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Protein responses in blue mussels (*Mytilus edulis*) exposed to organic pollutants: A combined CYP-antibody/proteomic approach

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Abstract

Polyclonal antibodies were raised against highly conserved, trans-metazoan sequences of cytochrome P450 (CYP) families 2 and 4 and used to investigate responses in the common blue mussel (*Mytilus edulis*) exposed to various organic contaminants. The results were evaluated by means of cross-reacting proteins on Western blots of both one- and two-dimensional electrophoresis gels, and by scanning spectroscopy measurements of total CYP content. Furthermore, a proteomic approach was applied aimed at elucidating exposure-related protein changes in a more general term. Identities of isolated proteins were searched by means of peptide mass fingerprints obtained from MALDI-TOF MS analyses.

The results demonstrated that both antibodies rendered several cross-reactive bands when probed on Western blots. The most obvious cross-reaction of the CYP2 antibody was with a strongly expressed protein of size ≈57 kDa, p*I* 4.5–4.6, whereas the CYP4 antibody cross-reacted with a protein of size ≈55 kDa, p*I* 5.6. However, expression of cross-reacting proteins did not change as a result of the exposures, and resulted only in small and insignificant fluctuations in total CYP content. As a contrast, silver-stained 2DE gels showed that several microsomal proteins were affected in individuals exposed to diallylphthalate as well as crude oil, with and without a spike of alkylphenols and PAHs. Mass spectrometry based analyses of excised, trypsin-digested spots did so far not decipher the identities of the proteins affected by the exposures, nor of those cross-reacting with CYP2 and CYP4 antibodies.

This study has underlined the power of the proteomic approach in environmental toxicology, although protein identification was not successful. The missing identities of the proteins cross-reacting with the CYP2- and CYP4-antibodies does not enable a clear conclusion as to whether or not these peptides actually represent CYP iso-enzymes.

Keywords: Cytochrome P450; CYP2; CYP4; Mussel; Oil; Phthalates; Proteomics; Two-dimensional electrophoresis

1. Introduction

Cytochromes P450 enzymes (CYPs) form the terminal component of the microsomal mixed function mono-oxygenase system, which is involved in synthetic and catabolic reactions of both endobiotic and xenobiotic substrates (Simpson, 1997). CYPs are expressed in all life forms, including bacteria (Nelson, 1998). In animals, CYP expression tend to be highest in glandular tissues involved in the metabolism of ingested food particles and steroid hormones, represented by the liver in vertebrates (Omura and Sato, 1964), the hepatopancreas in crustaceans (James, 1989), and the digestive gland (DG) in bivalves (Livingstone and Farrar, 1984). Induction of CYP enzymes by xenobiotics often lead to perturbation of endogenous pathways, with associated pathological consequences (Waxman, 1999), e.g. induction of CYP1A in fish exposed to organic contaminants has received a lot of attention (reviewed in Goksøyr, 1995). Little is still known about the CYP system in bivalves, but two fully sequenced protostomes, the bacterivorous nematode *Caenorhabditis elegans*, and the herbivorous fruit fly *D. melanogaster* express 80 and 86 CYP iso-forms, respectively, and any complex animal is considered needing a battery of 50–80 different CYP genes to cope with endogenous and exogenous chemical challenges (Nelson, 1999).

Increases in total CYP content have been demonstrated in *Mytilus* sp. after exposure to organic contaminants (e.g. Livingstone et al., 1985; Michel et al., 1993). One CYP4 fragment, named CYP4Y1 by the CYP nomenclature committee, has been described in *Mytilus galloprovincialis*, with decreasing gene expression as a result of exposure to β-naphtoflavone

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(Snyder, 1998), and degraded oil (Snyder et al., 2001). Another fragment of a CYP4 species has been sequenced from the freshwater mussel *Unio tumidus*, but transcription of this gene was unaffected by exposures to PCBs and a phthalate (Chaty et al., 2004). Furthermore, the complete cDNA sequence of a unique family, CYP30, has been cloned from the clam *Mercenaria mercenaria* (Brown et al., 1998), and a short CYP2-like fragment was recently reported in the oyster *Crassostrea rhizophorae* (NCBI Acc. no. AAP60020). Besides in bivalves, the CYP4 family is confirmed in gastropods such as *Haliotis refescens* (Snyder, 1998), while full cDNA sequences of the unique families CYP320A1 in *Biomphalaria glabrata* (NCBI Acc. no. AAX73196), and CYP10 in *Lymnaea stagnalis* (NCBI Acc. no. AAB23599) render a total of seven described mollusc iso-forms from five different CYP families, among which only the CYP2 and the CYP4 families are also expressed in vertebrates.

The CYP4 family, whose iso-forms are involved in hydroxylation of fatty acids, is predicted to be represented in all animal phyla (Nelson, 1998). Expression of CYP4 iso-forms is generally little affected by exposure to xenobiotics, with the exception of so called peroxisome proliferators, including phthalates (Waxman, 1999). Peroxisomal proliferation has also been reported in bivalves, with elevated peroxisomal acyl-CoA oxidase activity and increased peroxisomal volume in blue mussels transplanted to a contaminated field site (Cajaraville et al., 2003), but unlike vertebrates, no link has yet been established between peroxisomal proliferation and CYP4-induction in mussels.

While CYP2 iso-enzymes of sub-classes 2C, 2D, and 2E are important targets for pharmaceutical drugs in mammals (Zuber et al., 2002), little is known about the functions of described CYP2 iso-forms in invertebrate phyla. In rodents, developmental and reproductive toxicity has been reported as a result of exposure to high doses of BPA (Reel et al., 1997), which has been indicated to be linked to inhibition of steroid-metabolising CYP iso-enzymes belonging to the CYP2- and CYP3-families (Hanioka et al., 1998, 2000).

Like PCBs, many congeners among the poly-brominated diphenyl ethers (PBDEs) are disrupters of the thyroid hormone homeostasis in mammals, the most potent congeners being tetra- and penta-BDEs (Hallgren et al., 2001). Several studies indicate that PBDEs have some potential to induce CYP1A in vertebrates (reviewed in Hakk and Letcher, 2003), although the results deviate (Boon et al., 2002). Furthermore, treating female rats with congener BDE-47 rendered mixed type induction (Hallgren and Darnerud, 2002).

The aim of the present study was to employ polyclonal CYP-antibodies made against sequences that are conserved in invertebrate phyla, to mussels exposed to organic contaminants that are known to affect CYP in vertebrates. Moreover, total CYP content was quantified to enable monitoring of induction events among the CYP system as a whole, while the proteomic-based

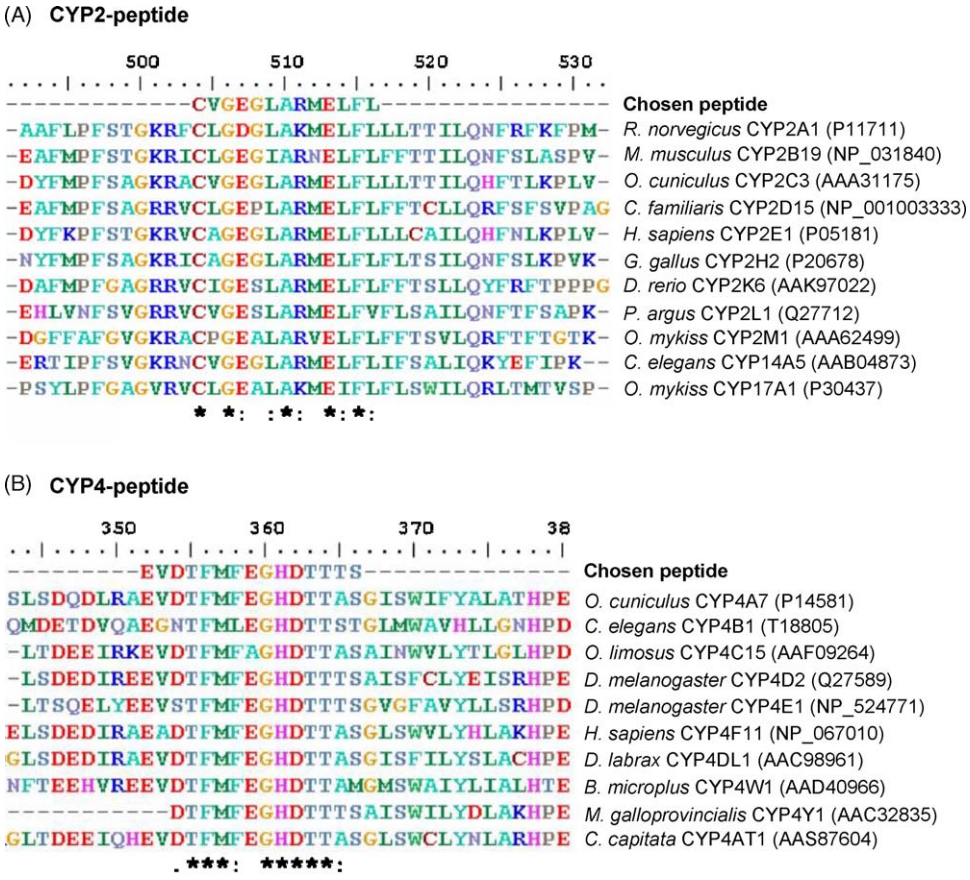

Fig. 1. Antigen sequences. ClustalX alignments displaying the conserved sequences within the CYP2 family, as well as within some other two-clan families (A), and the CYP4 family (B), that were used for immunisation of rabbits (NCBI Acc. nos. in parentheses).