Environmental Toxicology and Pharmacology 40 (2015) 300–309

Contents lists available at ScienceDirect

Environmental Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/etap

Sequence analysis of novel CYP4 transcripts from *Mytilus galloprovincialis*

Sanda Ravlić[a,*], Jurica Žučko[b], Mirta Smodlaka Tanković[a], Maja Fafanđel[a], Nevenka Bihari[a]

[a] Center for Marine Research, Ruđer Bošković Institute, Giordano Paliaga 5, 52210 Rovinj, Croatia
[b] Faculty of Food Technology and Biotechnology, University of Zagreb, Pierottijeva 6, 10000 Zagreb, Croatia

ARTICLE INFO

Article history:
Received 14 March 2015
Received in revised form 29 May 2015
Accepted 2 June 2015
Available online 19 June 2015

Keywords:
Cytochrome P450
cDNA cloning
Phylogenetic analysis
Environment
Bivalvia

ABSTRACT

Cytochrome P450 enzymes (CYPs) are essential components of cellular detoxification system. We identified and characterized seven new cytochrome P450 gene transcript clusters in the populations of bivalve mollusc *Mytilus galloprovincialis* from three different locations. The phylogenetic analysis identified all transcripts as clusters within the CYP4 branch. Identified clusters, each comprising a number of transcript variants, were designated CYP4Y1, Y2, Y3, Y4, Y5, Y6 and Y7. Transcript clusters CYP4Y2 and Y7, and CYP4Y5 and Y6 showed site specificity, while the transcript clusters CYP4Y1, Y3 and Y4 were present at all investigated locations. The comparison of transcripts deduced amino acid sequences with CYP4s from vertebrate and invertebrate species showed high conservation of the residues and domains essential to the putative function of the enzyme, as terminal ω-hydroxylation and prostaglandin hydroxylation. Our results suggest the great expansion of the CYP4Y cDNAs indicative of CYP4 proteins in the mussel *M. galloprovincialis* presumably as a response to different environmental conditions.

1. Introduction

The cytochrome P450 family (CYPs) is by number and function the largest and most diverse protein superfamily found in nature (Estabrook, 2003). CYP enzymes use molecular oxygen to modify numerous substrates involved in a huge number of physiological, ecological and toxicological processes. CYPs substrates include exogenous compounds such as polycyclic aromatic hydrocarbons (PAH), pesticides and plant allelochemicals, as well as endogenous compounds such as steroids, fatty acids and eicosanoids (Feyereisen, 1999; James and Boyle, 1998; Whalen et al., 2010).

The presence of CYPs in all living beings ranging from bacteria to plants and animals is implying the existence of a common ancestral gene, which has undergone consecutive gene duplications and subsequent divergence evolution resulting in the formation of a supergene family (Nebert and Gonzalez, 1987; Nelson and Strobel, 1987). Based on phylogenetics and according to sequence identity, CYP genes are classified into clans, families and subfamilies (Nelson et al., 1996). To date, 12 456 CYPs are named with about 6000 more that are known, but not yet named, all belonging to over 1000 families that have been identified in all three domains of life. In animals alone, 11 distinct clades have been described, encompassing 4088 named sequences placed in 156 CYP gene families. Vertebrate CYPs are distributed in 19 families and a growing number of subfamilies, e.g. 57 genes in humans and 102 genes in mouse (Nelson, 2011; Nelson et al., 2004). Increasing genomic information available for invertebrates already data – mined for their P450s, like sea anemone *Nematostella vectensis* (Goldstone, 2008), purple sea urchin *Strongylocentrotus purpurea* (Goldstone et al., 2006), owl limpet *Lottia gigantea* (Gotoh, 2012), points to a greater diversity of invertebrates CYPs than the one observed in vertebrates.

The induction of CYP1A transcripts, protein or enzyme activity has long been employed as a biomarker of exposure of fish and other vertebrates to anthropogenic contaminants in aquatic environments. A number of studies have searched for CYP responses in bivalve molluscs similar to those found in vertebrates. Results interpretation of the studies on molluscs have been complicated by cross – reactivity of antibodies to vertebrate CYP1As with non – P450 molluscan proteins, or low enzymatic activity with known CYP substrates (Livingstone, 1991; Livingstone et al., 2000; Peters et al., 1998; Shaw et al., 2004; Snyder, 2000). While evidence of deuterostome (e.g. tunicate and sea urchin) CYP1A – like proteins and CYP1A – like genes exist (Goldstone et al., 2007), to date no full length CYP1 – like sequences have been reported for molluscs (Rewitz et al., 2006). However, results of the insects CYP4 genes studies indicated their involvement in some forms of insecticide

* Corresponding author.
E-mail address: sravlic@irb.hr (S. Ravlić).

http://dx.doi.org/10.1016/j.etap.2015.06.005
1382-6689/

resistance and their induction by alkaloids and phenobarbital suggests that CYP4 isoform of cytochrome P450 is responsible for the metabolism of xenobiotics in invertebrates (Danielson et al., 1998).

The CYP4 family is considered one of the most ancient CYP families, having evolved from the steroid synthesizing CYPs and since diverged into an array of subfamilies and genes encoding enzymes acting on diverse substrates (Simpson, 1997). In vertebrates, CYP4 genes are predominantly fatty acid ω-hydroxylases, engaged in preventing lipotoxicity by hydroxylating eicosanoids, prostaglandins and leukotrienes (Hardwick, 2008). Although little is known about the functions of the CYP4 family in bivalves, complete CYP4 cDNA have been identified in *Chlamys farreri* (Miao et al., 2011), *Venerupis philippinarum* (Pan et al., 2011), whereas partial sequences have been cloned in *Unio tumidus* (Chaty et al., 2004), *Mytilus galloprovincialis* (Snyder, 1998) and *Perna viridis* (Zhou Chi, 2010).

There is a need for new knowledge of CYP genes and their function in bivalves, partly to understand CYP gene evolution in this old invertebrate class, and partly for the reason that these animals posses biological features excellent for pollution monitoring. Bivalve molluscs are sessile filter feeders and have been known to accumulate foreign organic chemicals (Bihari et al., 2007; Stegeman and Teal, 1973) and metals (Peric et al., 2012), and have been employed extensively as sentinels in monitoring programs, e.g., the Mussel Watch Program in the USA and MEDPOL programs in Europe.

In the present study we described novel cDNA sequences coding for CYP4 proteins in the mussel *M. galloprovincialis*. Furthermore, we investigated whether the environmental conditions as well as seasonal changes influence the presence of CYP4 transcript variants and their distribution. The identification and characterization of novel CYP4 transcript variants would contribute to our current knowledge regarding the metabolic detoxification metabolism in bivalve molluscs, while the analysis of mussels expressed CYP4 genes distribution could become useful in programs for detection of environmental pressures on a local, regional or even worldwide scale.

2. Materials and methods

2.1. Animals collection

Mussels *M. galloprovincialis* Lamarc 1891 (Mollusca: Bivalvia) average mass (10 ± 2 g) and length (4 ± 1 cm) were collected from natural populations at three different locations in Northern Adriatic Sea (Fig. 1). 10 mussel specimens were collected from each sampling site every second month, from September 2012 until May 2013. Sampling site Budava (uncontaminated sampling site) is protected area known for mariculture production. Sampling site Pula is in the inner part of the Pula harbor, in the close vicinity of highly urbanized area and industrial facilities, and is influenced by industrial and/or urban runoff from a shipyard and urban waste. Sampling site Dina is in the close vicinity of the Organic Petrochemical industry, and according to Croatian National Institute of Public Health no impact of the industry was determined on the quality of seawater and sediment ("Report on monitoring the impacts of the DINA Petrokemija d.d. Omišalj on the environment in 2008"; in Croatian). Mussels were transported in seawater tanks to the laboratory and the digestive glands were dissected within 1 h following collection, than immediately processed further.

2.2. RNA extraction and cDNA synthesis

Total RNA was isolated from 100 mg of individual mussel digestive gland tissue using TRI Reagent® Solution (Ambion, Austin, USA) according to the manufacturer's protocol. RNA quantity, purity and integrity were verified by both native RNA electrophoresis on 1% agarose gel in 1X TAE (Tris–acetate–EDTA, pH 8.6) buffer, and the UV absorbance ratios (A_{260}/A_{280}, A_{260}/A_{230}) were quantified using the NanoPhotometer™ Pearl (Implen GmbH, München, Germany). First strand cDNA was reverse transcribed from 2 μg of total RNA using the anchored-oligo $(dT)_{18}$ and random hexamer primers according to the Roche protocol for Transcriptor First Strand cDNA synthesis Kit (Roche, Basel, Switzerland).

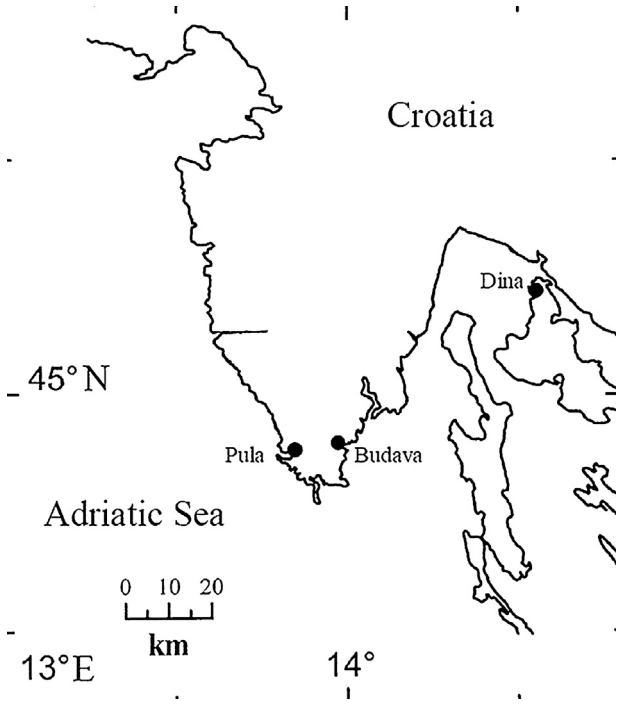

Fig. 1. *Mytilus galloprovincialis* sampling sites in the Northern Adriatic Sea, Croatia. Black circles indicate the locations where animals were collected.

2.3. Rapid amplification of cDNA ends (RACE)

In order to obtain a full length CYP4 cDNA, 5′ and 3′ RACE-PCRs (polymerase chain reaction) were carried out on the total RNA using the GeneRacer™ Kit (Invitrogen, Waltham, USA) according to manufacturer's protocol. Gene specific primers (Frace, Rrace; Table 1) were designed based on the partial sequence of the *M. galloprovincialis* CYP4Y1 (AF072855.1). For 3′ RACE of CYP4, amplification of first strand cDNA was conducted using the Frace, forward gene-specific primer (Table 1), and the GeneRacer™ 3′ Primer. Amplification conditions for touchdown PCR were the following: 2 min denaturation at 94 °C, 10 cycles of 94 °C for 30 s, 65 °C for 30 s, 72 °C for 1 min, 15 cycles of 94 °C for 30 s, 62 °C for 30 s, 72 °C for 1 min, 10 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min, followed by an additional extension at 72 °C for 10 min. The amplified

Table 1
Oligonucleotide primers used in this study.

Primer name	Primer sequence (5′–3′)
Frace	CAGAATATCAGAAAATGTGTCAGAATGAA
Rrace	GGAGACCATATATGTTGATACCGAAAA
R2	AAGAGTCCATCTTTGTAGCATTG
3-1	GTTCCTTTCTGCGTTTTGC
5-1	GGCGAACATAAGCTTTTTGTC
GeneRacer™ 3′Primer	GCTGTCAACGATACGCTACGTAACG
GeneRacer™ 5′Primer	CGACTGGAGCACGAGGACACTGA
pUC/M13 forward	CCCAGTCACGACGTTGTAAAACG
pUC/M13 reverse	AGCGGATAACAATTTCACACAGGAA