Study of Ecdysone receptor and Ultraspiracle in the salmon louse (*Lepeophtheirus salmonis)*

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SELECTED ABBREVIATIONS

Abbreviation	Full Name
Amp	Ampicillin
AD	Activation domain
cDNA	Complementary DNA
DBD	DNA binding domain
DEAE	Diethylaminoethyl cellulose
E.coli	*Escherichia coli*
EcR	Ecdysone receptor
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
H	Hour
IEC	Ion Exchange Chromatography
IMAC	Immobilized Metal Ion Affinity Chromatography
IPTG	Isopropyl β-D-1-thiogalactopyranoside
ITC	Isothermal Titration Calorimetry
LB	Luria-Bertani
LBD	Ligand binding domain
LBP	Ligand binding pocket
Ls	*L. salmonis*
Min	Minute
ON	Overnight
ORF	Open reading frame
PCR	Polymerase Chain Reaction
Rpm	Revolutions per minute
RT	Room temperature
SAP	Shrimp Alkaline Phosphatase
Sec	Second
SEC	Size Exclusion Chromatography
SOB	Super Optimal Broth
SOC	Super Optimal Broth with Catabolite repression
TEV	Tobacco Etch Virus
USP	Ultraspiracle
Ω	Ohm (resistance)

ABSTRACT

The salmon louse (*Lepeophtheirus salmonis*) is a parasite living on mucus, skin and blood of salmonids fishes. *L. salmonis* causes lesions and infections on fish fins and skins and such physical damages often lead to other diseases. The global salmon farming industry faces huge economic losses caused by the prevalence of salmon lice and is struggling to contain frequent salmon lice outbreaks. Chemical treatments have been a traditional way to combat salmon lice problem, but increased resistance of salmon lice to currently available chemicals leave the salmon aquaculture communities with fewer options. Therefore, it is warranted to search for new, efficient and environment-friendly drugs which are based on molecular studies of nuclear receptors of salmon lice. Investigation of ecdysone receptor (EcR), which acts as a receptor for the ecdosteroid hormone, is one of such molecule-based new drug searches. The ecdosteroid hormone plays an important role during molting, maturation and reproduction processes of crustaceans. Ecdysteroid agonists for EcR that disrupt these processes could be novel pesticides to control salmon lice.

In this study, expression constructs of *L. salmonis* ecdysone receptor (EcR) and ultraspiracle (USP), which forms a heterodimer with EcR, were made and they were expressed in *E. coli.* The EcR constructs (both ligand-binding domain and full-length) were expressed well, but the full-length USP construct was not expressed. Immobilised metal ion affinity chromatography (IMAC) was used to purify EcR proteins. The two EcR proteins, i.e., ligand-binding domain (LBD) and full-length EcR, bound very poorly to the Ni-resin. The reason can be that the 6x His tag was buried inside of the MBP-attached EcR protein, thus it was not available to the Ni-resin. To circumvent this challenge, ion-exchange chromatography (IEC) was employed. At a very low salt concentration (6.7 mM NaCl), the EcR proteins were eluted as flowthrough, whereas much of impurities remained in the column, hence achieving substantial purification. As the last step of purification, size exclusion chromatography (SEC) was used. The proteins were eluted at near the void volume, suggesting they are in a form of aggregates under the experimental conditions. With partially purified EcR-LBD, a binding study between EcR-LBD using isothermal titration calorimetry (ITC) was attempted.

1 INTRODUCTION

1.1 Background

Currently most of commercially available salmon come from salmon farming, which is dominated by just a few countries including Norway and Canada, and total world-wide farmed salmonids production was around two million tonnes (HOG) in 2013 (Salmon Farming Industry Handbook, 2014). One of the major threats to salmon farming is the sea louse *Lepeophtheirus salmonis*, which belongs to marine copepods of Caligidae family (Johnson et al., 1991). They are natural ectoparasites and commonly found in farmed (but also in wild) salmonids. The presence of sea lice was first recorded in 17th century and zoologist Henrik Nikolai Krøye in 1837 first named them. With the introduction of cage farming system in 1970, the spread of sea lice has recently become a major threat to salmon farming with frequent and economically devastating outbreaks. For example, around £305 millions in 2006 alone were spent world-wide for sea lice treatment (Costello, 2009). Norway, which is a major aquaculture (especially salmon farming) country, bears significant loss due to sea lice, with direct economic loss of more than 500 million NOK (Institute of Marine Research, Norway-2013). The sea lice problem has exacerbated further recently. Recent estimation by Giskeodegard and Tonnessen shows that sea lice-related cost (mainly management and disease control) per kg of salmon in Norway has increased 4 NOK in last 4 years (Undercurrentnews, 2015).

However, despite that sea lice cause a major problem to farmed salmonids, the effective drugs against sea lice are very limited. Furthermore, excessive use of these drugs has rapidly increased the resistance against them among sea lice and reduced drugs' sensitivity. This obvious dilemma has led to a search for new approaches against sea lice. One is a molecular approach, which aims to find novel risk-free and environment-friendly drugs. The other is a biological approach using predators. Among fishes eating sea lice are lumpfish and wrasse while wrasse has recently become more popular among fish farmers. The aforementioned sea lice problems are not limited to farmed fish. In fact, wide infestation of sea lice in farmed salmon also affects wild salmonid population and causes ecological imbalance (Bjorn et al., 2001; Krkosek et al., 2013).

1.*2 Salmon louse (Lepeophtheirus salmonis)*

Salmon louse is the member of phylum Arthropoda, sub-phylum Crustacea, subclass Copepod, order Siphonostomatoida and family Caligidae. Salt-sensitive salmon lice use salmonids as their host and survive in high-salinity sea water (Hahnenkamp et al., 1985; Tucker et al., 2000). They have 8 stages (Fig 1.2.1) in their life cycle and each stage is separated by moulting (Hamre et al., 2013). The entry stage of life cycle begins when matured female release egg-strings. Matured female *L. salmonis* can release on average ten pairs of egg strings during their life cycle and the egg numbers per string can be one hundred to several hundred (Heuch et al., 2000). The life cycle begins when planktonic Naupli hatch from egg-strings. Naupli stage consists of 2 stages nauplius 1 and 2. Nuaplius 1 persist for 9 h to 52 h and duration of Nuaplius 2 is 170 h to 36 h. From nauplius they enter into infective copepodid stage and this stage persist for 2 to 14 days depending on the temperature. At this stage, salmon louse searches for the host and depends on the fat reservoir for survival. When they get the host, attach themselves on the fins of the fish or the scales and enter into chalimus stages. At chalimus stages (Chalimus stages 1 and 2), louse attach to the host with frontal filament and then followed by stage pre-adult 1 and 2. Genital development occurs during the pre-adult stage (Johnson and Albright, 1991; Schram, 1993). After pre-adult stage, they transform into adult. During pre-adult and adult stage louse can move freely on the host surface but more commonly found on the head and fins. The mean length of matured sea louse is around 6-7 mm and female is bit larger than male.

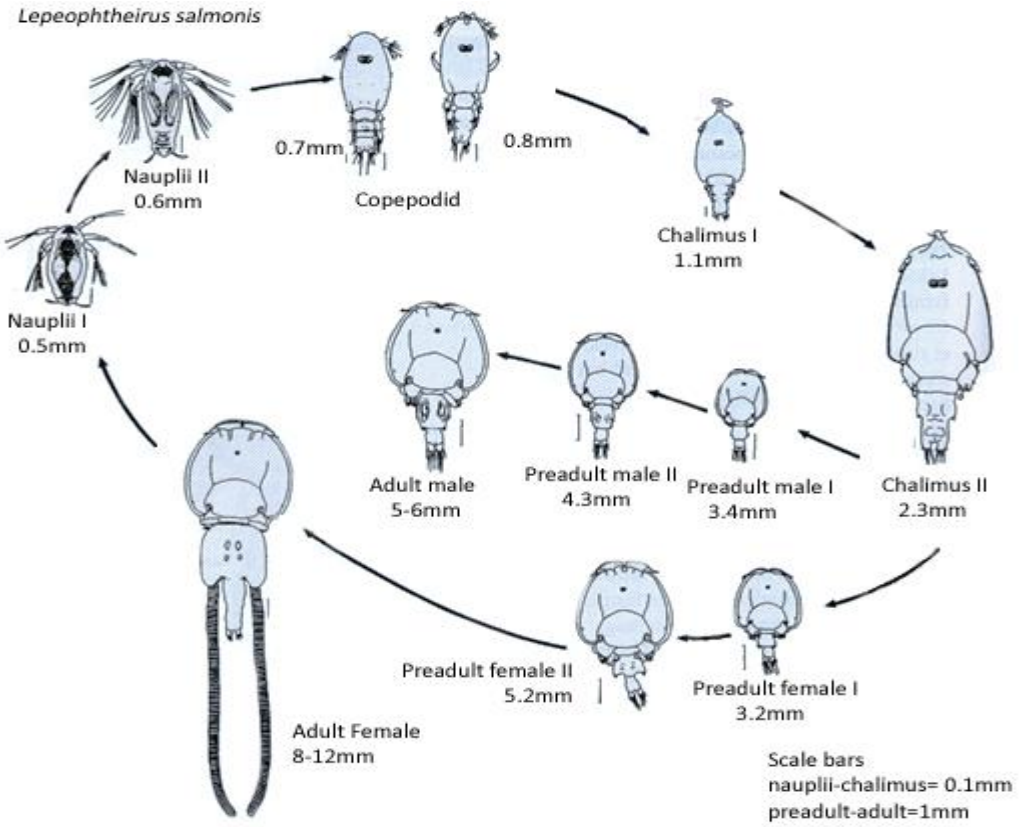


Figure 1.2.1 Salmon louse lifecycle. The approximate length is in millimetre (MM) and each transition state is indicated by arrow (Maran et al., 2013, originally taken from Schram, 1993).

1.3 Infection by salmon louse and host response

As previously mentioned, copepodid attach themselves with host via second antennae and first maxilla. Chalimus start to consume skin and mucus from the frontal filament region of the host. With the time, salmon louse becomes adult and start moving freely on surface of host using their maxilla and cephalothorax. Adult louse can also move to a new host especially when the host density is high. To get a host, louse use positional and chemical cues (Mordue and Birkett, 2009). During infectious stage, they normally cause skin erosion to the host and the damage depends on the level of infection (Johnson and Albright, 1991). If the infection level is high, skin erosion turn into large open wound and cranial bones become visible (Wootten et al., 1982). This large open wound often leads to pathway for other secondary pathogen like bacterial or fungal (Egidius, 1985). During pre-adult and adult stage when lice attached and feed on host, some clinical signs appear like Edema, hyperplasia, inflammation, damage of epidermal cell etc. (Jonsdottir et al., 1992).

At adult stage, for survival, lice consume mucus, skin tissue and blood of salmonids. During feeding they secret low molecular weight proteins and some other molecules like trypsin, prostaglandin E2 (PGE2) etc. Trypsin is digestive peptidases which serve for digestion of food and some cases to avoid immune response of host (Fast et al., 2005; Wagner et al., 2008). PGE2 inhibits interleukin-2 expression in the host which is a signalling molecule in the immune system. PGE2 may also acts as anti-hemostatic, anti-inflammatory (Riveiro et al., 1985; Aljamali et al., 2002).

Salmon louse infected host immediately responses to infection by changing mucus consistency, electrolyte balance, cortisol release, epithelium damage etc. As a result immune response decreases and make susceptible to other diseases. Physical activities of host like reproduction, homeostasis are also deeply affected in the host (Johnson and Albright, 1992; Ross et al., 2000). Some study has shown that salmon louse may also act as carrier to salmon for other infectious bacteria and virus like *Aeromonas salmonicida*, *Salmon anemia* virus etc (Nylund et al., 1993).

1.4 Chemical treatment

Chemical treatment to the infected fish is given either as bath treatment or medicated food. Chemicals that are delivered to the fish as bath treatment are known as pesticides and those that are delivered as medicated food known as drugs (Department of Fisheries and Oceans, 2013). Commonly used pesticides during bath treatment are organophosphates, pyrethroids, hydrogen peroxide, chitin synthesis inhibitors etc. In bath treatment all fish get exposed to the pesticides equally. Simultaneously non-target species can also be affected when these pesticides get release to the environment which is the important drawback of bath treatment. (Haya et al., 2005). In medicated food, drugs are delivered to the fish with food. Most widely used drugs are emamectin benzoate, benzoyl ureas, dichlorvos etc. As the drugs are given with food, some fish may have over dose of drugs due to consuming more food and other fishes may have under dose of drugs due to consuming less food (Grant, 2002; Norwegian Food Safety Authrority, 2013). Dependence on chemical treatments and excessive uses are reducing their sensitivity among sea lice.

1.5 Salmon louse management

To control salmon louse, integrated pest management programs have been recommended in several countries. Using of cleaner fish is a widely adopted biological control method to combat salmon louse infection. Cleaner fish develops symbiotic relationship with other fish where both partner become benefitted. In 1987, Asmund Bjordal first observed the cleaning of Atlantic salmon by wrasse (Costello and Bjordal, 1990). Wrasse is a small carnivore marine fish. They can efficiently eat and remove dead skins and ectoparasites from the surface of other fish. Sometimes they also feed on healthy tissue and mucus of symbiotic partner which brings health hazard for partner. During winter season, mortality rate of wrasse increases which makes problem for maintenance of them for next year use (Torrissen et al., 2013; Imsland et al., 2014).

Biological control is environmental friendly but it has maintenance problems and high economic cost. On contrast, chemical treatment is effective but resistance to chemical treatment is increasing alarmingly. That's why researchers are trying to develop new medicine against salmon louse specially inhibiting developmental process of insects. Some nuclear receptors play important regulatory role in sea lice development. Designing of drug targeting these specific nuclear receptors may open new era in controlling salmon louse.

1.6 Nuclear receptor

Nuclear receptors are transcription factors. When a ligand binds to the receptor, specific genes are expressed and regulate important physiological activities of organism like development, homeostasis, and metabolism etc. (Solt et al., 2011). For the nuclear receptor, ligands are steroid hormones, vitamin D, ecdysone, retinoic acids and thyroid hormones. According to recent studies, there are also some other ligands for nuclear receptor like fatty acids, oxysterols, farnesolmetabolites, leukotriene B4 and prostaglandin J2 (Forman et al., 1995; Kliewer et al., 1995; Devchand et al., 1996; Janowski et al., 1996; Serhan, 1996). A nuclear receptor can also be without any ligand which is known as orphan receptor. It is not yet confirmed by the researcher whether orphan receptors have undiscovered ligand or not (Moore, 1990; Laudet et al., 1992; O'Malley & Conneely, 1992; Enmark &Gustafsson, 1996). Depending on the structure, function and phylogenetic analysis, nuclear receptor superfamily has been classified into six subfamilies (Table 1.6.1). Receptors for known ligand are found in first 3 subfamilies, but orphan receptors can be found in any of the subfamilies. There is another subfamily which is different from the other six as this family member does not have

the common nuclear receptor structure in 4-5 functional domains (Laudet et al., 1992; Escriva et al., 1997; Laudet, 1997; Auwerx et al., 1999). Nuclear receptors are present in animals and absent in protists, algae and fungi (Escriva et al., 1998). Humans have 48 nuclear receptors (Zhang et al., 2004) while Nematode *C. elegans* contains large number of nuclear receptors which is around 270 (Bridgham et al., 2010).

Table 1.6.1 Nuclear receptor superfamily with selected members. Adopted from Table of Nuclear Receptors (NRs) - **http://nrresource.org/general_information/nrs.html**. **Cited 15.04.2015.**

NR Superfamily	**Subfamily**	**Name**	**NR Superfamily**	**Subfamily**	**Name**
1	A	TRα	3	A	ERa
	B	RAR			ERb
	C	PPAR		B	ERR
	D	HZF2		C	GR
	F	ROR			MR
	H	UR			PR
	I	VDR			AR
2	A	HNF4	4	A	NURR1
	B	RXR			NGFIB
	C	TR2	5	A	SF1
	E	TLL	6	A	RTR
	F	COUP-TFI	0	B	SHP

1.7 Structure and function of nuclear receptor

Nuclear receptors have 4-5 common structural domains (Fig 1.7.1) (Martín, 2010). The N-terminal region (A/B domain) is poorly conserved and contains activation function-1 (AF-1) domain. The AF-1 acts as ligand independent transcriptional activator. The DNA binding domain (C domain) is highly conserved which works to recognize specific sequence in promoter/enhancer regions of target gene. A short motif named P-box present in DBD gives the specificity of binding. Flexible domain D is present in between DBD and LBD domain and connects them together. Domain D has role in nuclear localization. The largest domain of

nuclear receptor is ligand binding domain (LBD, E) which sequence is comparatively less conserved but secondary structure is highly conserved. It contains ligand dependent activation function 2 (AF-2) which is a transcriptional activator. Some nuclear receptor may have highly variable F domain at the C-terminus of the E domain. Function of F domain has not yet clarified by the researcher (Mangelsdorf et al., 1995; Glass et al., 1997).

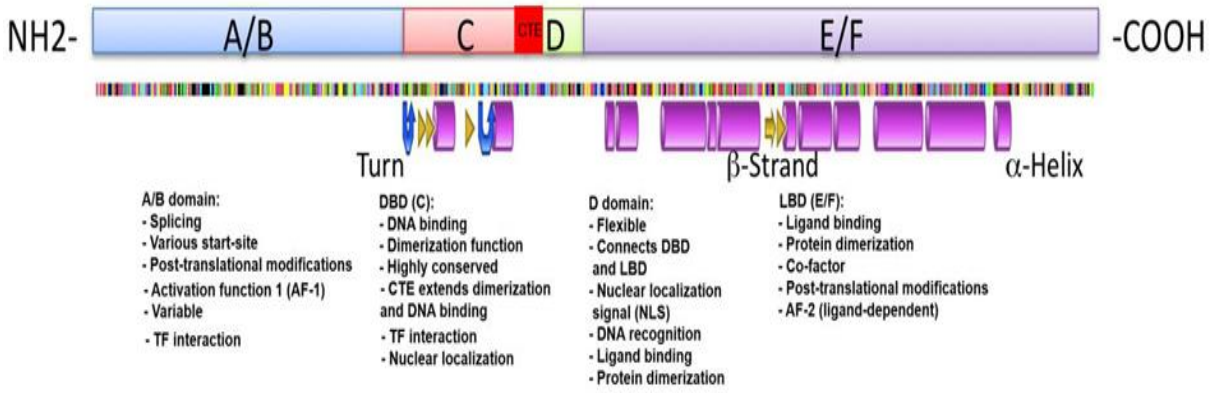


Figure1.7.1 General structure of nuclear receptor. Common domains of NR and their function with secondary structure of DBD(C) and LBD (E/F) are depicted. Adapted from Nuclear receptor resource: "Structure of NRs" - **http://nrresource.org/_Media/structure-of-nrs-2.png. Cited 02.04.2015.**

1.8 Ligand dependent activation of nuclear receptor

There are 12 α-helices (H1-H12) and a short two-stranded antiparallel β-sheet (S1 and S2) in crystal structures of retinoid receptor LBD (Fig 1.8.1). These α-helices and β-sheets are arranged in a three-layered sandwich like structure and a ligand-binding pocket (LBP) is present in the lower part of domain. When inducing ligand (ATRA; 9-cis retinoic acid) is bound to the LBP, helix H12 changes in the ligand-binding cavity and allows the recruitment of transcription coactivator (CoA). Nevertheless, if antagonists bound to the LBP, H12 become displaced. Then instead of transcription CoA, transcription corepressor (CoR) is recruited. Depending on the ligand biding, LBD maintains active or repressive state (Nagy and Schwabe, 2004; Bourguet et al., 2010).

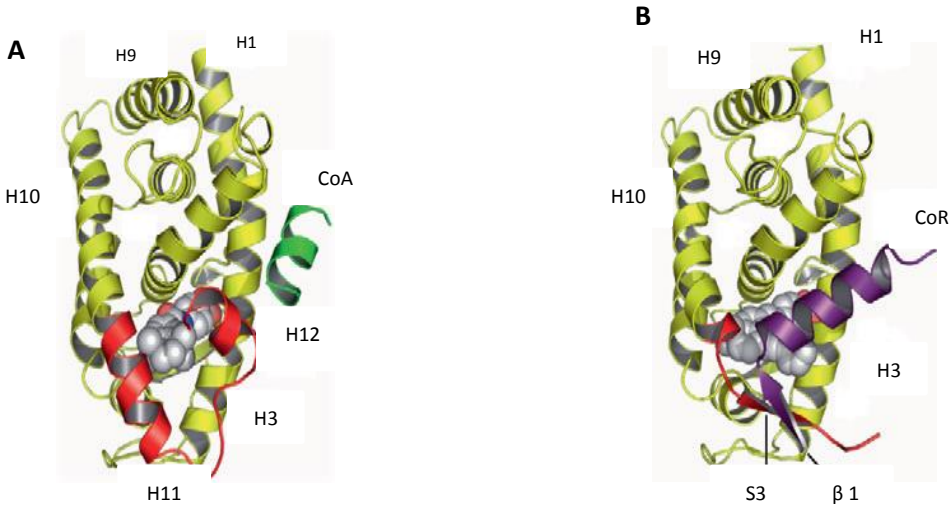
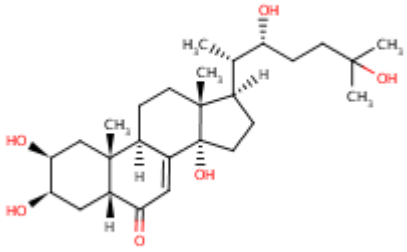


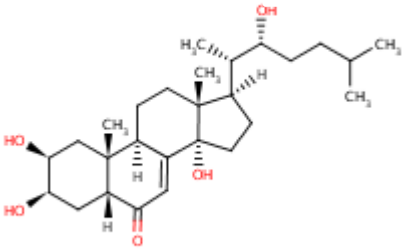
Figure1.8.1 Crystal structures of LBD (RAR α). (A) Ligand induces LBD and H12 allows incorporation of coactivator (CoA, green). **(B)** Antagonists displace H12 and allow the recruitment of corepressor (CoR, violet) (Adopted from Bourguet et al., 2010).

1.9 Ecdysone in arthropods

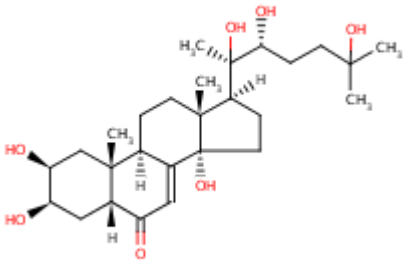
The ecdysone receptor present in arthropods is a member of nuclear receptor superfamily. It operates as a heterodimer protein of ecdysone receptor (EcR) protein and ultraspiracle protein The USP is homolog of the vertebrate retinoid X receptor. The ligand for ecdysone receptor is ecdysteroid which regulates moulting event in crustaceans and insects (Nakagawa et al., 2009). Ecdysone, 25-deoxyecdysone, 20-hydroxyecdysone (20-E) and Ponasterone A (25-deoxy-20-hydroxyecdysone, Pon A) are the most common hormones of this steroid (Figure 1.9.1.). A ligand binding pocket is formed within the LBD of EcR when EcR and USP heteromized. Several ligands such as 20-E, PonA can bind to this pocket (Billas et al., 2003; Carmichael et al., 2005; Browning et al., 2007; Iwema et al., 2007). Although ligands can directly bind to EcR receptor, binding is greatly enhanced by the addition of USP. In the presence of ligand, heterodimer EcR/USP complex become more stabilized and binding affinity for ecdysone response elements in the promoter region is also increased.



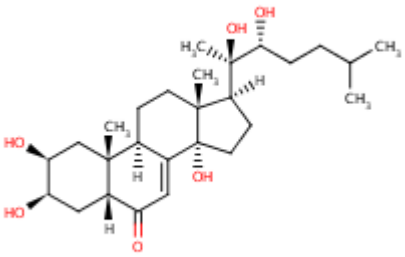
Ecdysone



25-deoxyecdysone



20-hydroxyecdysone (20E)



25-deoxy-20-hydroxyecdysone (PonA).

Figure 1.9.1 Chemical structure of four ecdysteroids (Ecdysone, 25-deoxyecdysone, 20-hydroxyecdysone & 25-deoxy-20-hydroxyecdysone). **Structure from ChEBI: http://www.ebi.ac.uk/chebi/init.do. Cited 13.04.2015.**

1.10 Secretion of ecdysteroids

In insects, ecdysteroid pathway begins with the secretion of prothoracicotropic hormone. This hormone makes ring gland to synthesize and release the steroid hormone ecdysone (E) (Gilbert et al., 2002). Then, cytochrome P-450 enzyme ecdysone-20-monooxygenase catalyses the conversion of E into biologically active metabolites Pon A and 20-E (Gilbert, 2004). Researchers also reported that ecdysteroids can also be produced in Y-organs by some crustaceans. Y-organs secrete ecdysteroids into peripheral tissues where these ecdysteroids

are also modified into active metabolites (Mykles, 2011). As Y-organs are not present in *L. salmonis,* it is assumed that hypodermis may act as main source of ecdysteroids (Hopkins, 2009)

1.11 Ecdysone pathway in arthropods

Ecdysteroids control target gene transcription by binding to EcR receptor which heterodimerizes to USP. In the absence of bound ecdysteroid, EcR/USP complex may also bind with hormone response elements (HREs) and repress the target gene expression by interacting with co-repressors (Hu et al., 2003). Ligand binding to receptor promotes the release of these co-repressors (Schubiger and Truman 2000; Tsai et al., 1999) and also contributes to the formation of binding site for coactivators in EcR/USP complex. Following ligand binding, EcR/USP complex binds to its HREs in a repeat sequence of reverse position containing a single intervening nucleotide. EcR/USP complex is placed in the promoter region of the ecdysteroid responsive genes by response element. Many of these ecdysteroid responsive genes represent transcription factors NRs which play important role in complex signalling pathway (Reviewed by King-Jones and Thummel, 2005).

1.12 Ecdysone receptors as targets for insecticides and pesticides

The ecdysteroid signalling pathway in arthropod species regulates important cellular events and also natural targets for pesticides. Researchers have found that some plants induce premature molting in insects by Pon A to protect themselves (Browning et al., 2007). Premature molting to insect leads to death and this principle has inspired to develop synthetic ecdysteroid molting accelerating compounds like tebufenozide, methoxyfenozide, chromafenozide and halofenozide to control various insect species (Dhadialla et al., 1998). The effects of synthetic ecdysteroid molting accelerating compounds on non-target arthropods are not clear yet (Kato et al., 2007). Study of the receptor system by cloning LBD of EcR, EcR and USP can help to develop more efficient and safe insecticides to control insects.

1.13 Protein expression

Advancement in recombinant DNA technology and cloning has made it easier to express and isolate the target protein. For different purpose like medicine and food, large amount of protein production is desired (Biotechnology learning hub, 2014). There are many expression systems to produce the target protein in large amount. Some of the most widely used expression systems are bacteria, yeast, insect or mammalian system. The choice of expression system depends on time to be spent for expression, required amount of protein, where expressed protein will be secreted, type of post-translational modifications, how easy to handle the expression system etc. Among different expression systems, gram-negative bacterium *Escherichia coli* (*E. coli*) is the most commonly used system for protein production. Bio-physical feature of *E. coli* allow themselves to adjust at different temperature and easy to manipulate genetically (Storz and Hengge-Aronis., 2000). *E. coli* is more suitable because it can be grown easily, low culture cost, and rapid biomass accumulation (Baneyx and Mujacic, 2004).

E. coli provides different strains to produce protein. Among different strains, BL21 is more popular because it causes less protein degradation during purification as it does not contains outer membrane (OmpT) proteases. Within BL21 strain, T7 RNA polymerase containing system like BL21(DE3) is more commonly used for protein production (Baneyx, 2004; Sanderson and Skelly, 2007). BL21(DE3) provides high-level expression of non-toxic recombinant protein from T7 promoter-based expression system. For toxic recombinant protein, BL21(DE3)pLysS is preferable as it has T7 lysozyme gene to reduce basal level expression and allow to produce more toxic protein (Studier, 1991). *E. coli* BL21(DE3) has T7 RNA polymerase controlled by *lac*UV5 promoter (Bashiri et al., 2015). In pET vector, target gene is controlled by T7 promoter. To express the target protein, expression of T7RNAP has to be induced which can be done using non-metabolisable lactose analog IPTG (Isopropyl β-D-1-thiogalactopyranoside). IPTG will turn on *lac* operon and then protein expression will be induced (Bashiri et al., 2015).

There are some major challenges of protein expression in bacterial expression system like over or very poor expression of protein, insoluble aggregation etc. Over expression gives the protein inactive, misfolded form and they accumulated through non-covalent hydrophobic or ionic interactions or a combination of both which is known as inclusion bodies. Inclusion

bodies can be solubilized using detergents and denaturants, like urea or guanidinium and then can be refold into the native and active conformation of the protein. Changing experimental conditions like temperature, cell strains, media condition or using the fusion partner, solubility of expressed protein can be improved (Mogk et al., 2002). In some cases, protein expressed as inclusion body is desired to obtain functional and active form of protein (Sorensen and Mortensen, 2005).

1.14 The pET expression system

Expression system carry the desired gene in a host and make thousands copy of that gene. For successful protein production, expression system needs a promoter compatible with host cell and ribosome binding site. Most commonly used expression system for protein production is T7 based pET expression system (Novagen, 2003). Number of commercially available different pET plasmids are around 40. These types of plasmids possess multiple cloning sites, promoters, protease cleavage sites, *lac*I gene which codes for the *lac* repressor protein, *lac* operator, an f1 origin of replication, antibiotic resistance gene etc. (Blaber, 1998).When target gene is incorporated into the vector and transformed into a host *E. coli* strain, T7 RNA polymerase starts to transcribe if *lac* operator is not repressed.

1.15 Fusion partner

In expression system, different fusion partner may be used with target gene. This fusion partner makes the purification and expression of recombinant proteins simpler. For rapid and efficient purification of proteins some commonly used fusion partners are His-tag (6-10 histidine), GST (glutathione-s-transferase-211 aa), MBP (Maltose binding protein-396 aa) etc. Due to small size and almost no effect on target protein, His-tag is more widely used as fusion partner for rapid purification (Carson et al., 2007). A His-tag is fused with desired protein either in N or C terminus. Although His-tag can be short or long, generally six histidine residues are widely used which provides optimal interaction with matrix.

To get soluble form of expressed protein in bacterial expression system is one of the major challenges. There are different fusion partners available with different characteristics to enhance the solubility of target protein. Some of the widely used fusion partner for improve solubility are MBP, NusA, GB1, Trx etc. MBP is a large fusion partner (43 kD), its efficiency to improve solubility is competitively higher than other tags. Although GB1 is a small in size

(56 residues) it is also a strong solubility enhancer (Dyson et al., 2004; Kataeva et al., 2005 and Zhou et al., 2010).

1.16 Position of fusion partner

The position of tag can be either at N or C terminus of a target protein. More than one tag can also be used for improved purification and solubility. Some tag can be used for both purification and solubility purpose like glutathione-s-transferase (GST) tag, maltose binding protein (MBP). There are some positive sides of placing the tag at the N terminus site. Protease can remove the tag form N terminus more efficiently and some solubility enhancing tags like MBP, Trx, and NusA are more efficient when they are at N terminus (Sachdev and Chirgwin, 1998).

1.17 Removal of the fusion partner

A small linker connects the fusion partner to the target gene. This linker contains recognition site for specific endoprotease enzyme. TEV (tobacco etch virus) protease is commonly used to remove the fusion partner from target protein due to its high specificity. Fusion partner can also be removed chemically using cyanogen bromide (CNBr), hydroxylamine etc. But chemical cleavage requires solvents and denaturing condition which is harmful that's why this type of cleavage is not highly preferable (Dobeli et al., 1998; Fairlie et al., 2002).

1.18 Protein purification

Isolation of desired protein from complex mixtures as pure protein by different techniques is known as protein purification. The degree of desired purity depends on where protein is going to be used. If protein is going to be used for medical or food purpose it has to be highly purified. There is no certain technique to purify protein. Depending on the protein properties like solubility, charge, size etc. purification techniques are selected.

Immobilized metal ion affinity chromatography (IMAC) is a widely used and reliable protein purification method which is based on the interaction between proteins and metal ions. The imidazole ring of histidin acts as electron donor and exhibits the strong interaction with metal ion (Co^{2+}, Ni^{2+}) on matrix. When protein solution is passed through the column, His-tag containing proteins are retained in column matrices which can be eluted later either changing pH or using high concentred imidazole to the column buffer (Porath, 1992; Cutler, 2004).

Depending on the reversible interaction between charged proteins and oppositely charged column materials, protein can be separated which is known as ion exchange chromatography. Protein samples are applied to an oppositely charged matrix in a column and then the proteins containing the opposite charge of matrix are bound to the matrix. The matrix of column can be either positively charged (anion exchange chromatography) or negatively charged (cation exchange chromatography). When the net charge of protein is negative then positively charged matrix is used in the column and if the net charge of protein is positive then negatively charged matrix is used. The net charge of protein is surrounding pH dependent. Bound proteins can be eluted by increasing the ionic strength or varying the pH of the elution buffer (Roe, 2001).

In a gel filtration column, proteins are separated based on their sizes. Column is packed with porous matrix and then protein sample are run on the column. Large molecule will be eluted quickly from the column but small molecule will be eluted later as the small molecule can diffuse into the porous matrix (Porath and Flodin, 1959; Cutler, 2004).

1.19 Study ligand analyte interaction by Isothermal Titration Calorimetry (ITC)

Isothermal titration calorimetry (ITC) is a technique that allows the direct measurement of the binding affinity, Gibbs free energy of binding, enthalpy and entropy of binding interaction between two molecules depending on the heat changes (Perozzo et al., 2004). When binding occurs between two molecules either heat is released to the surroundings or absorbed from the surroundings depending on the bond type. During protein-ligand interaction generally non-covalent interaction like hydrogen bonds and van der Waals occur (Freyer et al., 2008).

In an ITC machine, there are two cells- a reaction cell and a reference cell. The reaction cell contains sample solution (analyte) and the reference cell contains either water or buffer. When ligands are added from the injection syringe to the sample solution, interaction between ligand and analyte occurs which accompanied by heat changes. In constant temperature this heat change can be monitored through power compensation. Depending on the power compensation, signal will be generated by ITC instrument as peak. Generated data through power compensation are used to study the interaction between ligand and analyte using different binding models (Freyer et al., 2008; Duff et al., 2011).

Besides ligand- analyte interaction, there are some other non-specific sources of heat change during ITC experiments which are avoided by performing control experiment (Bronowska, 2011).

1.20 Aim of the study

The functional ecdysone receptor is formed by heterodimeraztion of EcR and USP and regulates several physiological processes. The ligand for this receptor are ecdosteroids for example, 20-hydroxyecdysone (20-E) and ponasterone A (25-deoxy-20-hydroxyecdysone, PonA) etc. Interaction patterns between ligand and *L. salmonis* ecdysone receptor are not resolved clearly yet. To better understand the mechanism of ligand binding and specifically to identify new possible ligands for ecdysone receptor from sea lice, the ecdysone receptor will be expressed, purified and used in ligand interaction assays. The aims of this project are:

1. Cloning of EcR-LBD, full length EcR (LBD+DBD) and full length USP (LBD+DBD).
2. Expression in suitable expression vector
3. Optimization of protein purification.
4. Interaction study of ligand e.g. Pon A with EcR-LBD and with heterodimer EcR/USP complex in ITC experiment.
5. Structure modelling and structure determination of EcR as an ultimate goal.

2. MATERIALS

2.1. Chemicals

2.1.1. General chemicals

Chemical Name	Formula	Supplier
DEAE (Diethylaminoethyl) cellulose		Sigma
Ethanol (96%)	C_2H_6O	Kemetyl
Ethidium bromide	EtBr	Sigma-Aldrich Norway
Glycerol	$C_3H_8O_3$	VWR
HiLoad 16/600 Superdex 200 prep grade column		GE Healthcare Life Sciences
Magnesium Chloride x $6H_2O$	$MgCl_2$ x $6H_2O$	Merck
Magnesiumsulfate-7-hydrate	$MgSO_4$ x $7H_2O$	Riedel-deHaen. LABOGLASS
Ni-resin		Sigma
Sodium chloride	NaCl	Merck
Sodium hydroxide	NaOH	Merck
Trisma®base	$C_4H_{11}NO_3$	Sigma® Life Science
Protease inhibitor cocktail		Sigmafast

2.1.2. Solutions and compounds

Name	Supplier
Advantage® 2 PCR buffer (10x)	Clontech
Advantage ® 2 polymerase mix (50x)	Clontech
Agar-Agar	MERCK
Agarose	Sigma® Life Science
Bacto™ trypton	Bacton, Dickinson and Company
Bacto™ yeast extracts	Bacton, Dickinson and Company
Bovine Serum Albumin (BSA)	New England Biolabs
Gel Loading Dye Blue 6x	New England Biolabs
Nucleotides (dATP, dTTP, dCTP, dGTP)	TaKaRa BIO INC.
Triton-X-100	Sigma-Aldrich

2.1.3. Antibiotics

Name	Supplier
Ampicillin	Bristol-Meyers Squibb
Kanamycin	Bristol-Meyers Squibb

2.2. Commercial kits

Name	Supplier
Advantage® cDNA PCR Kit	Clontech
GoTaq® Flexi DNA polymerase kit (MgCl solution, 5x Green PromegaGoTaq® Flexi buffer, GoTaq® DNA polymerase)	Macherey-Nagel
NucleicBond® Xtra Midi. Nucleic Acid and Protein purification kit	Macherey -Nagel
NucleoSpin® Gel and PCR Clean-up kit	Macherey -Nagel
NucleoSpin® Plasmid. Nucleic Acid and protein purification kit	Macherey- Nagel
TOPO TA Cloning® Kit for Sequencing	Invitrogen™ by life technologies™

2.3. Buffers and solutions used for protein purification

2.3.1 Buffer for EcR-LBD, EcR and USP protein purification

2.3.1.1 Lysis buffer (pH 7.5)

50 mM Tris HCl
150 mM NaCl
1.5 mM $MgCl_2$
1% glycerol
1X EDTA free protease inhibitor
1 mM DTT

2.3.1.2 Buffer for immobilized metal ion affinity chromatography

Elusion 1: 50 mM Tris HCl (pH 7.5), 10 mM Imidazole and 150 mM NaCl

Elusion 2: 50 mM Tris HCl (pH 7.5), 20 mM Imidazole and 150 mM NaCl

Elusion 3: 50 mM Tris HCl (pH 7.5), 40 mM Imidazole and 150 mM NaCl

Elusion 4: 50 mM Tris HCl (pH 7.5), 100 mM Imidazole and 150 mM NaCl

Elution 5: 50 mM Tris HCl (pH 7.5), 350 mM Imidazole and 150 mM NaCl

2.3.1.3 Buffer for ion exchange chromatography

Elusion 1: 0.01 M Tris-HCL (pH 7.5)
Elusion 2: 0.01 M Tris-HCL (pH 7.5), 0.02 M NaCl
Elusion 3: 0.01 M Tris-HCL (pH 7.5), 0.1 M NaCl
Elusion 4: 0.01 M Tris-HCL (pH 7.5), 0.5 M NaCl

2.3.1.4 Buffer for SEC column

50 mM Tris and 150 mM NaCl (pH 7.5)

2.3.2 Buffer for TEV protease purification

Lysis buffer
50 mM NaPi, 300 mM NaCl, pH 7.0.
Wash buffer
50 mM NaPi, 300 mM NaCl, 20 mM imidazole, pH 7.0.
Elusion buffer
50 mM NaPi,300 mM NaCl, 20 mM imidazole, pH 7.0

2.4 Ligand

Name	**Supplier**
25-deoxy-20-hydroxyecdysone (Ponasterone A - Pon A)	Santa Cruz Biotechnology, Inc

2.5 Growth medium, agar plate and other solution

Luria-Bertani (LB) medium	**LB-Agar plates**
1 % Bacto trypton	1 % Bacto trypton
0.5 % Bacto yeast extracts	0.5 % Bacto yeast extract
0.5 % NaCl	0.5 % NaCl
	1.5 % agar
	Autoclaved before adding antibiotic
	100 μg/ml ampicillin or
	50 μg/ml kanamycin

SOB	**SOC**
2 % Bacto trypton	10 mM $MgCl_2$
0.5 % Bacto yeast extracts	10 mM $MgSO_4$
10 mM NaCl	20 mM Glucose
2.5 mM KCl	In SOB

2.6 Enzymes

General enzyme	Supplier
Shrimp Alkaline Phosphatase (SAP)	TaKaRa
T4 Ligase + buffer	TaKaRa

Restriction Endonuclease	Recognition site	Supplier
BamHI	5' - G↓GATCC- 3' 3' - CCTAG↑G- 5'	TaKaRa
Nco1	5' - C↓CATGG- 3' 5' - GGATC↑C- 3'	TaKaRa

2.7 Primers

2.7.1 Primers used for amplification of DNA

Primer name	Used for	Sequence (5'-3')
EcR-FullFwd	EcR full-length	ATAGCCATGGTGGAAAATG
LBD-Fwd	EcR-LBD	ATAGCCATGGCTTCTTTTCCTAAAAGAC
LBD-Bwd	EcR full-length & EcR-LBD	TATGGATCC TCAGAT GTCCCAAATTTC CATGAG
USP-fullFwd	USP full-length	TGA GTT GGC GCC ATG GAT CCC AC
USP-fullBwd	USP full-length	TATGGATCCTCAGCAGCACTCTTCCAG

2.7.2 Primers used for sequencing

Primer name	Sequence (5'-3')
T7 primer (Used for pETGB1)	TACGACTCACTATAGGGGAATTG
pETMBP forward (Used for pETMBP)	GATCCACGTATTGCCGCCAC
pET reverse (Used for pETMBP and pETGB1)	GTTATTGCTCAGCGGTGGC

All primers were from Sigma® Life Science

2.8 Agarose gels for electrophoresis of nucleic acids

5x TBE	1 % agarose	Loading buffer (6X)
0.45 M Trisma® base	1 % agarose in 0.5x TBE	0.25 %
0.45 M boric acid	EtBr	bromophenol blue
0.01 M EDTA	40 % sucrose	ddH_2O

2.9 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel

2.9.1 12% running gel and 5% stacking gel for protein gel electrophoresis

Name	**12% running gel (For two minigels)**	**5% stacking gel (For two minigels)**
dH_2O (ml)	4.98	4.54
30% Acrylamide mix (ml)	6	1.3
1.5 M Tris, pH8.8 (ml)	2.5	
0.5 M Tris, pH6.8 (ml)		2
10% SDS (µl)	75	40
10% APS (µl)	150	80
TEMED (µl)	4	8

2.9.2 SDS-PAGE sample buffer (2X)

Tris-HCl (100 mM, pH 6.8)
Bromophenol blue (0.02%)
DTT (200 mM)
Glycerol (20%)
SDS (4%)

2.9.3 SDS-PAGE gel staining reagent

Name	**Supplier**
Imperial Protein Stain	Thermo SCIENTIFIC

2.10 Molecular weight marker

Name	**Marker Range**	**Supplier**
2-log DNA ladder	0.1 - 10.0 Kb	TaKaRa
Precision Plus Protein Dual Color Standards	10 - 250 kD	BIO-RAD

2.11 plasmid vectors

Name	**Supplier**
pETMBP	Novagen
pETGB1	Novagen

2.12 Bacterial strain

Escherichia coli XL-1-Blue

2.13 Consumables

Name	Supplier
1.5 ml Eppendorf-tube	Eppendorf
15 ml reaction tube	Cellstar ® greiner bio-one
50 ml reaction tube	Sarstedt
Petri dish (100 ml)	Sarstedt
Pipette tips	Axygen Scientific
Tissue	KIMTECH Science

2.14 Apparatus

Apparatus Category	Name	Supplier
Block heaters	DRI-BLOCK® DB•2A	Techne
Centrifuges	Avanti™ J-25 centrifuge + rotors (JA 14 and JA 25.50)	Bechman Coulter™
	Mini centrifuge C-1200 220V/50 Hz	NATIONAL LABNET CO
	HERAEUS FRESCO 21 Centrifuge	Thermo SCIENTIFIC
Electroporation machine	Gene Pulser ™ and pulse controller	BIO-RAD
Homoginzer	Frencepess	
Imager	Gel Doc™ EZ imager Gel Doc™ EZ imager	BIO-RAD
PCR	GeneAmp® PCR system 2700	Applied Biosystems
Power-source electrophoresis	Powerpac 300	BIO-RAD
Printer	Gel image printer	Mitsubishi P93D
Incubators	37°C	Termaks
	18°C 250 rpm. HT INFORS	Tamro MED-LAB
	37°C 250 rpm. HT INFORS	Tamro MED-LAB
Spectrophotometer	NanoDrop® ND-1000 Spectrophotometer	Fisher Scientific
Vortexer	Whirlmixer	Fisons Scientific equipment
Water-distiller	Milli-Q Advantage A10, Milli-Q Q-POD, 0.22 µm MILLIPAK®40 sterile	MILLIPORE lab-tec

2.15 Computer software

Software	Purpose
BLAST	Sequence analysis
ClustalW2	Sequence alignment
ClustalX2	Phylogenetic tree
ExPASy proteomics tool	Sequence characterization/translation
Image-lab™ Software	Gel imaging
NanoAnalyze Software v3.4.0	ITC experiment set up
TreeviewX	Processing of phylogenetic trees

3. METHODS

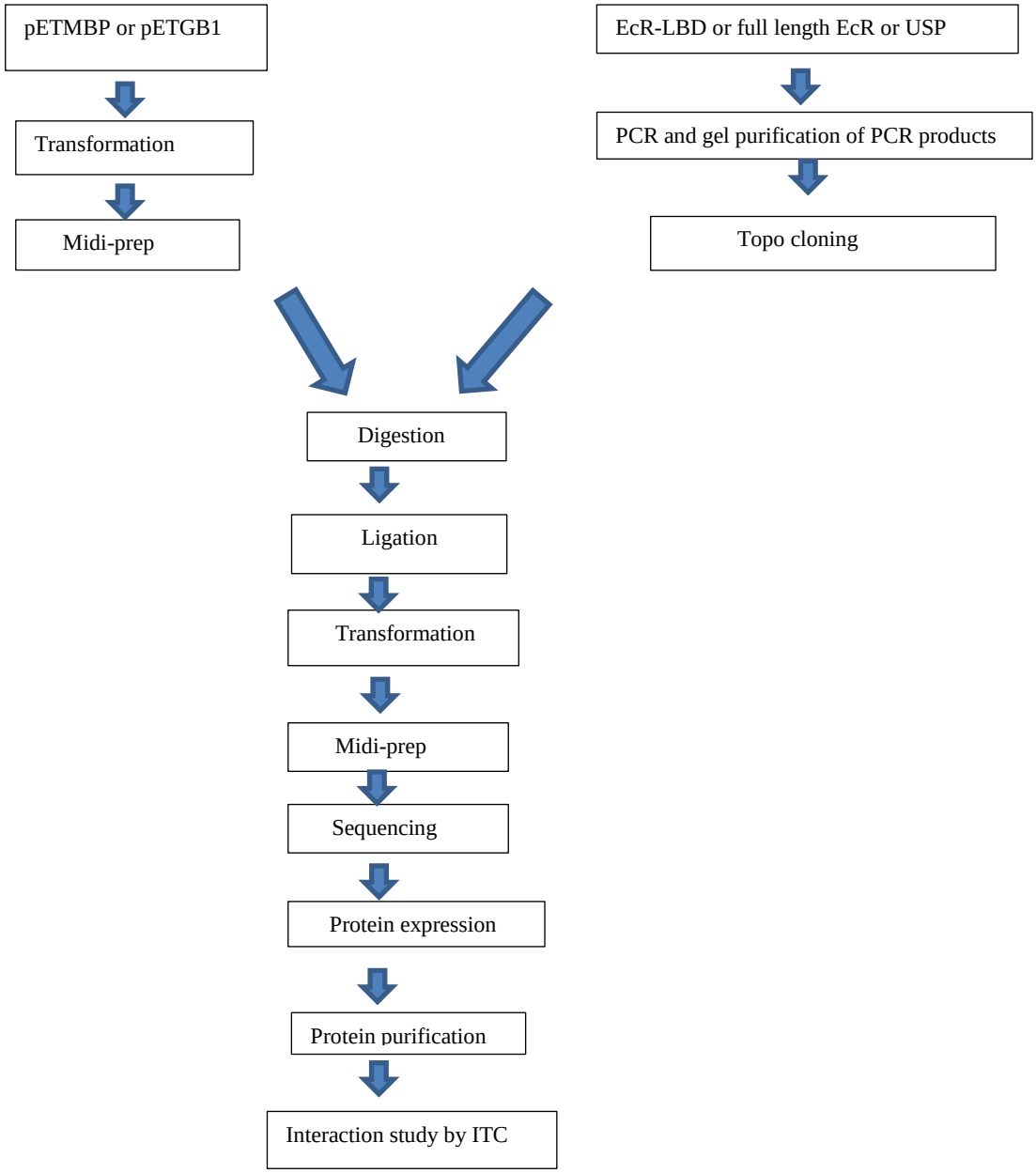


Figure 3.1 Outline of methods used in this study.

3.1.1 Polymerase Chain Reaction

Polymerase Chain reaction is a technique to amplify specific DNA fragment. It was developed by Kary Mullis in 1983 where sequence specific primer binds to the end of specific DNA sequence and by repeating cycles of heating and cooling it generates thousands of copies of that sequence (Bartlett and Stirling, 2003).

For PCR, two types of kits were used depending on the requirements. The Advantage® cDNA PCR kit was used when PCR products would be used for sequencing and to generate probes because of its proof reading capability. GoTaq® flexi DNA polymerase kit was used during colony selection to check the desired DNA fragments.

Advantage® cDNA PCR kit

1x Advantage polymerase buffer
0.2 mM dNTP
0.2 µM forward primer
0.2 µM reverse primer
1 µl plasmid DNA
1 µl Advantage® cDNA polymerase mix
Mili-Q H_2O to desired volume

GoTaq® flexi DNA polymerase kit

1x green Go Taq Flexi Buffer
2.5 mM $MgCl_2$
0.4 mM dNTP
0.4 µM forward primer
0.4 µM reverse primer
0.2 µl Go Taq polymerase
1 colony
Mili-Q H_2O to desired volume

The PCR reactions volume was between 10 µl to 50 µl.

PCR thermo-profile (Advantage® cDNA PCR kit)

Denaturation: 94°C - 5 min
Denaturation: 94°C - 30 sec
Annealing: X°C - 30 sec
Elongation: 72°C - Y sec
} X 25 cycles
Elongation: 72°C - 7 min
∞ : 4°C

PCR thermo-profile (GoTaq® flexi DNA polymerase kit

Denaturation: 94°C - 2 min
Denaturation: 94°C - 30 sec
Annealing: X°C - 30 sec
Elongation: 72°C - Y sec
} X 25 cycles
Elongation: 72°C - 7 min
∞ : 4°C

The annealing temperature was calculated using the following equation:

T annealing = T melting – 4°C, with T melting = 2°C x (n adenine bases + n thymine bases) + 4°C (n guanine bases+ n cytosine bases) – Equation 1

The elongation time was set at 1 min per 1000 bp PCR product

3.1.2 Agarose gel electrophoresis

To analyze the DNA fragments and PCR products, ethidium bromide containing 1% agarose gel in 0.5X TBE buffer was used. When an external electric field is applied to the gel, DNA molecules are separated according to their size in the agarose gel matrix. Ethidium bromide interacts with DNA and exhibits fluorescence activities under ultraviolet light which allow detection of DNA (Brody and Kern, 2004). The agarose gel electrophoresis was run at 80V and continued until loading buffer dye reached two thirds of the gel. The gel pictures were taken using Gel Doc™ EZ imager.

3.1.3 Extraction and Purification of DNA from agarose gel

Using NucleoSpin® Gel and PCR Clean-up kit, DNA was extracted and purified from agarose gel. In this method, when DNA with other impurities are added into a column (provided with NucleoSpin® Gel and PCR Clean-up kit), DNA binds to the silica membrane of column. After several washing steps, DNA was eluted with 30 µl elution buffer (NE). All centrifugations steps were done at 12,000×g (Heraesus Biofuge pico centrifuge). The concentration of eluted DNA was measured using nanodrop and also checked with gel-electrophoresis.

3.2 Topo cloning

To check the PCR products, TOPO cloning was done. The protocol of TOPO TA cloning® kit from Invitrogen was used. This kit contains special type of linearized plasmid vector (pCR™4-TOPO®) which has single overhanging 3´ deoxythymidine (T). In TOPO cloning, Taq polymerase adds a single deoxyadenosine (A) to the 3'-end of the PCR products which allow ligation with vector and this vector construct then can be transformed by heat shock into competent bacterial cells (Untergasser, 2006). For the TOPO cloning, reaction mixture components were 4 µl gel purified PCR product, 1 µl salt solution and 1 µl TOPO vector.

3.3. Mini-prep

The protocol of NucleoSpin® Plasmid Nucleic Acid and protein purification kit was used for small scale plasmid DNA purification from bacterial culture. One colony was inoculated in 5 ml LB medium containing appropriate antibiotic at 37°C and 250 rpm overnight. From the overnight bacterial culture, 3 ml was used to isolate plasmid DNA from the bacteria. All centrifugation was done at 12,000 x g speed with HeraesusFresco 21 centrifuge and elusion

was done with 50 ml AE buffer provided with kit. Concentration of extracted plasmid was measured in nanodrop and also checked by running on 1% agarose gel.

3.4.1 Digestion of DNA with restriction enzymes

Restriction endonucleases digest the double standard DNA at specific point and create either "blunt" or "sticky end. To make recombinant construct, plasmid and insert were cut with same two sticky-end restriction endonucleases (BamHI and NcoI) at 37°C for overnight.

Insert digestion reaction mix	**Plasmid digestion reaction mix**
1 µg insert (x µl)	10 µg plasmid (x µl)
2 µl 10 x TaKaRa buffer (K buffer)	2 µl 10 x TaKaRa buffer (K buffer)
2 µl 10 x BSA	5 µl 10 x BSA
4.5 U BamHI	15 U BamHI
4.5 U NcoI	15 U NcoI
dH_2O to 20 µl	dH_2O to 50 µl

Digestion was checked by running on 1% Agarose gel. After digestion, plasmid DNA was dephosphorylated to prevent re-ligation by adding 1 U shrimp alkaline phosphatase into reaction mix followed by incubation at 37°C for 30 minutes. Then heat-shock was given to both insert and plasmid reaction mix by placing in heating block at 65°C for 15 minutes to deactivate enzymes. DNA gel purification was done with NucleoSpin® Gel and PCR Clean-up kit and protocol.

3.4.2 Insert and Plasmid ligation

T4 DNA Ligase catalyse ligation by forming phosphodiester bond between insert and plasmid. Total 150 ng plasmid (Nano drop concentration) was used for ligation. Depending on the requirements, vector and insert ratio for ligation reaction was either 1:3 and 1:8 or both. The reaction was carried out at RT (19°C) for overnight.

Ligation reaction mix

150 ng vector (x µl)
x ng insert (y µl)
1 µl 10x ligation buffer
1 µl T4 DNA ligase (350 U/µl)
dH_2O to 10 µl

3.4.3 Transformation by Electroporation

In electroporation, high voltage electric pulse transiently changes the cell membrane structure of host by disturbing phospholipid bilayer and creates temporary pores. Foreign DNA can enter into the host by this pore (Shigekawa and Dower, 1988). Plasmid was diluted 1:100 in ddH_2O and 2 µl was added to 40 µl of competent cells (*E. coli* XL-1- Blue) then placed in ice for 1 minute. Solution was transferred to a 0.2 cm cuvette which was placed in an electroporator. GenePulserTM and Pulse controller were set to 2.5 kV, 200 Ω and 25 µF. The electric pulse was carried out for few seconds through the sample. Then 1 ml SOC medium was added to the sample into the cuvette. Solution was transferred to an eppendorf tube and incubated at 37°C and 250 rpm for 45 minutes. After incubation, 100 µl of this sample was plated out on agar-plates containing an appropriate antibiotic and incubated at 37°C overnight. Then colony shaking was done to select the right colony using GoTaq PCR. Selected colonies were further processed by midi-prep.

3.5 Midi-prep

For midi-prep, pre-culture was done by taking one colony in 20 ml LB medium containing appropriate antibiotic and then incubated at 37ºC and 250 rpm for 8 h. 5 ml of the pre-culture was added to the 200 ml LB containing appropriate antibiotic and then incubated overnight at 37°C and 250 rpm for overnight. To extract plasmid DNA from cell pellet, NucleicBond® Xtra Midi Nucleic Acid and Protein purification kit and protocol was used. All centrifugations were done at 15 000 g, 4°C with AvantiTM J-25 centrifuge (JA-14 and JA-25.50 rotor) from Beckman Coulter. Extraxted DNA was resuspended in 300 µl of dH_2O. The DNA quality was checked on 1 % agarose gel and the concentration was determined with nanodrop.

3.6 Sequencing reaction

To determine the sequence, DNA sequencing was done using Sanger method. In this method, DNA polymerase selectively incorporates chain terminating fluorescently labelled dideoxynucleotides (ddNTPs). These modified ddNTPs lack 3'-OH group which is required for the formation of a phosphodiester bond to extend the PCR fragment (Sanger et al., 1977). For each template, two reactions were made where one contained forward primer and other contained reverse primer.

Components for each sequencing reaction

Big Dye		1 µl
Sequencing buffer		1 µl
Forward or Reverse primer (1 µM)		3.2 µl
Plasmid (200-400 ng, Nanodrop concentration)		X µl
Milli-Q dH_2O	to	10 µl.

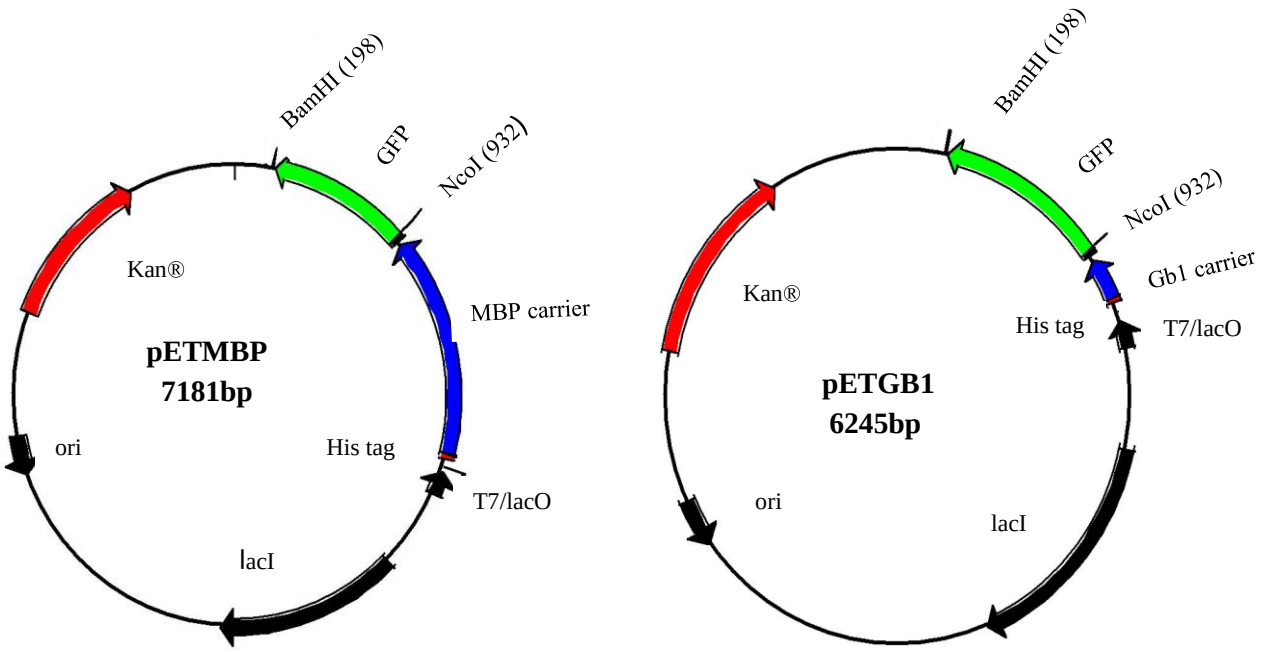
PCR thermo-profile

94°C for 5 minute
94°C for 10 second
50°C for 5 second } X 27 cycles
60°C for 4 minute
∞ 4°C

After sequencing reaction, in each PCR tube 10 µl ddH_2O was added and the final volume was 20 µl. Then remaining part of sequencing was performed at sequencing lab of Institute of Molecular Biology at the University of Bergen. The nucleotide sequences obtained from the sequencing lab were translated by using ExPASy. From the Basic Logical Alignment Search Tool (BLAST) of National Centre for Biotechnology Information (NCBI), related sequences were found. Selected sequences were aligned using ClustalW2 (Larkin et al., 2007) and phylogenetic tree called Bootstrap Neighbour-joining (N-J) trees were made using ClustalX2 (Larkin et al., 2007). The trees were edited using TreeViewX (Page, 1996).

3.7 Transformation into protein expression system

Vector (Fig 3.7.1) containing desired gene was transformed into *Escherichia coli* strain BL21Star(DE3) by heat shock. For heat-shock, 10 µl competent cells were taken out from the -80°C freezer and placed in ice. Cells were allowed to thaw in ice. 2 µl of plasmid DNA (Total 16 ng) was transferred into tube containing competent cells and then incubated in ice for 30 minutes. Heat shock was done at 42°C for 33 seconds and tube was placed in ice for 2 minutes. Then 60 µl SOC medium was added to the tube which followed by incubation for 60 minute at 37°C. Cells were plated on LB-kanamycin plate and incubated for overnight at 37°C. After overnight incubation, colonies on the plate were counted.



T7 Promoter	His tag	MBP or GB1	TEV site	EcR-LBD or full length EcR or USP

Figure 3.7.1 pETMBP and pETGB1 vector maps. Structure from: http://babel.ucmp.umu.se/cpep/web_content/pdf/vector%20maps/. Cited 10.02.2015.

3.8 Protein expression

Around 60-70 colonies containing the desired gene were inoculated in kanamycin containing 5 ml LB medium at 37°C and 250 rpm for overnight. After overnight incubation, 2.5 ml of culture was added to the kanamycin containing 1 liter LB medium and incubated at 37°C and 250 rpm shaking (around 2-4 hours) until OD (at 600nm) was 0.8 to 1. When OD was around 0.8 then 500 µl IPTG (1 M) was added to flask to induce the protein expression and incubated at 18°C and 250 rpm shaking for overnight (16h). Samples were collected at different hours (at 0h, 1h, 2h, 3h, 4h and 16h) after IPTG induction. After overnight incubation, cells were harvested by centrifugation (JLA 9, 1000) at 5180 X G for 15 min. Supernatant was removed and pellet was resuspended in same flask using around 7 ml LB medium. Then, centrifugation

was done at 3700 rpm (JA 25.50 rotor) for 15 minutes. Supernatant was removed and the pellet was frozen at -80°C for further use.

3.9 Cell lysis

3.9.1 Lysis by French press

Expressed protein in bacterial cell can be extracted either by enzymatic method or physical method. Enzymatic method includes use of different enzymes, detergent, solvents etc. and physical methods are French press, sonication etc. In French press, cell suspension is placed within the cylinder and an external hydraulic pump drives a piston within the cylinder. As a result, sample comes out through a outlet valve and cell breakage occur due to shear stress (Ludmil and Al-Ibraheem, 2002).

First, cell pellets (2-3 gram) from -80° C were resuspended in lysis buffer (10 ml lysis buffer for 1 gram pellet). Then, French press was performed at 1000 psi for 2 times and followed by addition of 0.2 % Triton-X 100 to the solution and incubation in a giroshaker for 30 minute at room temperature.

3.9.2 Lysis by sonication

Another physical method is sonication that uses ultrasonic frequencies (>20 kHz) to breakdown the bacterial cells. When the cells are subjected to high-frequency sound waves with a vibrating probe, vibrations are generated which cause mechanical shearing of the cell wall. As a result, proteins come out from the cell inside. (Benov and Al-Ibraheem, 2002).

After resuspension of cells, lysozyme was added to a final concentration 1 mg/ml of lysate and followed by incubation in ice for 20 minutes. Then sonication was performed at 60 % intensity for total 2 minutes. Each time sonication continued for 15 second and then 30 second interval to avoid overheating.

After French press or sonication, lysate was centrifuged at 20000 rpm (JA 25.50 rotor) and 4°C for 30 minutes. Then supernatant was collected and purified using metal ion affinity chromatography column, ion exchange chromatography column and size-exclusion chromatography column.

3.10 Protein purification

3.10.1 Immobilized Metal Ion Affinity Chromatography (IMAC)

2 ml of Ni-resin (binding capacity 15 mg/ml) was taken into a column containing 45 µm filter. After resin sedimentation, the column was equilibrated with buffer and then the supernatant was loaded on the column. Several elusion steps were carried out in column with 2x column volume elution buffers containing sequentially increasing concentration of imidazole. (All buffer compositions in materials section).

3.10.2 Ion exchange chromatograph (IEC)

For ion exchange chromatograph purification, 6 ml DEAE (Diethylaminoethyl) cellulose resin slurry was taken into a column containing 45 µm filter. After resin sedimentation, the column was equilibrated with buffer and then the supernatant was loaded on the column. Several elusion steps were performed to sequentially increase the concentration of NaCl. To check which ionic strength gives better purification for proteins, using different concentrations of NaCl in lysate buffer, IEC were repeated several times.

3.10.3 Size Exclusion Chromatography (SEC)

HiLoad 16/600 Superdex 200 prep grade column (Column volume 120 ml) was equilibrated with buffer. Then protein sample was loaded into the column and the protein sample volume was 2% of the column volume. Fraction was collected at 1 ml/min rate and in 1 ml aliquots.

3.11 Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE is a widely used technique to characterize protein. In SDS-PAGE, anionic detergent sodium dodecyl sulphate (SDS) is used to linearize protein by breaking secondary and non-disulphide linked tertiary structure of the protein. To break disulphide linked tertiary structure a reducing agent (like dithiothreitol (DTT) or 2-mercaptoethanol) is used. SDS also gives negative charges to linearized proteins and these negative charges on protein are equally distributed per unit mass. As a result, during electrophoresis protein will move to the anode according to their size and relative molecular weight. During gel formation, acrylamide is polymerized by ammonium persulfate (APS). APS acts as source of free radicals and initiate the gel formation. N, N, N', N' tetramethylethylenediamine (TEMED) stabilizes free radicals and better polymerization. Polymerized acrylamide are cross-linked by bisacrylamide (Shapiro et al., 1967).

A gel chamber of 10-well comb was prepared where a 12% running gel and a 5% stacking gel were used. To prepare 12% running gel and 5% stacking gel dH_2O, acrylamide mix, SDS, APS and TEMED and Tris were used (Used amount in each gel are in the materials section). 5 µl of precision plus protein dual color standards from BIO-RAD was used as Marker.

3.12 Expression and purification of TEV protease and TEV digestion

The TEV protease was expressed and purified following the procedures by Berg et al. (2006). Berg et al. (2006) used mutant TEV protease gene as starting material and Gateway system (Invitrogen) for recombinant cloning where pTH24 and pTH31 were vectors. Clones containing mutant TEV protease gene were transformed into Rosetta(DE3)pLysS and protein production was induced by adding IPTG (Final concentration 1mM) when OD (at 600nm) was 0.6. After overnight (16h) incubation at 20ºC, TEV protease proteins were purified by IMAC. The eluates TEV from IMAC were desalted and added glycerol to a final concentration of 10%. The aliquot of 0.2 ml was stored at -80^{o}C until use. For TEV digestion, only fresh aliquots were used. MBP-fused EcR-LBD was digested with the TEV protease for overnight (16h). For 10 ml protein samples 30 µl TEV protease (Nanodrop concentration 2ng/µl) were used.

3.13 Isothermal Titration Calorimetry (ITC)

ITC experiments were done on a Nano ITC low volume from TA instruments. Sample cell and injection syringe were cleaned several times with distilled water and degassed buffer. The sample cell was filled with 300 µl of IEC purified MBP-EcR-LBD protein with a concentration of 2 µM. The injection syringe was filled with 50 µl of Pon A solved in ethanol with a concentration of 8.6 µM, the syringe was then placed in the burette handle and inserted in the machine.

The experiment was set up using the ITC run software (NanoAnalyze Software v3.4.0). During the experiment a stirring rate of 250 rpm and a temperature of 25°C were used, and a total of 20 injection of 2.03 µl with and interval of 180 seconds were completed. A control experiment where Pon A was injected into buffer using the same parameters was also done.

4. RESULTS

The result section consists of four parts. Sequence analyses and phylogenies of *L. salmonis* ecdysone receptors and ultraspiracle protein from representative species including *L. salmonis* are presented at the first segment. The second part describes the protein expression studies of EcR (both EcR-LBD and full-length EcR) and USP. The purification of EcR-LBD and EcR proteins using different techniques are presented at the third segment and the last part presents the interaction study between EcR-LBD and Pon A using ITC.

4.1 Sequence analysis and phylogeny

The *L. salmonis* cDNAs containing EcR-LBD, EcR (LBD+DBD) and USP (LBD+DBD) were PCR-amplified using respective primer sets. The amplified products were gel purified, cloned and sequenced. Shown are nucleotides and deduced amino acid sequences of *L. salmonis* EcR-LBD, EcR and USP (Fig 4.1.1.1 and 4.1.2.1). The BLAST alignment was performed to compare various sequences and multiple sequence alignments were used to make phylogenetic trees.

4.1.1 Sequence analysis and phylogeny of EcR

EcR amino acid sequences from six selected species were aligned (Table 4.1.1.1). All species showed high degrees of conservation for DBD (Fig 4.1.1.2), but less conservation for LBD (Fig 4.1.1.3). Among the species aligned, the copepod *Tigriopus japonicas* showed the highest (69%) amino acid sequence identity. A neighbour-joining (N-J) tree confirmed that *L. salmonis* is most closely related to the copepod *T. japonicas* (Fig 4.1.1.4).

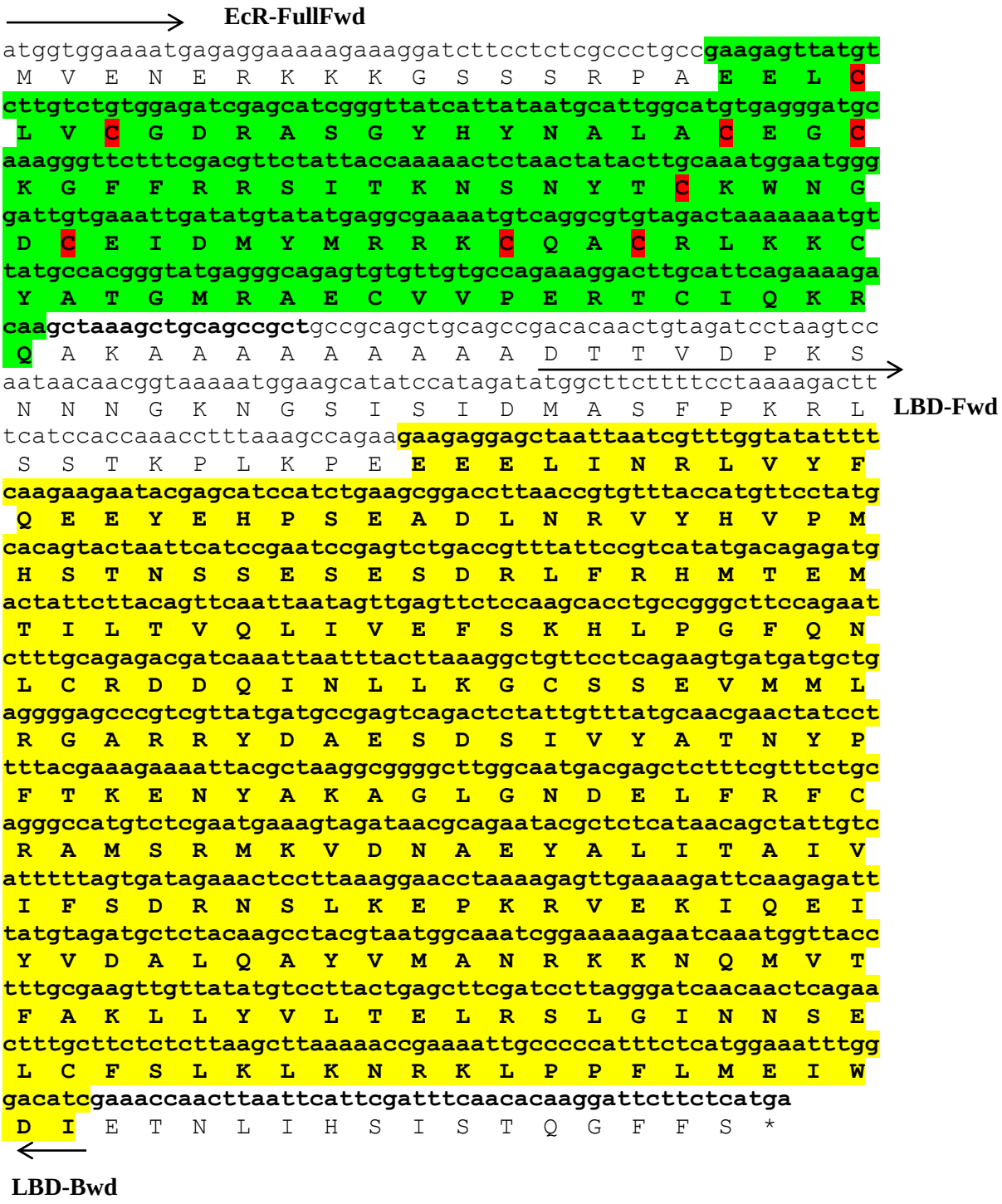


Figure 4.1.1.1 Nucleotide and deduced amino acid sequences of *L. salmonis* EcR. DNA-binding domain (DBD) is marked in light green and ligand-binding domain (LBD) is marked in yellow. Cysteine residues (C) in DBD where zinc-ion binds are highlighted (Red). The unshaded region between DBD and LBD is the D-domain. The position of primers used to create full-length EcR (LBD+DBD) and EcR-LBD constructs are marked by black arrows

Table 4.1.1.1 EcR sequences from different species used in amino acid alignment

Classification	Species (common name)	NCBI Accession number	Sequence Identity (LBD+DBD)	Size (aa)
Crustacea	*Lepeophtheirus salmonis, L.S.* (salmon louse)	AIZ04022.1		536
	Tigriopus japonicas, T.J. (copepod)	ADD82902.1	69%	546
Nematoda: Adenophorea	*Trichuris trichiura, T.T.* (whipworm)	CDW58186.1	48%	754
Nematoda: Secernentea	*Toxocara canis, T.C.* (dog roundworm)	KHN78537.1	48%	465
Arthropoda: Insecta	*Locusta migratoria, L.M.* (migratory locust)	AAD19828.1	62%	541
Mollusca: Bivalvia	*Crassostrea gigas, C.G.* (pacific oyster)	EKC19773.1	39%	471

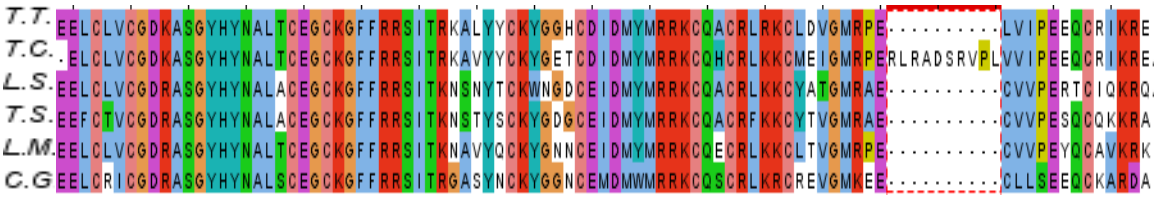


Figure 4.1.1.2 Multiple alignment of *L. salmonis* EcR-DBD with EcR-DBD from different species. NCBI BLAST search for EcR-DBD sequence was used for selection of comparable protein sequences. ClustalW2 multiple alignment was performed with obtained EcR-DBD sequences from NCBI BLAST. The alignment was edited in Jalview (Waterhouse et al., 2009) and coloured. Similar amino acids are shown with same background colour. Sequence of *L. salmonis* (L.S) was aligned to *Tigriopus japonicas* (T.J.), *Trichuris trichiura* (T.T.), *Toxocara canis* (T.C.), *Locusta migratoria* (L.M.) and *Crassostrea gigas* (C.G.). The sequence of the nematoda T.C. differed from the other compared species in that it contained 10 extra residues (red box).

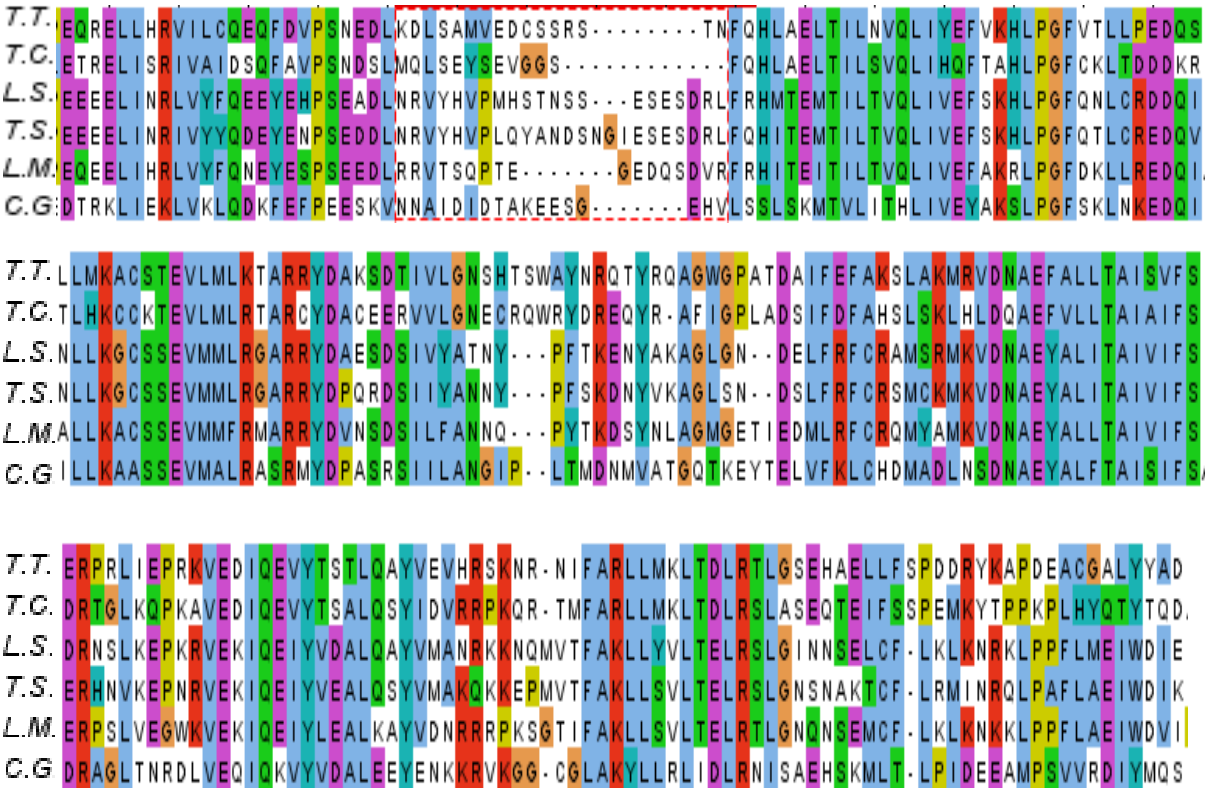


Figure 4.1.1.3 Multiple alignment of *L. salmonis* EcR-LBD with EcR-LBD from different species. NCBI BLAST search for EcR-LBD sequence was used for selection of comparable protein sequences. ClustalW2 multiple alignment was performed with obtained EcR-LBD sequences from NCBI BLAST.The alignment was edited in Jalview (Waterhouse et al., 2009) and coloured. Similar amino acids are shown with same background colour. Sequence of *L. salmonis* (L.S) was aligned to *Tigriopus japonicas* (T.J.), *Trichuris trichiura* (T.T.), *Toxocara canis* (T.C.*), Locusta migratoria* (L.M.) and *Crassostrea gigas* (C.G.). Most diverged region throughout the ligand-binding domain among different species is marked (red box).

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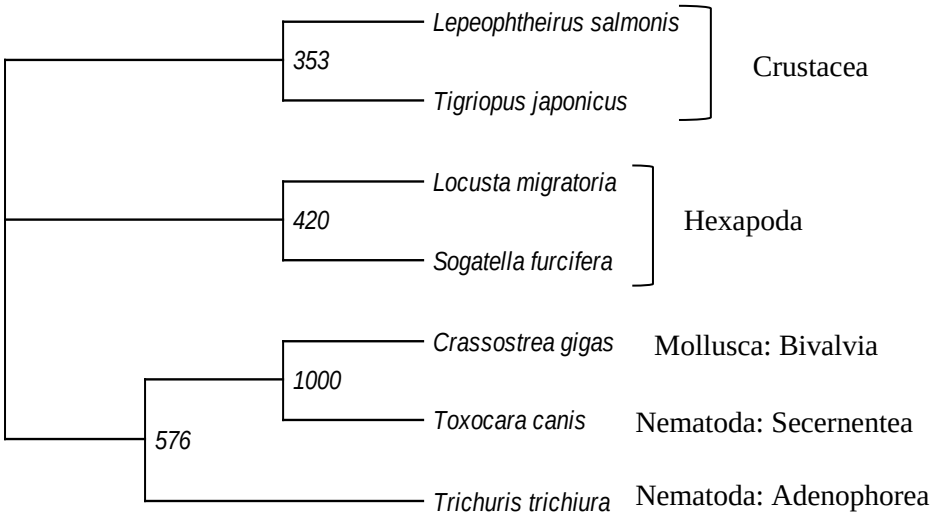

Figure 4.1.1.4 Phylogenetic relationship of *L. salmonis* EcR and other species. Related sequences were selected from NCBI BLAST against *L. salmonis* EcR (LBD+DBD). ClustalX2 (Larkin et al., 2007) was used to align selected sequences and create a Bootstrap N-J tree excluding positions with gaps at 1000 bootstrap trials. The tree was edited using TreeViewX (Page, 1996). Selected species were *Trichuris trichiura* (whipworm), *Crassostrea gigas* (pacific oyster), *Tigriopus japonicas* (copepod), *Toxocara canis* (dog roundworm), *Lepeophtheirus salmonis* (salmon louse), *Locusta migratoria* (migratory locust) and *Sogatella furcifera* (whitebacked planthopper). Full overview of selected species, their classification and accession numbers are presented in Appendix A.

4.1.2 Sequence analysis and phylogeny of USP

USP amino acid sequences from five selected species were aligned (Table 4.1.2.1). All species showed high degrees of conservation for DBD (Fig 4.1.2.2), but less conservation for LBD (Fig 4.1.2.3). Among the species aligned, the copepod *Tigriopus japonicas* showed the highest (58%) amino acid sequence identity. A neighbour-joining (N-J) tree was made from multiple sequence alignments (Fig 4.1.2.4).

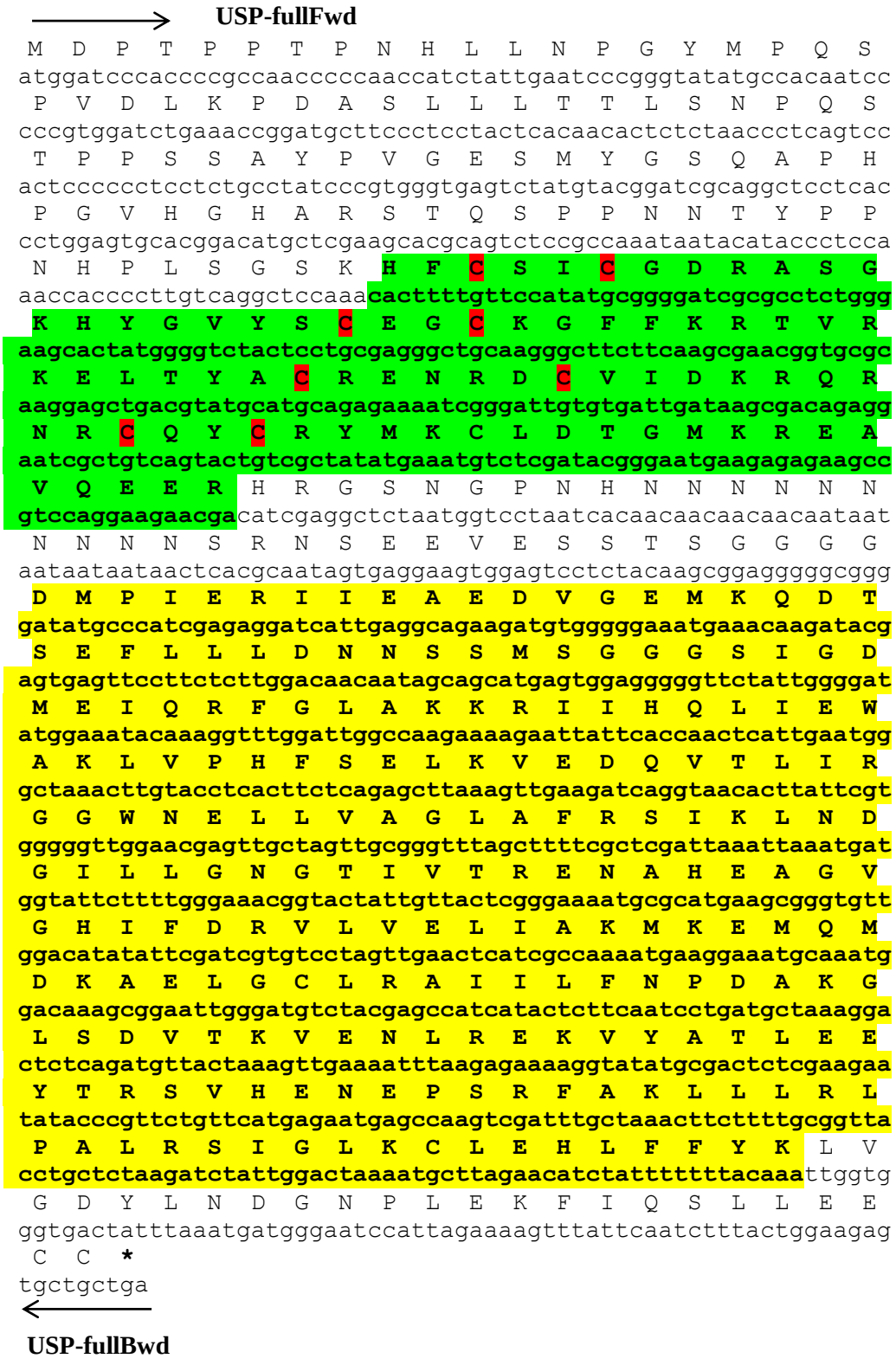

Figure 4.1.2.1 Nucleotide sequence and deduced amino acid sequences of *L. salmonis* USP. DNA-binding domain **(**DBD) is marked in light green and ligand-binding domain (LBD) is marked in yellow. Cysteine residues (C) in DBD where zinc-ion binds are highlighted (Red). The unshaded region between DBD and LBD is the D-domain. The position of primers used to create full-length USP (LBD+DBD) construct are marked by black arrows

Table 4.1.2.1 USP sequences from NCBI BLAST of different species used in amino acid alignment

	Species with common name	NCBI Accession number	Sequence Identity	Size (aa)
Crustacea	*Lepeophtheirus salmonis, L.S.* (salmon louse)	AIE45497.1		442
	Tigriopus japonicas, T.J. (copepod)	AID52845.1	58%	449
Hexapoda	*Tribolium castaneum, T.C.* (red flour beetle)	CAL25729.1	54%	407
Chelicerata	*Liocheles australasiae, L.A.* (wood scorpion)	BAF85823.1	56%	410
Mollusca	*Reishia clavigera, R.C.* (sea snail)	AAU12572.1	53%	431

Figure 4.1.2.2 Multiple alignment of *L. salmonis* USP-DBD with DBD from different species. NCBI BLAST search for DBD sequence was used for selection of comparable protein sequences. ClustalW2 multiple alignment was performed with obtained DBD sequences from NCBI BLAST. The alignment was edited in Jalview (Waterhouse et al., 2009) and coloured. Similar amino acids are shown with same background colour. Sequence *of L. salmonis* (L.S) was aligned to *Tigriopus japonicas* (T.J.), *Tribolium castaneum* (T.C.), *Liocheles australasiae* (L.A.) and *Reishia clavigera* (R.C.)

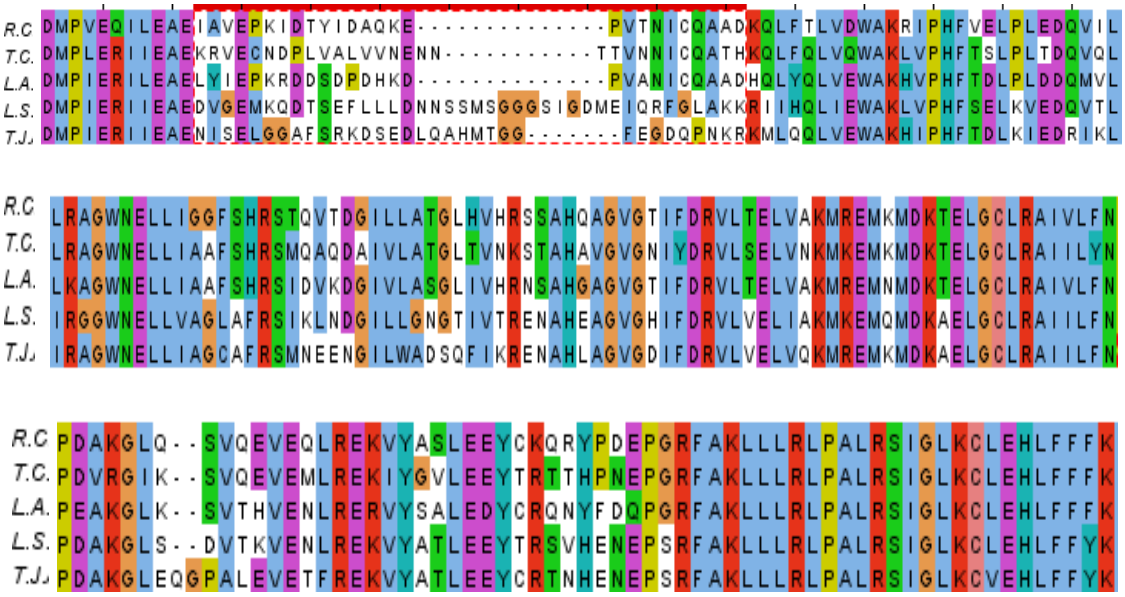

Figure 4.1.2.3 Multiple alignment of *L. salmonis* USP-LBD with USP-LBD from different species. NCBI BLAST search against *L. salmonis* USP-LBD sequence was run and selected comparable protein sequences.ClustalW2 (Larkin et al., 2007) multiple alignment was performed with obtained LBD sequences from NCBI BLAST. The alignment was edited in Jalview (Waterhouse et al., 2009) and coloured. Similar amino acids are shown with same background colour. Sequence of *L. salmonis* (L.S) was aligned to *Tigriopus japonicas* (T.J.), *Tribolium castaneum* (T.C.), *Liocheles australasiae* (L.A.) and *Reishia clavigera* (R.C.). Most diverged region throughout the ligand-binding domain among different species is marked (red box)

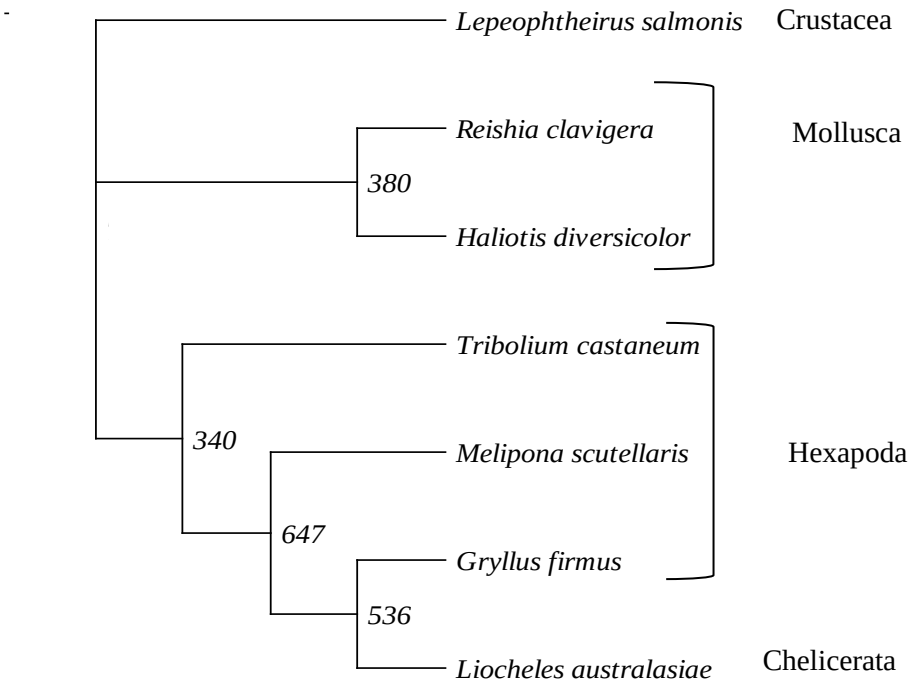

Figure 4.1.2.4 Phylogenetic relationship of USP sequences from *L. salmonis* and other species. Related sequences were selected from NCBI BLAST against *L. salmonis* USP. ClustalX2 (Larkin et al., 2007) was used to align selected sequences and create a Bootstrap N-J tree excluding positions with gaps at 1000 bootstrap trials. The tree was edited using TreeView X (Page, 1996). Selected species were *Tribolium castaneum* (red flour beetle), *Liocheles australasiae* (wood scorpion), *Reishia clavigera* (sea snail), *Haliotis diversicolor* (abalone), *Gryllus firmus* (sand field cricket) and *Melipona scutellaris* (stingless bees). Full overview of selected species, their classification and accession numbers are presented in Appendix B.

4.2 PROTEIN EXPRESSION

4.2.1 Expression of EcR-LBD, EcR and USP

The expression constructs containing EcR-LBD (both in pETMBP and in pETGB1), EcR (in pETMBP) and USP (in pETMBP) were transformed into the *E. coli* strain BL21(DE3). Desired proteins were induced by adding IPTG to the bacterial cultures of optical density of 0.8 to 1 and the protein production was monitored at different time points (Figs. 4.2.1). Both GB1-fused EcR-LBD (expected size 38 kD; Fig. 4.2.1.1) and MBP-fused EcR-LBD (expected size 72 kD; Fig. 4.2.1.2) were clearly detectable after 3 hours induction with IPTG and the levels of expression increased and peaked at 16 hours after the induction. The expression profile of MBP-fused EcR (86 kD, containing both DBD and LBD) was also similar to the EcR-LBD constructs, although MBP-EcR was not as strongly expressed as MBP-EcR-LBD (Fig. 4.2.1.3). Meanwhile, MBP-fused USP (93 kD) did not expressed at all under the same experimental conditions (Fig 4.2.1.4).

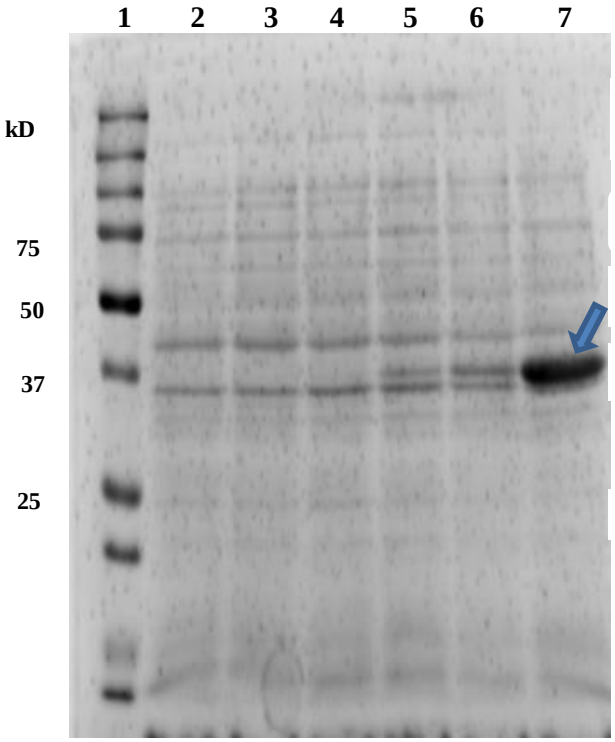

Figure 4.2.1.1 SDS-PAGE analysis of expression of GB1-fused EcR-LBD protein (38 kD). Gel was stained with Imperial Protein Stain. 5 µl sample + 5 µl 2x loading buffer were loaded on lanes 2-7. Lane 1, protein ladder. Lanes 2, 3, 4, 5, 6 and 7: samples at 0h, 1h, 2h, 3h, 4h and overnight (16h) after adding IPTG, respectively. The arrow indicates the GB1-fused EcR-LBD.

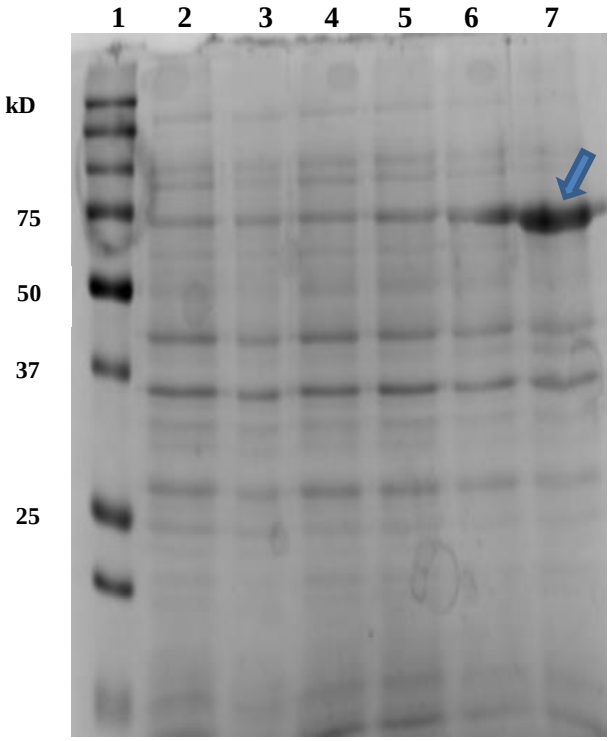

Figure 4.2.1.2 SDS-PAGE analysis of expression of MBP-fused EcR-LBD protein (72 kD). Gel was stained with Imperial Protein Stain. 5 µl sample + 5 µl 2x loading buffer were loaded on lanes 2-7. Lane 1, protein ladder. Lanes 2, 3,4,5,6 and 7: samples at 0h, 1h, 2h, 3h, 4h and overnight (16h) after adding IPTG, respectively. The arrow indicates the MBP-fused EcR-LBD.

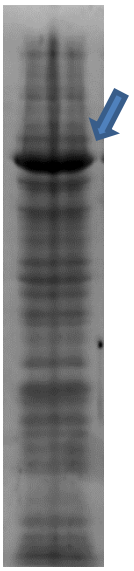

Figure 4.2.1.3 SDS-PAGE analysis of expression of MBP-fused EcR protein (86 kD) at 16h after IPTG induction. Gel was stained with Imperial Protein Stain. 5 µl sample + 5 µl 2x loading buffer was loaded on the lane. The arrow indicates the MBP-fused EcR.

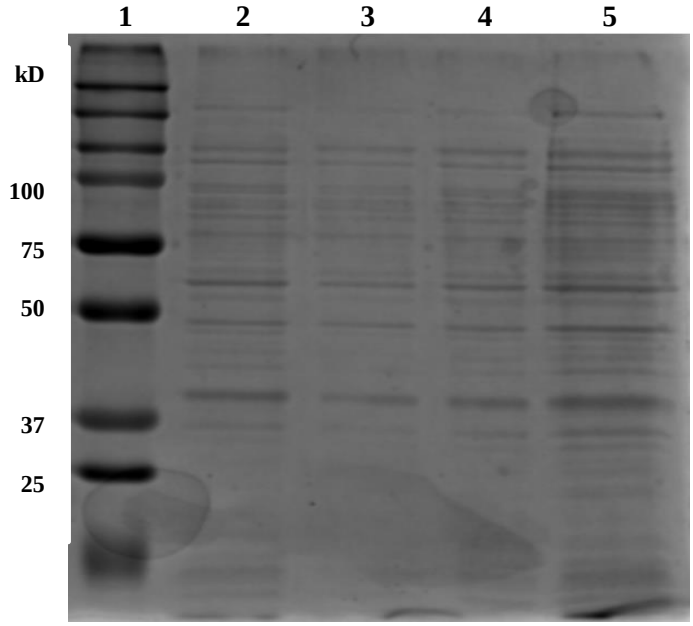

Figure 4.2.1.4 SDS-PAGE analysis of expression of MBP-fused USP protein (93 kD). Gel was stained with Imperial Protein Stain. 5 µl sample + 5 µl 2x loading buffer were loaded on lanes 2-5. There was no visible expression of USP protein. Lane 1, protein ladder. Lanes 2, 3, 4 and 5: samples at 0h, 2h, 4h and overnight (16h) respectively after IPTG induction, respectively.

4.3 Purification of *L. salmonis* EcR-LBD, EcR and USP

4.3.1 Purification of EcR-LBD by immobilized metal ion affinity chromatography

E. coli cells expressing MBP-fused EcR-LBD protein was harvested by centrifugation and the pellet was lysed and supernatant was subjected to IMAC (Fig 4.3.1.1). (The GB1-fused protein was mostly found in pellets, thus not further used (Fig 4.3.1.2). The binding of His-tag to the Ni-resin was very poor and most MBP-fused EcR-LBD was eluted as a flowthrough and the remaining protein was eluted during the elusion step using low imidazole containing buffers (Fig 4.3.1.1).

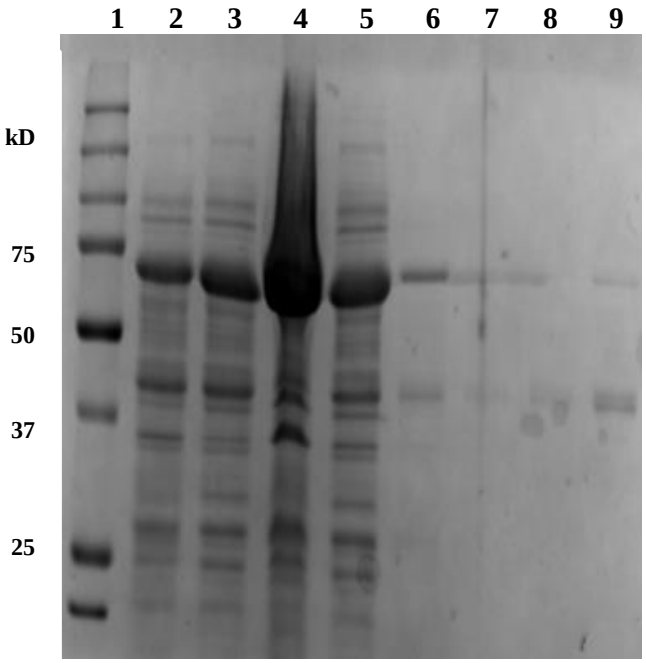

Figure 4.3.1.1 SDS-PAGE analysis of MBP-fused EcR-LBD protein purification by IMAC. Gel was stained with Imperial Protein Stain. 5 µl sample + 5 µl 2x loading buffer were loaded on lanes 2-9. Lane 1, protein ladder. Lane 2, lysate. Lane 3, supernatant. Lane 4, pellet. Lane 5, flowthrough. Lanes 6-9: elusion at 20 mM, 40 mM, 100 mM and 350 mM imidazole, respectively.

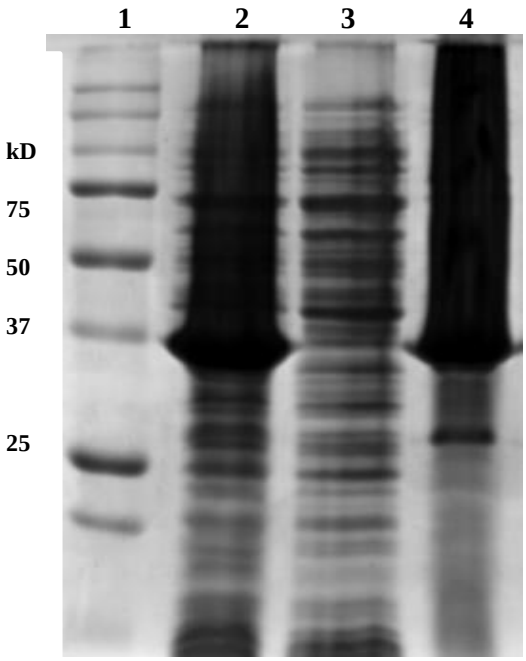

Figure 4.3.1.2 SDS-PAGE analysis of lysis of GB1-fused EcR-LBD protein. Gel was stained with Imperial Protein Stain. 5 µl sample + 5 µl 2x loading buffer were loaded on lanes 2-4. Lane 1, protein ladder. Lane 2, lysate. Lane 3, supernatant. Lane 4, pellet.

4.3.2 Purification of MBP-fused EcR by immobilized metal ion affinity chromatography

The MBP-fused EcR protein was subjected to IMAC, and like MBP-fused EcR-LBD, MBP-fused EcR also did not bind to the Ni-resin (data not shown).

4.3.3 Expression and purification of TEV protease and TEV digestion of MBP-EcR-LBD

The TEV protease was expressed and purified following the procedures by Berg et al. (2006) (Fig 4.3.3.1). A strong, single band of around 32 kD was eluted with from the IMAC (Fig 4.3.3.1, lane 9). MBP-fused EcR-LBD was digested with the TEV protease, and further purified by IMAC (Fig 4.3.3.2). After overnight incubation, the digestion has proceeded about 50% (Fig 4.3.3.2, lane 5). The digestion mixture was subjected to IMAC. EcR-LBD without MBP (Fig 4.3.3.2, lane 6, lower arrow) along with undigested i.e. MBP-EcR-LBD (Fig 4.3.3.2, lane 6, upper arrow) was eluted as flowthrough (Fig 4.3.3.2, lane 6), whereas most of freed MBP (Fig 4.3.3.2, lane 8), was eluted with 350 mM imidazole. However, after TEV digestion MBP-less EcR-LBD precipitated (data not shown).

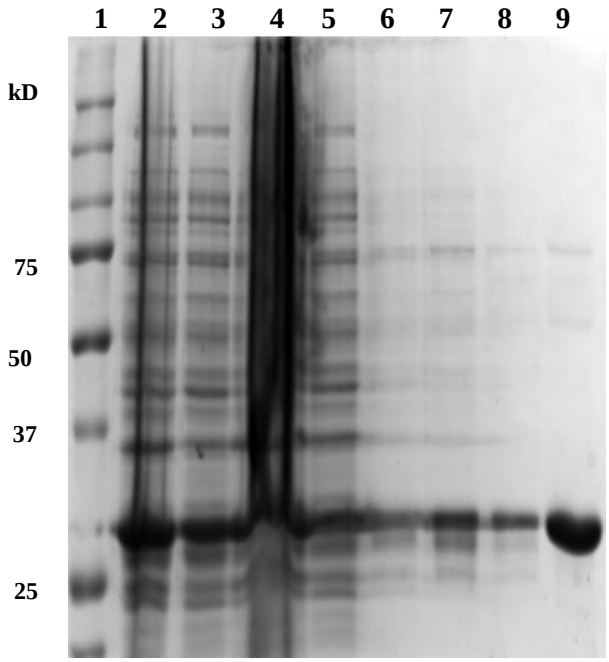

Figure 4.3.3.1 SDS-PAGE analysis of TEV (around 32 kD) purification by IMAC. Gel was stained with Imperial Protein Stain. 5 µl sample + 5 µl 2x loading buffer were loaded on lanes 2-9. Lane 1, protein ladder. Lane 2, lysate. Lane 3, supernatant. Lane 4, pellet. Lane 5, flowthrough. Lanes 6-9: elusion at 20 mM, 40 mM, 100 mM and 350 mM Imidazole , respectively.

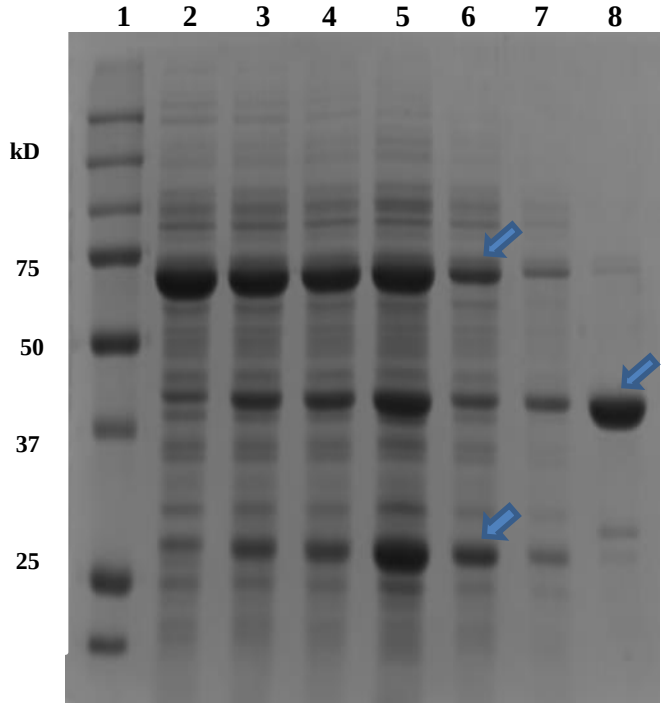

Figure 4.3.3.2 SDS-PAGE analysis of MBP-fused EcR-LBD protein purification by IMAC after TEV digestion. Gel was stained with Imperial Protein Stain. 8 µl sample + 8 µl 2x loading buffer were loaded on lanes 2-8. Lane 1, protein ladder. Lanes 2-5: samples at 0h, 1h, 2h, and overnight (16h) after adding TEV protease to MBP-fused EcR-LBD protein, respectively. Lane 6, flowthrough. Lanes 7-8: elusion at 20 mM and 350 mM Imidazole, respectively.

4.3.4 Purification of MBP-fused EcR-LBD by ion exchange chromatography

Since both MBP-fused EcR-LBD and EcR bound very poorly to the Ni-resin and most of EcR-LBD and EcR proteins were eluted as flowthrough in IMAC, IEC was chosen instead to purify the MBP-fused EcR-LBD protein. At 150 mM NaCl, which was the salt concentration of cell lysis, MBP-EcR-LBD eluted as flowthrough without any binding to the DEAE matrix (data not shown). Also, most of MBP-EcR-LBD was eluted as flowthrough at much lower salt concentration of 20 mM NaCl (Fig 4.3.4.1), although some binding of MBP-fused EcR-LBD did occur, and this was accompanied by removing much of impurities from the MBP-fused EcR-LBD containing fraction (Fig 4.3.4.1, lanes 7 and 8). However, MBP-fused EcR-LBD bound to the DEAE matrix well at 6.7 mM NaCl (Fig 4.3.4.2) and it was eluted with the buffer containing 100 mM NaCl (Fig 4.3.4.2, lane 7).

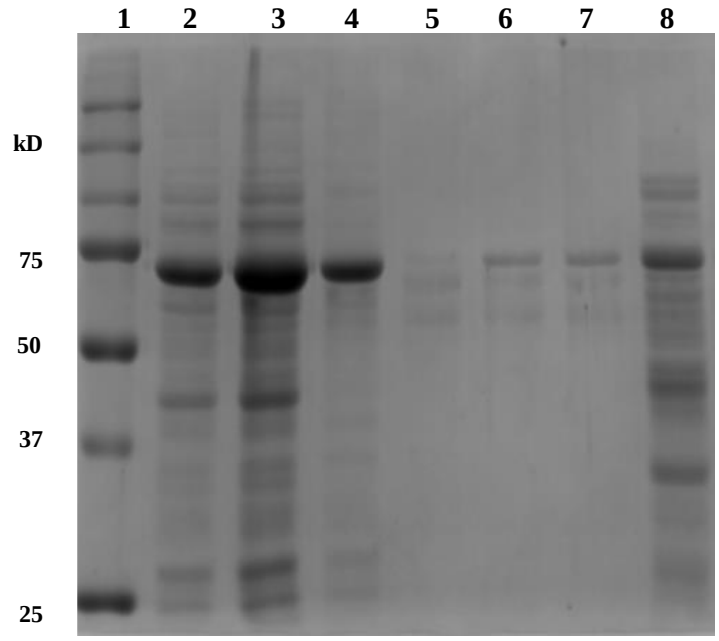

Figure 4.3.4.1 SDS-PAGE analysis of MBP-fused EcR-LBD protein purification by IEC (2nd IEC column and NaCl was 20 mM). Gel was stained with Imperial Protein Stain. Lysate was run on IEC column (Lysate buffer contains 150 mM NaCl). Flowthrough from first IEC column was diluted from 150 mM to 20 mM NaCl and re-run on IEC column. In gel picture, lane 3 should have come first and then lane 2. Accidently interchange happened during sample loading into gel. 10 µl sample + 10 µl 2x loading buffer were loaded on lanes 2-8. Lane 1, protein ladder. Lane 2, dilution of first IEC column flowthrough at 20 mM NaCl. Lane 3, flowthrough from first IEC column at 150 mM NaCl. Lane 4, flowthrough from second IEC column at 20 mM NaCl. Lanes 5-8: elusion at 20 mM, 50 mM, 100 mM and 500 mM NaCl of second IEC column purification, respectively.

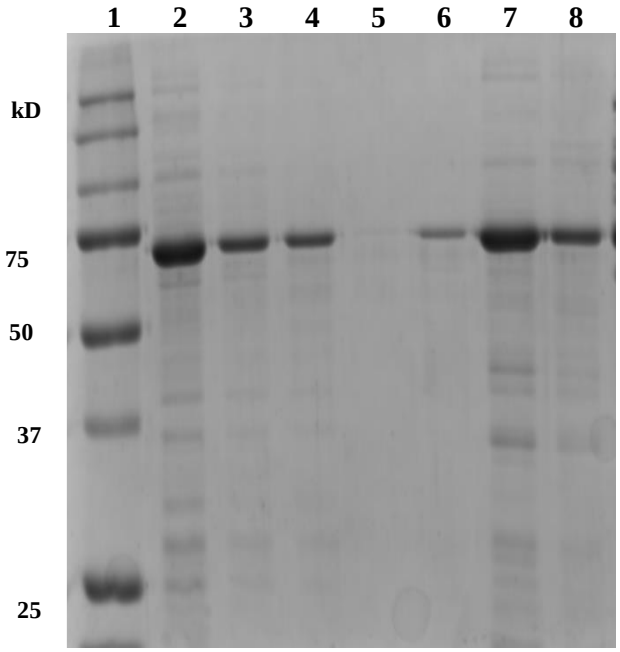

Figure 4.3.4.2 SDS-PAGE analysis of MBP-fused EcR-LBD protein purification by IEC column (3rd IEC column and NaCl was 6.7 mM). Gel was stained with Imperial Protein Stain. Flowthrough from second IEC column was diluted from 20 mM to 6.7 mM NaCl and again loaded on IEC column. 15 µl sample + 15 µl 2x loading buffer were loaded on lanes 2-8. Lane 1, protein ladder. Lane 2, flowthrough from second IEC column (20 mM NaCl). Lane 3, dilution of second IEC column flowthrough at 6.7 mM NaCl. Lane 4, flowthrough from third IEC column. Lanes 5-8: elusion at 20 mM, 50 mM, 100 mM and 500 mM NaCl of third IEC column purification, respectively.

4.3.5 Purification of MBP fused EcR-LBD by size exclusion chromatography

SEC, which separates proteins according to their apparent size, was chosen as the last step of purification. A Superdex-packed SEC column was equilibrated with a buffer containing 150 mM NaCl and then IEC-purified MBP-EcR-LBD protein was loaded on the column. The MBP-EcR-LBD was eluted right after the void volume (Figs 4.3.5.1 and 4.3.5.2), indicating the protein was aggregated. High salt column equilibration buffers (i.e., 0.5 M or 1 M NaCl) did not affect the elution profile (data not shown).

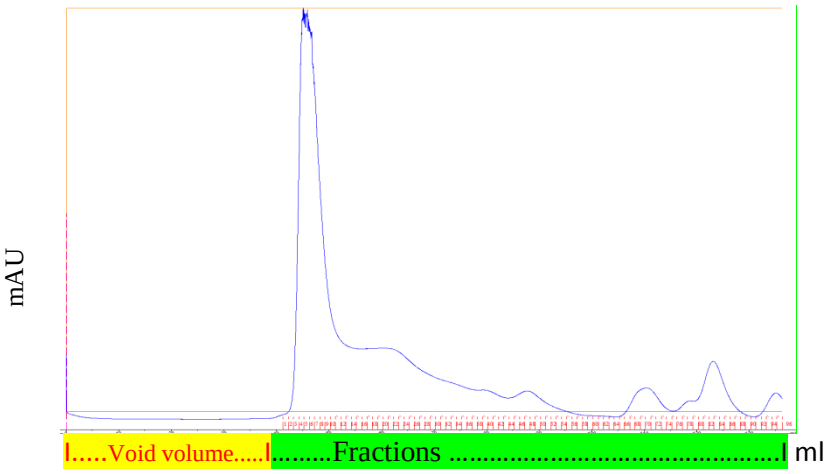

Figure 4.3.5.1 Chromatogram of MBP-fused EcR-LBD protein from SEC using HiLoad 16/600 Superdex 200 prep grade.

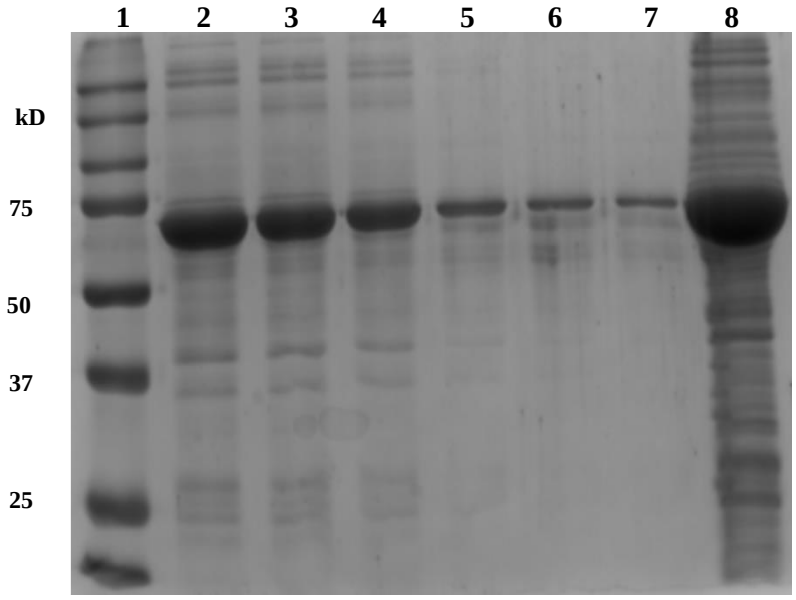

Figure 4.3.5.2 SDS-PAGE analysis of purification of MBP-fused EcR-LBD protein by SEC using HiLoad 16/600 Superdex 200 prep grade. Gel was stained with Imperial Protein Stain. 7 µl sample + 7 µl 2x loading buffer were loaded on lanes 2-8. Lane 1, protein ladder. Lanes 2-7: sequential samples of fraction number 4-9, respectively. Lane 8, supernatant.

4.4. Interaction study between MBP-EcR-LBD and Pon A using isothermal titration Calorimetry

The IEC-purified MBP fused EcR-LBD was used for interaction studies with Pon A. The sensogram (Fig 4.4.1) shows the heat developed with successive injections of Pon A into either buffer or MBP-EcR-LBD. The experiment shows that the heat generation is higher for Pon A injected into buffer alone compared to Pon A injected into MBP-EcR-LBD. This data suggest that there is no detectable interaction between MBP-EcR-LBD and its potential ligand Pon A.

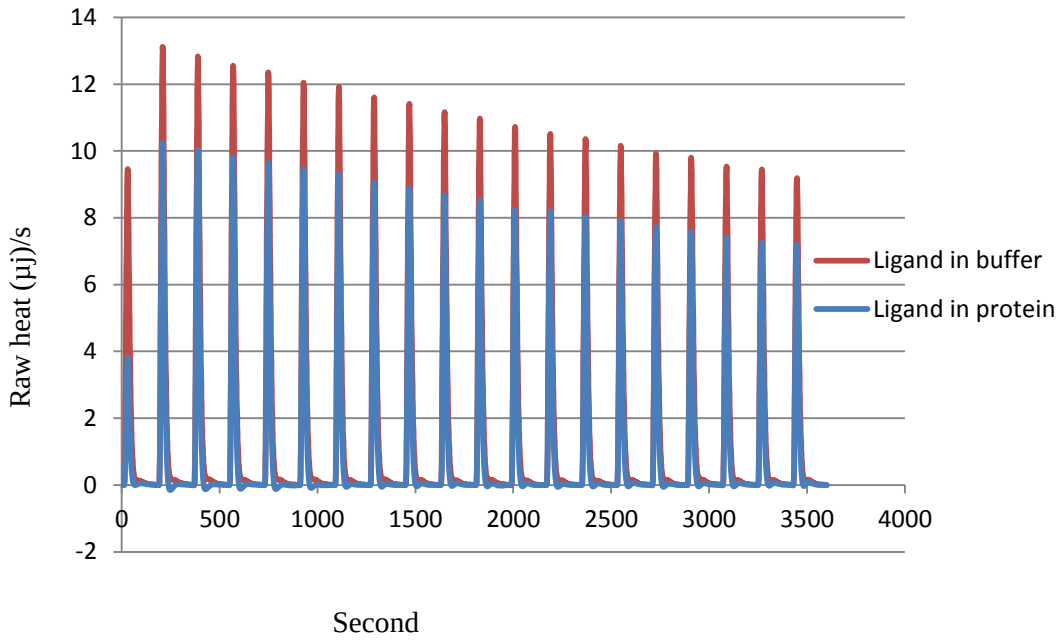

Figure 4.4.1 ITC analysis of Pon A (ligand) and MBP-EcR-LBD (analyte). ITC heat transfer curves of Pon A injections into either MBP-EcR-LBD or buffer alone. 20 injections of 8.6 µM Pon A into 2 µM MBP-EcR-LBD blue line. 20 injections of 8.6 µM Pon A into buffer alone red line.

5. DISCUSSION

The aims of this study were cloning of *L. salmonis* EcR-LBD, full length EcR (LBD+DBD) and full length USP (LBD+DBD) and then express them at optimum level using suitable expression vector, purification of expressed protein using different techniques and finally study the interaction with Pon A. The cloning of all three constructs was verified by sequencing. In this study, the expression vectors pETMBP and pETGB1 were used. From the previous experience of working with these vectors, protein production was induced at 18°C and the expression level for EcR-LBD and EcR was quite good. The process of protein purification technique selection is based primarily on trial and error. Several techniques were used with different parameters and this step took comparatively longer time. In IMAC, both EcR-LBD and EcR protein had similar problem. The binding between 6X-His and Ni-resin was very poor as His tag probably was hidden inside the protein. Assuming that EcR-LBD and EcR proteins behave almost in same way during purification as like as IMAC purification, only MBP-fused EcR-LBD protein was subjected further purification study. In ITC interaction study, there was no effective interaction between EcR-LBD and Pon A under experimental condition.

5.1 Sequence and Phylogenetic analyses

Sequence alignments of EcR and USP sequences from *L. salmonis* and other species showed that ligand-binding domains of both EcR and USP are less conserved than the respective DNA-binding domains (Figs. 4.1.1.3 and 4.1.2.3). The sequence divergence of LBD may open a way to design highly specific drugs targeting ligand-binding domains of each species, since any drug targeting the LBD of a species will be specific to the targeted species and may give minimal side-effect, if any, to other no-target species (Dhadialla et al., 1998, 2005).

5.2.1 Expression of EcR

The use of carrier proteins (such as MBP, GB1, GST) in protein expression constructs helps the desired protein to express better and to make it more stable and soluble (Kapust et al., 1999). In addition, they often provide efficient purification tags. In this study we used both MBP and GB1 to express EcR-LBD (and MBP for EcR 'full-length'). Both GB1- and MBP-fused EcR proteins were expressed strongly at 18^{o}C overnight (Figs. 4.2.1); however, since MBP-fused EcR proteins were more soluble than GB1-fused proteins, the former were used for further purification and binding studies. Our results on the function of MBP concurred

with earlier studies which showed that MBP provides better expression and yields more soluble proteins compare to other carriers due to its relatively large size and stability and the effect of using MBP is more pronounced with proteins expressed in bacterial systems (Kapust and Waugh, 1999; Fox et al., 2003).

5.2.2 Expression of USP

The USP expression construct did not yield a MBP-fused USP protein of expected size 92 kD (Fig 4.2.1.4), although under the same experimental conditions EcR proteins expressed very strongly (Figs 4.2.1.1, 4.2.1.3; also see Section 5.2).

In general, the instability of the mRNA, proteolysis of the target protein, improper protein folding, presence of rare codon in open reading frame, formation of hairpin at the 5'-side of the mRNA may inhibit the expression or very low expression of target protein in the *E. coli* strain (Kim et al., 2008). For *L. salmonis* USP, one could consider following two reasons. One is that the protein synthesis has stopped early on or the synthesis never has started, though we do not know what may have cause the stoppage. The other is that synthesised protein was degraded by the internal system, possibly due to faulty folding or to avoid the toxic effect of the newly synthesised protein. However, since USP proteins from other species have been expressed well in similar bacterial expression systems, these notions seem difficult to hold. The SDS-PAGE analysis of USP expression profile (Fig 4.2.1.4) reveals that the protein profiles are essentially the same between before and after IPTG induction. Especially, the same protein profiles of zero hour and 4 hours after induction (Lanes 2 and 4 in Fig 4.2.1.4) indicates no protein synthesis has occurred (or alternatively newly synthesised protein was degraded right after the synthesis).

Regardless of the causes of failed expression of USP in the bacterial system we employed, other ways of protein expression such as using insect cell-based system or cell-free in vitro system should be exploited.

5.3 PROTEIN PURIFICATION

5.3.1 EcR protein purification by IMAC

MBP-fused EcR proteins, which have 6X-His tag in the N-terninus of MBP, did not bind to the Ni-agarose resin and was instead eluted as a flowthrough fraction during IMAC-based purification (Fig. 4.3.1.1). Most likely reason for this seemed that the 6X-His tag was buried inside the protein and could not bind to the Ni-agarose resin. To test this possibility, we digested the fusion protein with the TEV protease and ran the digestion mixture on IMAC again under the same condition. As expected, now liberated MBP was bound to the Ni-resin and only eluted with very high (350 mM) imidazole (Fig 4.3.3.2, lane 8). The folding (or aggregation), which has prevented His-tag from binding to the Ni-resin, must have been very tight, as this did not loosen up neither at high salts (upto 1 M NaCl) nor with detergent (1.2% TX-100) (data not shown). Furthermore the overnight TEV digestion has completed only 50% (Fig 4.3.3.2, lane 5).

If the N-terminus His-tag was the cause of purification, one could make the fusion protein with a C-terminus His-tag. In this case, the fusion protein would be subjected to IMAC and eluted with a buffer containing high imidazole. (Later the MBP carrier could be cleaved with TEV and separated away from EcR by repeated IMAC.) However, the present aggregation problem is most likely due to extremely high hydrophobicity of the EcR protein (Fig. 5.1) hence changing the position of the His-tag may not solve the problem.

It is known that the size of protein directly influence the efficiency of His-tag binding to the Ni-resin, meaning the smaller protein is the tighter (or the more) binding occurs (Frangioni and Neel, 1993). However, neither the GBI-EcR-LBD fusion (size 38 kD) nor the two MBP fusions (EcR-LBD, size 72 kD; EcR-full, size 92 kD) had any differences and all eluted as flowthrough in IMAC. Therefore the size of EcR fusion proteins was not a factor in IMAC separation.

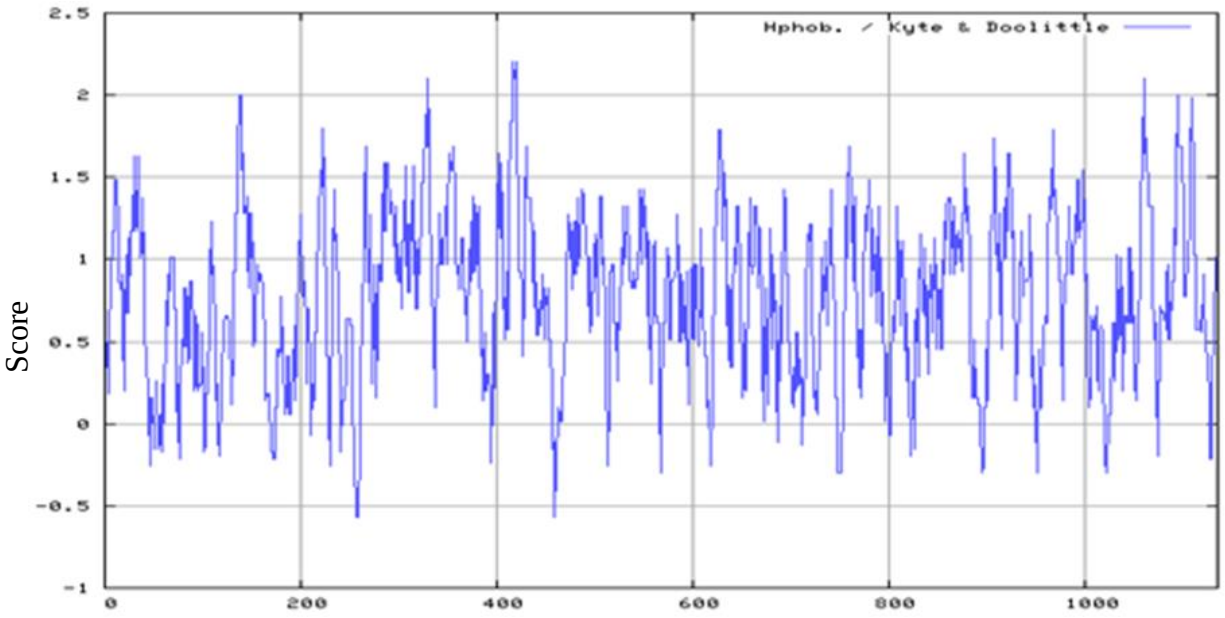

Figure 5.1 Hydrophobic plot of *L. salmonis* EcR using Kyte-Doolittle scale (1982). Positive value represents the hydrophobic region.

5.3.2 EcR protein purification by IEC using DEAE-cellulose

Anion exchange chromatography using weak positively charged DEAE cellulose gave thus far the only reliable mean to purify the aggregation-plagued MBP-EcR fusion protein. The procedures we employed were to run IEC at low salt (20 mM NaCl) first, at which MBP-EcR would elute as flowthrough whereas other impurities remain bound to the matrix. This flowthrough was further diluted to 6.7 mM NaCl and subjected another round of IEC with fresh DEAE-cellulose. At this very low salt condition, MBP-EcR did bind to DEAE-cellulose and the fusion protein was eluted with a buffer containing 100 mM NaCl. Using the successive IEC steps impurities could be removed first (at 20 mM NaCl) as bound form in DEAE, and second (at 6.7 mM NaCl) as flowthrough.

5.3.3 EcR protein purification by SEC

The size exclusion chromatography (SEC) is usually employed as the final step of recombinant protein purification where the affinity chromatography is often the first step. Our attempts to use SEC were not successful because the MBP-EcR fusion protein was eluted as high molecular aggregates without noticeable removal of impurities (Figs. 4.3.5.1, 4.3.5.2). Since these aggregates failed to loosen up or dissolved even in high salt buffer (1 M NaCl),

SEC could not be employed as a reliable mean for further purification of IEC-purified MBP-EcR fusion proteins. However, the fact that MBP-EcR elutes as very high molecular weight aggregates could be used to remove impurities with smaller molecular weight (likely 50 kD or less) and, if successful, this could yield MBP-EcR proteins with an acceptable level of purity.

5.3.4 Digestion of EcR-LBD protein with TEV

When MBP-EcR-LBD fusion protein was digested with the TEV protease and the MBP part including 6X His-tag was removed from the EcR-LBD protein (see Results; Fig 4.3.3.2), the EcR-LBD part was aggregated and became insoluble, which did not loosen up even at high salt of 1 M NaCl. Together with the fact that the digestion was completed only 50% made TEV digestion, thus removing the MBP carrier part for the eventual ligand-receptor binding studies, unachievable.

5.3.5 Interaction Study of EcR-LBD with Pon A

Under our experimental conditions EcR-LBD did not interact with its potential ligand Pon A (Fig. 4.4.1). One obvious possibility could be that the carrier protein MBP, which was fused to EcR-LBD, might have hindered the interaction. (The use of MBP-fused EcR-LBD, not MBP-less EcR-LBD, was necessary because without MBP the EcR-LBD protein precipitates.) Although we cannot rule out this possibility because the MBP only control assay has not been done, it is not likely that MBP was the reason for the non-interaction between EcR and Pon A. Other carrier proteins such as GST have shown that they had no influence on ligand binding to *Chironomus* EcR-LBD (Grebe and Spindler-Barth, 2002). More likely reason could be that purified EcR-LBD was either denatured or aggregated, thus the ligand was not able to bind it. This proposition is supported by a Kyte-Doolittle hydrophobicity plot (Fig 5.1), which shows that hydrophobic regions dominate over hydrophilic regions in most part of the protein. The excessive nature of hydrophobicity could not only make the purification very difficult, but also make purified protein more aggregation prone.

The use of USP in both purification and binding studies of EcR could be a way to overcome the challenges caused by the extraordinary hydrophobic nature of EcR proteins. Previous studies have shown that USP, which forms stable heterodimers with EcR, makes EcR more soluble and less aggregating upon purification of EcR (Li et al., 1997). Mammalian two-hybrid assay also showed that binding of ligand to the *L. salmonis* EcR is USP-dependent (Tolas, 2014). However, it should be noted that in *Drosophila* EcR could bind its ligands in

the absence of USP, albeit the binding affinities were lower than that of in the presence of USP (Grebe et al., 2003; Lezzi et al., 2002).

6. CONCLUSION AND FUTURE PERSPECTIVES

Expression constructs of the ecdysone nuclear receptor (EcR) from the salmon louse *L. salmonis* were made and expressed in a bacterial system. The expression level was very high for both MBP and GB1 constructs, with MBP-fused EcR proteins being more soluble, hence used in further studies. Both of MBP-fused EcR proteins (i.e., EcR-LBD and EcR 'full-length'), which had 6X His purification tag, did not bind to the Ni-resin upon separation using IMAC. The most likely reason could be that the His tag was hidden inside of the protein. The EcR proteins also seemed aggregated due to its very high hydrophobic nature and were eluted as a high molecular weight 'aggregates' in SEC. High salts (up to 1 M NaCl) failed to loosen them up. Unlike IMAC and SEC, IEC using DEAE under a very low salt concentration (6.7 mM NaCl) was able to remove most of impurities from the MBP-fused EcR proteins. Therefore, an IEC-based purification approach could be utilised for the purification of salmon louse EcR proteins or other highly hydrophobic proteins.

Another approach to purify and study EcR could be using USP. USP has been shown already to form stable heterodimers with EcR and they could be co-purified from the bacterial cultures. The EcR-USP dimers then can be subjected various studies including measuring interactions with potential ligands. One challenge to this approach is that the expression and purification of USP may require other expression system such as insect cells, which is not as facile as bacterial system. Nonetheless, using of insect cell-base expression system could be necessary as EcR (and USP) proteins expressed and purified from bacterial systems tended to be non-functional.

Besides ITC, Surface Plasmon Resonance (SPR) technique could also be used to study the interaction between ligand and analyte which allows to monitor the interaction between two molecules in real time.

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Appendix

A. Species used in phylogenetic analysis of EcR

Classification	Species	NCBI Accession number	Size (aa)
Crustacea	*Lepeophtheirus salmonis*	AIZ04022.1	536
	Tigriopus japonicas	ADD82902.1	546
Nematoda: Adenophorea	*Trichuris trichiura*	CDW58186.1	754
Nematoda: Secernentea	*Toxocara canis*	KHN78537.1	465
Hexapoda	*Locusta migratoria*	AAD19828.1	541
	Sogatella furcifera	AFC61183.1	569
Mollusca: Bivalvia	*Crassostrea gigas*	EKC19773.1	471

B. Species used in phylogenetic analysis of USP

	Species	NCBI Accession number	Size (aa)
Crustacea	*Lepeophtheirus salmonis*	AIE45497.1	442
Hexapoda	*Tribolium castaneum*	CAL25729.1	407
	Gryllus firmus	ADT64884.1	403
	Melipona scutellaris	AAW02952.1	427
Chelicerata	*Liocheles australasiae*	BAF85823.1	410
Mollusca	*Reishia clavigera*	AAU12572.1	431
	Haliotis diversicolor	ADK60866.1	441