, respectively and optimum temperature was found to be 30°C for both tissues. It has been observed that brain and liver AA have saturated at and above 2 µM DBF concentrations. Determined Vmax and Km values of brain and liver aromatase using Lineweaver-Burk graph have calculated 8,054±0,550 and 8,389±0,543 pmol/min/mg protein and 0,840±0,161 µM and 0,959±0,152 µM, respectively. Testosterone appeared to competitively inhibit the *Sparus aurata* brain and liver aromatase. In conclusion, the parameters of this assay that are reported for brain and liver aromatase in gilthead seabream could be useful to measure AA in other species.

Çipura (*Sparus aurata* L., 1758) Beyin ve Karaciğer Aromataz Enziminin Dibenzilfloresein Kullanılarak Karakterize Edilmesi

Anahtar Kelimeler
Aromataz
Çipura
Beyin
Karaciğer
Dibenzilfloresein.

Özet: Aromataz, androjenlerin ösrojenlere dönüşümünü katalizlemektedir. Doğal aromatazın katalitik aktivitesi ve enzim kinetikleri hakkında bilgi balıklarda çok azdır ve bu tür içi ve türler arasında farklı organlardan elde edilen aromataz aktivitesinin (AA) karşılaştırılmasını engellemektedir. Bu çalışmada, floresans aromataz metodu, florometrik bir substrat olan dibenzilfloresein (DBF) kullanılarak çipura (*Sparus aurata* L., 1758) beyin ve karaciğer mikrozomlarında AA'ni ölçmek için karakterize edilmiştir. Beyin ve karaciğer AA 30 dak reaksiyon zamanı boyunca sırasıyla 20 µg ve 40 µg protein miktarına kadar doğrusallık göstermiştir. Beyin ve karaciğerde enzimin optimum pH'sı sırasıyla 6,50 ve 8,25 olarak ve optimum sıcaklığı her iki doku için 30 ˚C olarak bulunmuştur. Beyin ve karaciğer AA'inin 2 µM DBF konsantrasyonu üzerinde doyuma ulaştığı gözlenmiştir. Lineweaver-Burk grafiği kullanılarak yapılan saptamalarda beyin ve karaciğer aromataz enzimi için V_{max} ve K_m değerleri belirlemişi ve sırasıyla 8,054±0,550 pmol/dak/mg protein, 8,389±0,543 pmol/dak/mg protein ve 0,840±0,161 µM, 0,959±0,152 µM olarak hesaplanmıştır. Testosteronoun *Sparus aurata* beyin ve karaciğer aromataz enzimini yarışmalı bir şekilde inhibe ettiği gözlenmiştir. Çipurada beyin ve karaciğer aromatazı için ilk defa rapor edilmiş bu metodun parametreleri diğer türlerde de AA'ni ölçmek için faydalı olabilecektir.

1. Introduction

Aromatase is a key enzyme in steroidogenesis and plays an important role in sexual differentiation, fertility, neuronal differentiation during development, neuronal function in the differentiated organism and carcinogenesis (Weisz, 1982). The *cyp19* encodes the P450 aromatase, a heme-binding protein of the enzyme complex that converts androgens into estrogens by aromatizing the A-ring of the C19 sex steroid. The enzyme complex is composed of the cytochrome P450 and the

* İlgili yazar: hancerdemet@gmail.com

flavoprotein NADPH-cytochrome P450 reductase. The reaction takes place in the endoplasmic reticulum. The *cyp19* gene that encodes aromatase was first cloned in human (Evans et al., 1986). Since then, full coding sequences of the aromatase *cyp19* gene were obtained from 11 other mammalian species (goat, horse, cow, sheep, pig, marmoset, macaque, mouse, rat, rabbit and white-sided dolphin) (Wilson et al., 2005). Most of the higher vertebrates possess a single *cyp19* gene (Simpson et al., 1994), excluding pig, in which multiple tissue-specific aromatase isoforms encoded by different *cyp19*genes are present (Choi et al., 1997). The control of the tissue-specific expression of *cyp19* in human has been extensively studied and shown to depend on the use of an alternative splicing of promoter and untranslated exon I, resulting in the generation of transcript variants with different tissue-specific 50-UTRs, but identical coding sequences (Harada et al., 1993). In contrast, two different *cyp19* genes are found in many teleosts, including channel catfish *Ictalurus punctatus* (Trant, 1994), japanese medaka *Oryzias latipes* (Fukada et al., 1996), nile tilapia *Oreochromis niloticus* (Kwon et al., 2001), goldfish *Carassius auratus* (Gelinas et al., 1998), japanese flounder *Paralichthys olivaceus* (Kitano et al., 1999), zebrafish *Danio rerio* (Kishida and Callard, 2001), eastern mosquitofish *Gambusia holbrooki* (Orlando et al., 2002), sea bass *Dicentrarchus labrax* (Blazquez and Piferrer, 2004), rubble goby *Trimma okinawae* (Kobayashi et al., 2004), black porgy *Acanthopagrus schlegeli* (Liu et al., 2004), wrasse *Halichoeres tenuispines* (Choi et al., 2005), killifish *Fundulus heteroclitus* (Greytak et al., 2005), pejerrey *Odontesthes bonariensis* (Strobl-Mazzulla et al., 2005), and mangrove rivulus *Rivulus marmoratus* (Lee et al., 2006). These genes encode two structurally and functionally different aromatase isoforms: CYP19A (CYP19A1) and CYP19B (CYP19A2), preferentially expressed in the ovary and the brain, respectively. The heme- and the steroid- binding sites are highly conserved among different species. However, the percentage of overall identity of the two aromatase sequences in a given species is around 60%, indicating that they have a long history as independent genes (Kishida and Callard, 2001). Ovarian P450arom expression (CYP19A) and subsequent P450arom activity is responsible for endogenous estradiol-17b (E2) biosynthesis in the gonads and thereby mediates the process of ovarian differentiation in several fishes (e.g., Kitano et al., 1999; Luckenbach et al., 2005). Previous studies also suggest that suppression of P450arom gene expression, P450arom activity, and E2 biosynthesis may be necessary for testicular differentiation (e.g., Kitano et al., 1999; Kwon et al., 2001; Luckenbach et al., 2005). Teleost fish are characterized by exceptionally high levels of aromatase activity per unit of protein in brain, 100–1000 times greater than in mammals, and have the ability to synthesize important amounts of estrogens. The biological significance for the elevated aromatase levels and thus the high production of neural estrogens in teleosts is still an unresolved issue although several hypothesis, including neuroprotection and neurogenesis have been proposed (Blazquez and Piferrer, 2004).

The gonads and the brain are the main sites of aromatase expression in most vertebrate species studied to date. However, in mammals aromatase is also found in a variety of tissues such as the liver, the kidney, the digestive tract, adipose and vascular tissues, in bone, skin, and the placenta (Harada et al., 1999). It has long been thought that in teleost fish CYP19A is only expressed in the ovary, while CYP19B can be found only in the brain (Kishida and Callard, 2001). Aromatase CYP19 activity in the brain of vertebrates is found most often in the forebrain areas related, among other functions, to the control of reproduction and sexual behavior (Balthazart and Ball, 1998). The sexually dimorphic pattern of cellular distribution of aromatase expression and/or activity is observed in mammals (Lephart, 1996) as well as in some teleost fish where males display higher activity than females in brain areas related to reproduction (Melo and Ramsdell, 2001).

Estrogens play an important role in breast and prostate cancers development (Ziegler, 2004). Natural and synthetic chemicals, including certain xenoestrogens, phytoestrogens, imidazole pesticides and organotin compounds, are able to inhibit AA on the protein level, both in mammals (Kellis and Vickery, 1984) and in fish (Noaksson et al., 2003). Phytoestrogens are estrogen-like molecules that have a diphenolic but nonsteroidal structure that are found in many plants, and are especially abundant in soy products. These estrogen mimics selectively bind estrogen receptors, are mild inhibitors of both the P450aro and 5α-reductase enzymes in peripheral tissues and appear to protect against age-related (i.e. cardiovascular disease and osteoporosis) and hormone-dependent cancers (i.e. breast and prostate cancers) (Adlercreutz et al., 1994).

In vitro assay methods have been developed to measure aromatase activity that are determined by measurement of the amount of ^{3}H-water released upon enzymatic conversion of RI-labeled androstendione (RI-method). However, these assays require the use of radioactive materials and specialized equipment for radiometric measurement. In the current study, we developed a novel nonradioactive aromatase assay. Therefore, the objective of this study was first to set up and validate the fluorometric assay to catalytically characterize *Sparus aurata* (*S. aurata*) aromatase enzyme activity. This assay was then used to measure AA under a variety of experimental paradigms in other species of teleosts. This is the first detailed comparison of the

catalytic properties of the brain and liver aromatase isozymes in *S. aurata*.

2. Material and Methods

Chemicals

Bovine serum albumin (BSA; A7906), Dibenzylfluoresein (D7191), fluorescein (32615), β-nicotinamide adenine dinucleotide phosphate (β-NADPH; N–7505), folin phenole reagent (F–9252), glycine (G–7126), glycerol (15524), calcium chloride–2-hydrade (12022), ethylene diamine tetra acetic acid disodium salt (EDTA; E–5134), phenil methane sulfonile fluoride (PMSF; P- 7626), sodium potassium tartarate (S–2377), tris (T–1378), N,N,N',N'-tetramethylene diamine (TEMED; T–8133) were purchased from Sigma Chemical Company (Saint Louis, Missouri, USA). Sodium hydroxide (06203) was purchased from Fluka Chemie (GmbH Industrie Strasse 25 CH–9471 Buchs/Switzerland). Potassium monohydrogen phosphate (1.05101), potassium dihydrogen phosphate (1.04873), triton X–100 (11869) were purchased from E. Merck (Darmstadt, Germany). All the other chemicals were of analytical grade and were obtained from commercial sources at the highest grade of purity available.

Fish

The fish used in this study, gilthead seabream (*S. aurata*) were obtained from the fish farm of Pinar Sea Products, Ildir, Izmir, Turkey. The fish used in this research weighed 250 ± 50 g and were about 24 months old. Fish were sacrificed by decapitation and tissues were dissected and transported to the laboratory in liquid nitrogene where they were used immediately or stored at -80 ºC until analysis.

Data analysis

All experiments were repeated at least three times. Three replicate determinations were used for each datapoint within each experiment. Values were expressed as mean ± standard error of the mean (SEM).

Assays

Microsomal fractions were prepared according to the method of Shenkman and Cinti (1978) as optimized by Schenkman and Cinti (1978). Proteins were determined by the Lowry method (Lowry et al., 1951) using BSA as standard. Brain and liver AA were determined using fluorescein as standard. Aromatase activity is quantified by measuring the fluorescent intensity of fluorescein, the hydrolysis product of dibenzylfluorescein (DBF) by aromatase. For measuring brain and liver AA, the reaction mixture consisting of 100 mM phosphate buffer (pH 6.5 for brain, and pH 8.25 for liver), 0,2 mM NADPH, 20 µg microsomal protein for brain and 40 µg microsomal protein for liver was added into a tube and preincubated at 30 ºC for 5 minutes. Then 50 µl of 20 µM DBF added into the tube and incubated at 30 ºC for 30 minutes with shaking. And then 600 µl of 1 N NaOH added into the tube and vortex and this mixture was then centrifuge at 12000 *xg* for 15 minutes and the supernatant was transferred to a new tube and incubated at 37 ºC for 2 hours. The mixture was read at Cary Eclipse (Varian Ltd., 28 Manor Road, Walton-on-Thames, Surrey KT12 2Qf, England) fluorometer (Ex= 485 nm, Em= 512 nm). AA was expressed as pmol of fluorescein per mg of protein per minute.

Statistical analysis

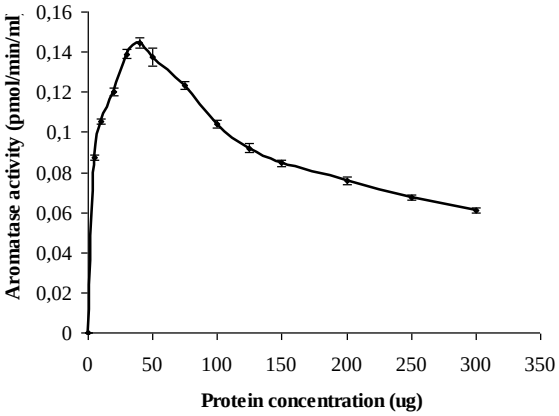
All experiments were carried out in triplicates. The samples collected from each replicate were tested for AA. Means of AA was calculated and significant differences were calculated by determining standard error.

3. Results

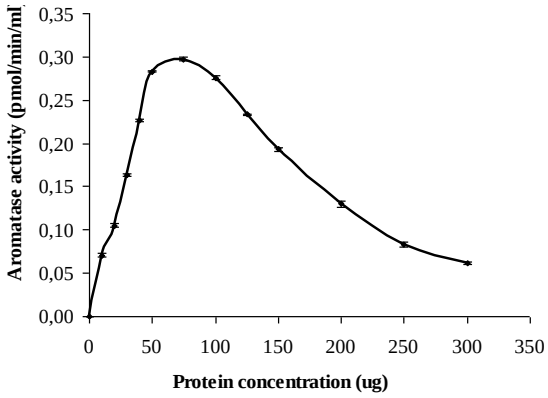
Preliminary experiments were conducted in order to estimate roughly the range of values of some key assay variables such as amount of tissue, pH, incubation temperature, substrate concentration, and incubation time. The results below reflect the order in which the experiments were conducted.

Effects of amount of tissue, pH of buffer, consantration of substrate, temperature of incubation, time of incubation, and testosterone

Variations in any of these six variables resulted in substantial changes in AA. Brain and liver AA was determined at different amounts of tissue (5, 10, 20, 30, 40, 50, 75, 100, 125, 150, 200, 250, and 300 µg) as shown in the Figure 1. (a) and (b). There is a basal fluorescein which we could not measure, so that brain AA didn't increase in a lineer fashion with protein concentration but partial lineerity increased up to 20 µg. Liver AA increased in a lineer fashion with protein concentration up to 40 µg.



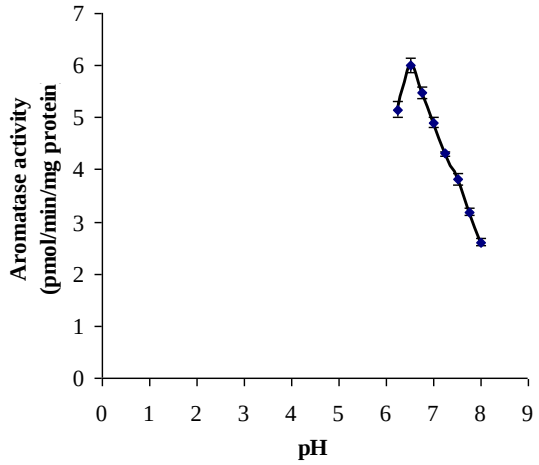
(a)



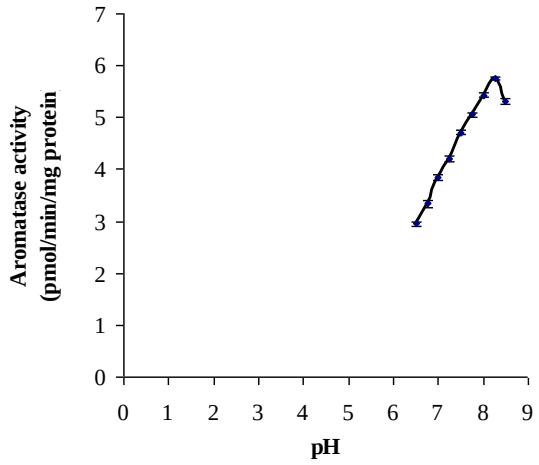
(b)

Figure 1. (a) Effect of protein concentration on brain AA of gilthead seabream, (b) effect of protein concentration on liver AA of gilthead seabream

Brain and liver AA was determined at different pH values (6.25, 6.50, 6.75, 7.00, 7.25, 7.50, 7.75, 8.00, 8.25, and 8.50) as shown in the Figure 2. (a) and (b). Maximum AA in brain and liver was obtained with pH 6.50 and 8.25, respectively.



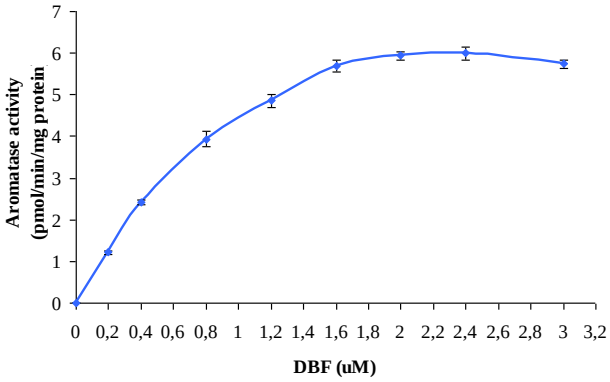
(a)



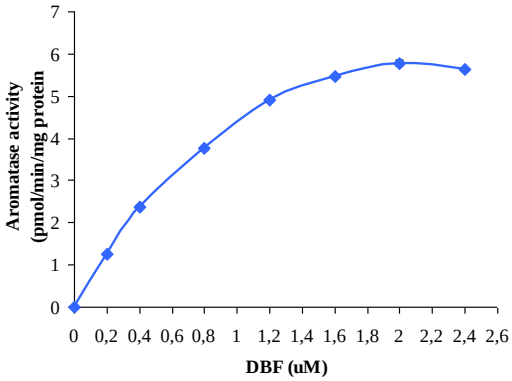
(b)

Figure 2. (a) Effect of pH on brain AA of gilthead seabream, (b) effect of pH on liver AA of gilthead seabream

Once assay conditions (pH, and amount of tissue) were set, the effect of substrate concentration (0.2, 0.4, 0.8, 1.2, 1.6, 2, and 2.4 µM) was studied in order to ensure saturating amounts available for the enzyme. Brain and liver AA became saturated with substrate concentrations at 2 µM and above as shown in the Figure 3. (a) and (b).



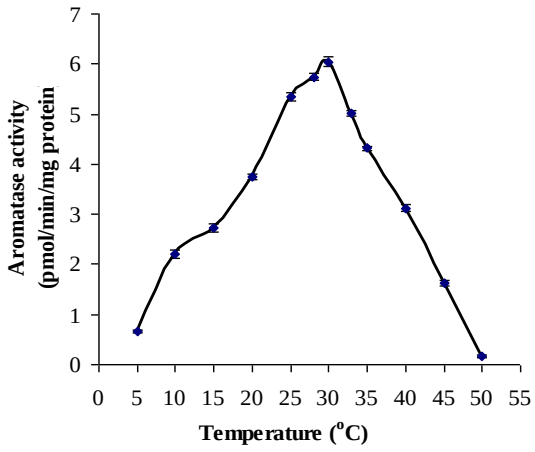
(a)



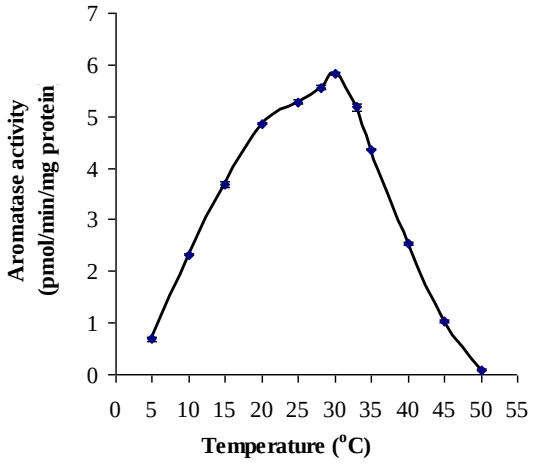
(b)

Figure 3. (a) Effect of substrat (DBF) on gilthead seabream brain AA, (b) effect of substrat (DBF) on gilthead seabream liver AA

Brain and liver AA was determined at different temperature degrees (5 ºC, 10 ºC, 15 ºC, 20 ºC, 25 ºC, 28 ºC, 30 ºC, 33 ºC, 35 ºC, 40 ºC, 45 ºC, and 50 ºC). Maximum AA both in brain and liver was obtained with incubation temperature of 30 ºC as shown in the Figure 4. (a) and (b).



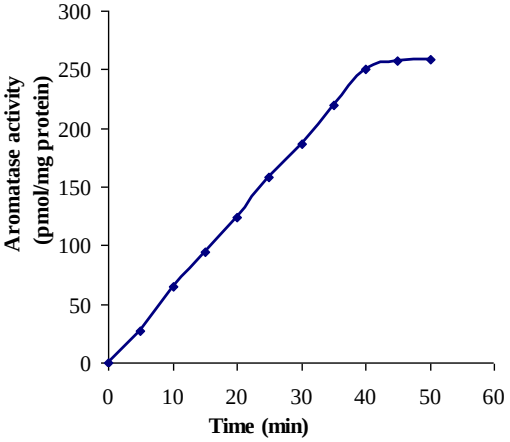
(a)



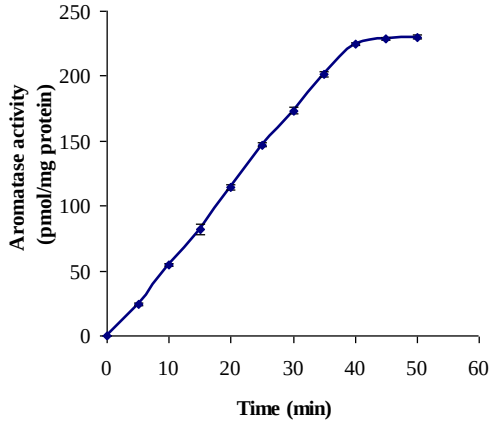
(b)

Figure 4. (a) Effect of temperature on brain AA of gilthead seabream, (b) effect of temperature on liver AA of gilthead seabream

Brain and liver AA was determined at different time courses (5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 min). With incubations up to 30 min, the brain and liver AA was time-dependent in a linear fashion as shown in the Figure 5. (a) and (b).



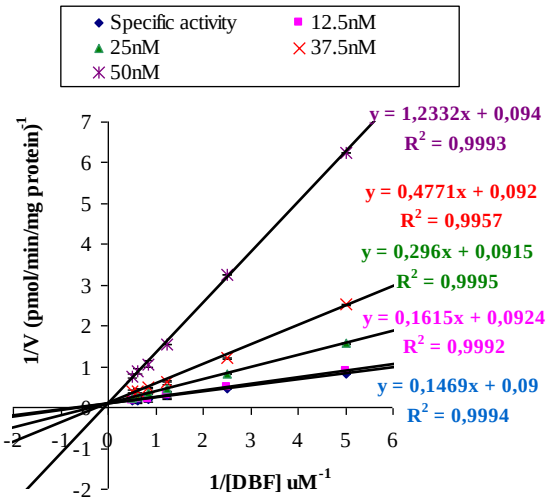
(a)



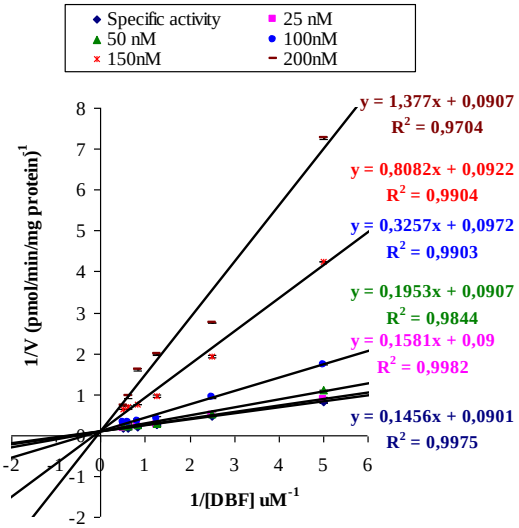
(b)

Figure 5. (a) Effect of time on brain AA of gilthead seabream, (b) effect of time on liver AA of gilthead seabream

In the presence of increasing concentrations of testosterone, the *Km* increased while the *Vmax* (maximal velocity of enzyme-catalyzed reaction) remained unchanged. Thus, testosterone appeared to competitively inhibit the fish brain and liver aromatase as shown in Figure 6. (a) and (b).



(a)



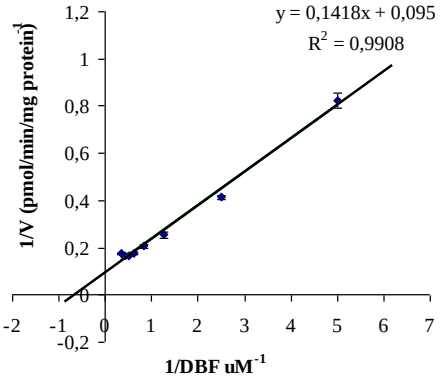
(b)

Figure 6. (a) Effect of testosterone on gilthead seabream brain AA, (b) effect of testosterone on gilthead seabream liver AA

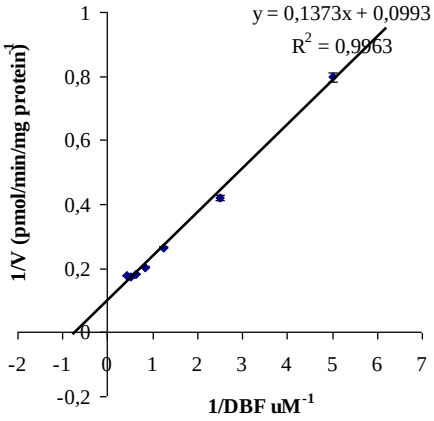
Application of the aromatase assay

The optimized assay was used to determine *S. aurata* aromatase affinity for DBF (*Km*) and its maximum reaction rate (*Vmax*) in both brain and liver microsomes. The brain *Km* (0,840±0,161 µM) and the liver *Km* (0,959±0,152 µM) were quite similar, demonstrating that *S. aurata* aromatase has similar affinity for DBF in both tissues. *Km* denoting a slightly lower affinity for DBF of the brain and liver aromatase. The *Vmax* values of *S. aurata* brain and

liver aromatase were 8,054±0,550 and 8,389±0,543 pmol/min/mg protein, respectively. These were determined by using Lineweaver-Burk plots shown in Figure 7. (a) and (b).



(a)



(b)

Figure 7. (a) Lineweaver- Burk plots of S. aurata brain aromatase, (b) Lineweaver- Burk plots of S. aurata liver aromatase

Tissue distribution

To investigate the overall distribution of AA in the *S. aurata*, several tissues, representative of different embryonic origins, were homogenized and assayed. These included the whole brain, liver, heart, spleen, testis and portions of the kidney, large intestine and small intestine. Background (obtained by incubating the substrate without tissue) is used for negative control. After dissection, the homogenization and incubation conditions were the same for all tissues. Results (Figure 8.) showed that AA per milligram of protein was detected, from higher to lower amounts, in the large intestine (6,465±0,033 pmol/min/mg protein), brain (6,017±0,102 pmol/min/mg protein), liver (5,734±0,039 pmol/min/mg protein), small intestine (5,081±0,053 pmol/min/mg protein), heart (3,425±0,057 pmol/min/mg protein), kidney (3,184±0,049 pmol/min/mg protein), spleen (2,918±0,044 pmol/min/mg protein) and testis (1,590±0,037 pmol/min/mg protein) as shown in Figure 8.

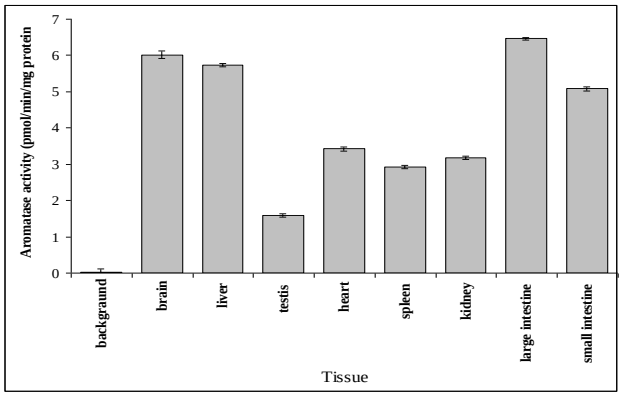


Figure 8. Tissue distribution of AA in the S. aurata

4. Discussion

In this study, fluorometric aromatase assay is successfully used and assay conditions are characterized for gilthead seabream brain and liver microsomes. In addition, AA was measured in different tissues of the gilthead seabream. Optimized assay conditions for brain and liver aromatase were: 20 µg and 40 µg protein; a pH of 6.50 and 8.25, respectively. Incubation temperature (30 ºC), incubation time (30 min) and substrate concentration (2 µM DBF) were found to be same for both brain and liver aromatase. There is a basal fluorescein which we could not measure, so that brain AA didn't increase in a lineer fashion with protein concentration but partial lineerity increased up to 20 µg. Liver AA increased in a lineer fashion with protein concentration up to 40 µg**.** In the tritiated water assay, at all concentrations of microsomal proteins tested (0.1, 0.2 and 0.5 mg), aromatase activities in brain and ovaries of rainbow trout (*Oncorhynchus mykiss*) were linear for up to 30 min. Based on these results, 200 µg of microsomal proteins and 30 min of incubation time were chosen as standard procedure for the subsequent measurements on brain and ovarian aromatase activities. In brain and ovarian microsomes, aromatase activity was maximal at 75 nM of radiolabelled androstenedione and above. This concentration was chosen to ensure that the substrate was not limiting (Hinfray et al., 2006). The tritiated water assay for the sea bass (*Dicentrarchus labrax*) was carried out using the following conditions: incubation time 30 min, and incubation temperature 30 ºC (Gonzales and Piferrer 2002). To our knowledge, this study is the first to report *Vmax* and *Km* values for both the brain and liver P450 aromatase in gilthead seabream. The brain *Km* (0,840±0,161 µM) and the liver *Km* (0,959±0,152 µM) were quite similar, demonstrating that *S. aurata* aromatase has similar affinity for DBF in both tissues. The *Vmax* values of *S. aurata* brain and liver aromatase were 8,054±0,550 and 8,389±0,543 pmol/min/mg protein, respectively.

In *S. aurata* AA was found highest in the large intestine (6,465 pmol/min/mg protein), followed by the brain (6,017 pmol/min/mg protein), liver (5,734

pmol/min/mg protein), small intestine (5,081 pmol/min/mg protein) and was detectable in heart (3,425 pmol/min/mg protein), kidney (3,184 pmol/min/mg protein), spleen (2,918 pmol/min/mg protein) and testis (1,590 pmol/min/mg protein). On the other hand, in southern flounder (*Paralichthys lethostigma*) P450arom mRNA was shown to be most abundant in the ovary and spleen and detected at much lower levels in the brain, testis, gill, and liver and was not detected in muscle, heart, intestine, or kidney (Luckenbach et al., 2005). In several species of birds, AA was found in brain and liver but not in testis, muscle and adrenal gland (Silverin et al., 2000). In the turtle *Chrysemys picta*, AA was maximum in brain, detectable in the gonads but negative in muscle, liver, and adipose tissue (Callard et al., 1977). In fish, data on the tissue distribution of aromatase are very scarce and limited to just two species. In the longhorn sculpin, *Myoxocephalus octodecemspinosus*, the brain and ovary exhibited high AA but other tissues tested, including liver, kidney, heart, muscle, skin, and gills, were negative (Callard et al., 1981). In the goldfish, *Carassius auratus*, brain AA per unit protein was ten times higher than in the ovary and a hundred times higher than in the mammalian ovary (Pasmanik and Callard, 1988). Furthermore, data on brain AA in relation to development or during the annual reproductive cycle are very scarce in fish. In two species of perch (*Perca fluviatilis* and *Rutilus rutilus*), Noaksson et al. (2001) reported that brain AA in sexually immature females was significantly lower (~0.5 pmol/mg prot/h) than that measured in sexually mature females (1.7–2.5 pmol/mg prot/h). In the very few previous studies carried out with teleosts, the liver was found to be negative for AA and expression (Callard et al., 1981; Kwon et al., 2001). In contrast, the presence of hepatic aromatase is well documented in mammals and has been found in both mature (Gonzalez, 1992) and immature rats (Katagiri et al., 1998). The liver is known for its high abundance of P450 enzymes in all vertebrates, but the precise function of aromatase in this organ in egg-laying vertebrates which is an established target for estrogen action, is not clear. The positive AA observed in the head kidney is consistent with the steroidogenic capacity of this organ in fish 0,959±0,152 (Borg, 1994). Similarly, the negative AA observed in the testis is consistent with male teleost having low or negligible levels of plasma estradiol (Borg, 1994), and with previous findings regarding aromatase mRNA expression (Kwon et al., 2001). However, locally-synthesized estrogens in the testes of lower vertebrates has been implicated, as is the case of mammals, in the autocrine or paracrine regulation of certain stages of spermatogenesis (Callard, 1992). In adult tissues of southern flounder, P450arom mRNA was most abundant in the ovary and spleen. P450arom mRNA was detected at much lower levels in the brain, testis, gill, and liver and was not detected in muscle, heart, intestine, or kidney (Luckenbach et al., 2005). One particularly interesting discovery in southern flounder was the relatively high levels of P450arom mRNA present in the spleen (Luckenbach et al., 2005). P450arom expression in the spleen has been previously reported in hirame (Kitano et al., 1999), nile tilapia (Kwon et al., 2001), a goby (*Trimma okinawae*, Kobayashi et al., 2004), and in the human fetus (Price et al., 1992). These findings suggest that the spleen may produce estrogens via actions of P450arom. However, the role of P450arom expression and any potential sexual dimorphism in spleen function remains unclear.

The effect of testosterone on aromatase catalyzed DBF was also investigated. In the presence of increasing concentrations of testosterone, the *Km* increased while the *Vmax* (maximal velocity of enzyme-catalyzed reaction) remained unchanged. Thus, testosterone appeared to competitively inhibit the fish brain and liver aromatase. The ovary-derived P450 aromatase (CYP19A) shares 68–72% sequence identity with ovarian aromatases of other fish species, but only 62% identity with the homologous brain-derived P450 aromatase (CYP19B) (Tchoudakova and Callard, 1998).

Aromatases CYP19A and CYP19B are crucial for regulation of estrogen levels in the fish during various stages of the life cycle. *cyp19* genes are key players in sexual differentiation and reproduction in teleosts. Aromatase CYP19B is also suggested to have a role in neuronal development and neuroprotection, as well as in regulation of male reproductive behavior. In addition, detection of aromatase CYP19 in tissues other than the major expression sites (brain and ovary) points to potential existence of other functions (Cheshenko et al., 2008).

Estrogens play an important role in breast cancer development. Aromatase inhibiting compounds like certain phytoestrogens, xenoestrogens and organotins are potent modulators of the activity of the aromatase enzyme. The phytoestrogen studies will help to determine which fruits and vegetables (those containing the appropriate phytoestrogens) should be included in the diet of postmenopausal women in order to reduce the incidence for breast cancer by inhibiting estrogen biosynthesis in breast tissue. For this purpose, our laboratory has been interested in the identification of Turkish traditional dietary plants that can effect AA. The effect of a variety of Turkish traditional dietary herbs on AA could be determined and findings could lead us to identify the diets that could be a potential dietary source for novel aromatase inhibitors.

The optimized aromatase activity assay with gilthead seabream microsomes developed in this study is a suitable test for screening for aromatase inhibiting activities of environmental chemicals or phytoestrogens. The assay is reproducible, and

inexpensive. In addition, the parameters of this assay that are reported here could be useful to measure AA in other species. Further studies are required to resolve whether observations can be generalized to other vertebrates.

Funding support

This work was funded by Pamukkale University Department of Scientific Research Projects, (Project No: 2006FBE002) Turkey.

Findings from this study were presented as a poster at the 32nd FEBS congress, July 7-12, 2007, Vienna, Austria.

References

Adlercreutz, H., Bannwart, C., Wahala, K., Makela, T., Brunow, G., Hase, T., Arosemena, P.J., Kellis Jr., J.T., Vickery, L.E. (1994). Inhibition of human aromatase by mammalian lignans and isoflavonoid phytoestrogens. The Journal of Steroid Biochemistry and Molecular Biology, 44, 147–153.

Balthazart, J., Ball, G.F. (1998). New insights into the regulation and function of brain estrogen synthase (aromatase). Trends in Neurosciences, 21, 243–249.

Blazquez, M., Piferrer, F. (2004). Cloning, sequence analysis, tissue distribution, and sex-specific expression of the neural form of P450 aromatase in juvenile sea bass (*Dicentrarchus labrax*). Molecular and Cellular Endocrinology, 219, 83–94.

Borg, B. (1994). Androgens in teleost fishes. Comparative Biochemistry and Physiology, 109, 219–245.

Callard, G.V., Petro, Z., Ryan, K.J. (1977). Identification of aromatase in the reptilian brain. Endocrinology, 100, 1214–1218.

Callard, G.V., Petro, Z., Ryan, K.J., Clairbone, J.B. (1981). Estrogen synthesis *in vitro* and *in vivo* in the brain of a marine teleost (Myoxocephalus). General and Comparative Endocrinology, 43, 243–255.

Callard, G.V. (1992). Autocrine and paracrine role of steroids during spermatogenesis: studies in *Squalus acanthias* and *Necturus maculosus*. Journal of Experimental Zoology, 261, 132–142.

Cheshenko, K., Pakdel, F., Segner, H., Kah, O., Eggen, R.I.L. (2008). Interference of endocrine disrupting chemicals with aromatase CYP19 expression or activity, and consequences for reproduction of teleost fish. General and Comparative Endocrinology, 155, 31-62.

Choi, I., Troyer, D.L., Cornwell, D.L., Kirby-Dobbels, K.R., Collante, W.R., Simmen, F.A. (1997). Closely related genes encode developmental and tissue isoforms of porcine cytochrome P450 aromatase. DNA and Cell Biology, 16, 769–777.

Choi, J.Y., Park, J.G., Jeong, H.B., Lee, Y.D., Takemura, A., Kim, S.J. (2005). Molecular cloning of cytochrome P450 aromatases in the protogynous wrasse, *Halichoeres tenuispinis*. Comparative Biochemistry and Physiology, 141, 49–59.

Evans, C.T., Ledesma, D.B., Schulz, T.Z., Simpson, E.R., Mendelson, C.R. (1986). Isolation and characterization of a complementary DNA specific for human aromatase-system cytochrome P-450 mRNA. Proceedings of National Academy of Sciences, 83, 6387–6391.

Fukada, S., Tanaka, M., Matsuyama, M., Kobayashi, D., Nagahama, Y. (1996). Isolation, characterization, and expression of cDNAs encoding the medaka (*Oryzias latipes*) ovarian follicle cytochrome P-450 aromatase. Molecular Reproduction and Development, 45, 285–290.

Gelinas, D., Pitoc, G.A., Callard, G.V. (1998). Isolation of a goldfish brain cytochrome P450 aromatase cDNA: mRNA expression during the seasonal cycle and after steroid treatment. Molecular and Cellular Endocrinology, 138, 81–93.

Gonzalez, F.J. (1992). Control of constitutively-expressed developmentally- activated rat hepatic cytochrome P450 genes. The Keio Journal of Medicine, 41, 68–75.

González, A., Piferrer, F. (2002). Characterization of aromatase activity in the sea bass: effects of temperature and different catalytic properties of brain and ovarian homogenates and microsomes. Journal of Experimental Zoology, 293, 500–510.

Greytak, S.R., Champlin, D., Callard, G.V. (2005). Isolation and characterization of two cytochrome P450 aromatase forms in killifish (*Fundulus heteroclitus*): differential expression in fish from polluted and unpolluted environments. Aquatic Toxicology, 71, 371–389.

Harada, N., Utsumi, T., Takagi, Y. (1993). Tissue-specific expression of the human aromatase cytochrome-P-450 gene by alternative use of multiple exons-1 and promoters, and switching of tissue-specific exons-1 in carcinogenesis. Proceedings of National Academy of Sciences, 90, 11312–11316.

Harada, N., Sasano, H., Murakami, H., Ohkuma, T., Nagura, H., Takagi, Y. (1999). Localized expression of aromatase in human vascular tissues. Circulation Research, 84, 1285–1291.

Hinfray, N., Porcher, J.M., Brion, F. (2006). Inhibition of rainbow trout (Oncorhynchus mykiss) P450 aromatase activities in brain and ovarian microsomes by various environmental substances. Comparative Biochemistry Physiology-Part C Toxicology and Pharmacology, 144, 252-262.

Katagiri, M., Tatsuta, K., Imaoka, S., Funae, Y., Honma, K., Matsuo, N., Yokoi, H., Ishimura, K., Ishibashi, F., Kagawa, N. (1998). Evidence that immature rat liver is capable of participating in steroidogenesis by expressing 17a-hydroxylase/17,20-lyase P450cl7. Journal of Steroid Biochemistry and Molecular Biology, 64, 121–128.

Kellis, J.T.J., Vickery, L. E. (1984). Inhibition of human estrogen synthetase (aromatase) by flavones. Science, 225, 1032–1034.

Kishida, M., Callard, G.V. (2001). Distinct cytochrome P450 aromatase isoforms in zebrafish (*Danio rerio*) brain and ovary are differentially programmed and estrogen regulated during early development. Endocrinology, 142, 740–750.

Kitano, T., Takamune, K., Kobayashi, T., Nagahama, Y., Abe, S.I. (1999). Suppression of P450 aromatase gene expression in sex-reversed males produced by rearing genetically female larvae at a high water temperature during a period of sex differentiation in the Japanese flounder (*Paralichthys olivaceus*). Journal of Molecular Endocrinology, 23, 167–176.

Kobayashi, Y., Kobayashi, T., Nakamura, M., Sunobe, T., Morrey, C. E., Suzuki, N., Nagahama, Y. (2004). Characterization of two types of cytochrome P450 aromatase in the serial-sex changing gobiid fish, *Trimma okinawae*. Zoological Science, 21, 417–425.

Kwon, J.Y., McAndrew, B.J., Penman, D.J. (2001). Cloning of brain aromatase gene and expression of brain and ovarian aromatase genes during sexual differentiation in genetic male and female Nile tilapia *Oreochromis niloticus*. Molecular Reproduction and Development, 59, 359–370.

Lee, Y.-M., Seo, J.S., Kim, I.-C., Yoon, Y.-D., Lee, J.-S. (2006). Endocrine disrupting chemicals (bisphenol A, 4-nonylphenol, 4-tert-octylphenol) modulate expression of two distinct cytochrome P450 aromatase genes differently in gender types of the hermaphroditic fish *Rivulus marmoratus*. Biochemical and Biophysical Research Communications, 345, 894–903.

Lephart, E.D. (1996). A review of brain aromatase cytochrome P450. Brain Research Reviews, 22, 1–26.

Liu, X., Liang, B., Zhang, S. (2004). Sequence and expression of cytochrome P450 aromatase and FTZ-F1 genes in the protandrous black porgy (*Acanthopagrus schlegeli*). General and Comparative Endocrinology, 138, 247–254.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L., Randal, R.J. (1951). Protein Measurement with the Folin Phenol Reagent. The Journal of Biological Chemistry, 193, 265-275.

Luckenbach, J.A., Early, L.W., Rowe, A.H., Boski, R.J., Daniels, H.V., Godwin, J. (2005). Aromatase cytochrome P450: Cloning, intron variation, and ontogeny of gene expression in Southern flounder (*Paralichthys lethostigma*). Journal of Experimental Zoology, 303, 643-656.

Melo, A.C., Ramsdell, J.S. (2001). Sexual dimorphism of brain aromatase activity in medaka: Induction of a female phenotype by estradiol. Environmental Health Perspectives, 109, 257–264.

Noaksson, E., Tjarnlund, U., Bosveld, A. T., Balk, L. (2001). Evidence for endocrine disruption in perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*) in a remote Swedish Lake in the vicinity of a public refuse dump. Toxicology and Applied Pharmacology, 174, 160–176.

Noaksson, E., Linderoth, M., Bosveld, A.T.C., Balk, L. (2003). Altered steroid metabolism in several teleost species exposed to endocrine disrupting substances in refuse dump leachate. General and Comparative Endocrinology, 134, 273–284.

Orlando, E.F., Davis, W.P., Guillette, L.J.J. (2002). Aromatase activity in the ovary and brain of the eastern mosquitofish (*Gambusia holbrooki*) exposed to paper mill effluent. Environmental Health Perspectives, 110, 429–433.

Pasmanik, M., Callard, G.V. (1988). A high abundance androgen receptor in goldfish brain: characteristics and seasonal changes. Endocrinology, 123, 1162–1171.

Price, T., Aitken, J., Simpson, E.R. (1992). Relative expression of aromatase cytochrome P450 in human fetal tissues as determined by competitive polymerase chain reaction amplification. The Journal of Clinical Endocrinology and Metabolism, 74, 879–883.

Schenkman, J.B., Cinti, D.L. (1978). Preparation of microsomes with calsium. Methods in Enzimology, 52, 83-89.

Silverin, B., Baillien, M., Foidart, A., Balthazart, J. (2000). Distribution of aromatase activity in the brain and peripheral tissues of passerine and nonpasserine

avian species. General and Comparative Endocrinolology, 117, 34–53.

Simpson, E.R., Mahendroo, M.S., Means, G.D., Kilgore, M.W., Hinshelwood, M.M., Grahamlorence, S., Amarneh, B., Ito, Y.J., Fisher, C.R., Michael, M.D., Mendelson, C.R., Bulun, S.E. (1994). Aromatase cytochrome-P450, the enzyme responsible for estrogen biosynthesis. Endocrinology Reviews, 15, 342–355.

Strobl-Mazzulla, P.H., Moncaut, N.P., Lopez, G.C., Miranda, L.A., Canario, A.V.M., Somoza, G.M. (2005). Brain aromatase from pejerrey fish (*Odontesthes bonariensis*): cDNA cloning, tissue expression, and immunohistochemical localization. General and Comparative Endocrinolology, 143, 21–32.

Tchoudakova, A., Callard, G.V. (1998). Identification of multiple cyp19 genes encoding different cytochrome P450 aromatase isozymes in brain and ovary. Endocrinology, 139, 2179–2189.

Trant, J. (1994). Isolation and characterization of the cDNA encoding the channel catfish (*Ictalurus punctatus*) form of cytochrome P450 arom. General and Comparative Endocrinolology, 95, 155–168.

Weisz, J. (1982). In vitro assays of aromatase and their role in studies of estrogen formation in target tissues. Cancer Research, 42, 8, 3295-3298.

Wilson, J.Y., McArthur, A.G., Stegeman, J.J. (2005). Characterization of a cetacean aromatase (CYP19) and the phylogeny and functional conservation of vertebrate aromatase. General and Comparative Endocrinolology, 140, 74–83.

Ziegler, R.G. (2004). Phytoestrogens and breast cancer. The American Journal of Clinical Nutrition, 79, 183–184.