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**EXPRESSION AND FUNCTIONAL CHARACTERIZATION OF CK-1: A  
PUTATIVE RAINBOW TROUT (*Onchorynchus mykiss*) CC CHEMOKINE**

By

Fatme Al-Anouti

A Thesis

Submitted to the Faculty of Graduate Studies and Research  
Through the Department of Chemistry and Biochemistry  
In Partial fulfillment of the Requirements for the  
Degree of Master of Science at the  
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Windsor, Ontario, Canada

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## **ABSTRACT**

Previous experiments by Dixon et al. (1997) succeeded in isolating a cDNA clone from rainbow trout (*Onchorynchus mykiss*) head kidney leukocytes that encodes an 8 kDa protein designated CK-1. This protein had structural similarities to molecules called chemokines identified in mammals and avians that attract leukocytes to inflammatory sites. It was similar to a certain family called CC chemokines and in particular the C6- $\beta$  subfamily because it had 6 cysteine residues. We have set a study to determine whether CK-1 was indeed a rainbow trout chemokine by testing for its function. We expressed CK-1 protein in prokaryotic expression vectors and purified it to homogeneity. When this protein was tested using seven different experiments of chemotaxis assay; which is the most conventional method to assay for chemotactic activity; it was chemotactic to rainbow trout leukocytes and in particular to lymphocytes. Another protein that we expressed under exactly identical conditions of CK-1 production called  $\beta$ 2 microglobulin ( $\beta$ 2m) was not chemotactic to trout leukocytes. Southern blot analysis of rainbow trout genomic DNA with CK-1 genomic fragments indicated that CK-1 was a single copy gene within rainbow trout genome. Tissue distribution and expression pattern of CK-1 transcript investigation revealed that CK-1 was an inducible gene like all other known chemokine genes. When rainbow trout was stimulated with a mitogen for 24 hours, CK-1 message was detected in the blood leukocytes, liver, head kidney and spleen upon northern blotting. A one hour stimulation failed to induce CK-1 transcription, however. CK-1 protein is the first teleost chemokine whose activity has been verified. The use of this chemokine could be helpful in aquaculture if used as a vaccine adjuvant against common fish pathogens.

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## I. INTRODUCTION

### Chemokine Subfamilies.

Studies involved in activation of leukocytes and chemotaxis have recently merged with searches for genes expressed in stimulated leukocyte subtypes to define a superfamily of inflammatory chemotactic cytokines called sis for small, inducible and secreted or intercrine (1). This array of molecules includes members like I-309, MCP-1 (monocyte chemotactic protein), IL-8 (interleukin-8), HC-14, C10 and many other molecules (1,2). All these members are small, inducible, basic, heparin binding and secreted proteins that are structurally related. One hallmark of members of this superfamily is the ability to induce chemotactic migration of specific cells such as lymphocytes and neutrophils and, hence, the name "chemokines". In addition to their chemotactic activity, these molecules can induce a wide variety of cellular responses: respiratory burst (in polymorphonuclear cells), change in adhesiveness of cells and increase in concentration of intracellular calcium. They are also involved in angiogenesis, regulation of cell proliferation and maturation and homing of migrating leukocytes (2,3).

All chemokines share 20-50% sequence similarity and have the same three dimensional structure consisting of three antiparallel  $\beta$  pleated sheets and an  $\alpha$  helical carboxy terminus (3). They are small: 60-80 amino acid residues with a typical molecular mass of 8-12 kDa. All chemokines are divided into  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$  subfamilies based on the presence of an intervening amino acid (a.a) located between the first two of four highly conserved cysteines. The first and third cysteines form a disulfide bridge while the second and the fourth form a second disulfide bridge (4). The  $\alpha$  chemokine subfamily (sometimes denoted CXC) contains a single a.a between the first two cysteine residues whereas in the  $\beta$  subfamily (sometimes denoted CC) the first two cysteines are not separated by an amino acid, but are adjacent to each other. The  $\gamma$  subfamily (sometimes denoted C) is unique because it lacks two of the four conserved cysteines retaining only cysteine 2 and 4. The most recently identified  $\delta$  subfamily (sometimes denoted CX3C) is so far represented by a single member called neurotactin or fractalkine which is an integral membrane protein with chemokine domain at its amino terminus

(4,5). This chemokine has only one cysteine motif in which the cysteine residues are separated by three amino acids and hence the name CX3C for its family (5).

The CC or  $\beta$  chemokine subfamily has different subgroups according to the total number of cysteine residues and their positions within the sequence. Some CC chemokines have only four cysteines like MCP-1 while others have additional cysteines. One subgroup called the C6- $\beta$  has 6 cysteines or 2 additional cysteine residues besides the conserved four like the human I-309 and CK- $\beta$ 8 and the mouse C10. The alignment of the two additional cysteines is conserved within this subgroup and there is always an aspartate residue preceding the first CC motif (2,3). Other  $\beta$  chemokines may have additional cysteine residues like the chemokine C6kine (which also has 6 cysteines) but do not belong to the C6- $\beta$  subgroup because they do not share the same alignment for their cysteines (3).

Members of the same subfamily share specificities for certain leukocytes. For example, the  $\alpha$  subfamily members IL-8, NAP-2 (neutrophil activating peptide-2), MGSA (melanocyte growth stimulating activity) all induce a plethora of activities in PMN cells including chemotaxis and granule release, but have no effect on monocytes (5). On the other hand, members of the  $\beta$  subfamily such as MCP-1, RANTES (regulated upon expression normally T cell expressed) and I-309 predominantly stimulate monocytes, T cells, basophils and eosinophils as well (3,4). Thus, CC members are responsible for chronic inflammatory responses whereas CXC members mediate acute inflammation (4). Members of the  $\gamma$  subfamily like lymphotactin specifically attract T cells and NK cells while fractalkine attracts lymphocytes but not granulocytes (6).

The expression pattern of these chemokines is also variable because some chemokines are produced by a wide variety of cells whereas others display a much more restricted pattern. IL-8 is for instance produced by monocytes, T cells, fibroblasts, endothelial cells, keratinocytes, neutrophils, chondrocytes and hepatocytes. Platelet factor-4 (PF-4) on the other hand, is only secreted by platelets (7).

Many of the known chemokines were first identified by molecular cloning of cDNA clones encoding protein of unknown function. It is only now that their biological properties are coming to light. Most techniques exploit mRNA induction by immune cells upon mitogenic stimulation (8). It is worth mentioning however, that although

inducibility is a feature of most chemokines, it is not a universal property (1,2). For instance, RANTES mRNA is actually reduced after activation of differentiated T cells and hence its name regulated upon activation normally T cell expressed and secreted (2).

### **Mammalian Chemokine Genes: Organization and Chromosomal Clustering.**

Genes encoding the chemokines are clustered in close physical proximity to each other. A large cluster of human CC chemokine genes resides on the long arm of chromosome number 17 although two members of this same subfamily: TARC and LARC have been mapped to chromosomes number 16 and 2 respectively (9). All CXC genes are clustered on chromosome number 4 in humans while lymphotactin (C subfamily) has been mapped to chromosome number 1. Fractalkine, a member of the CX3C subfamily has been mapped to human chromosome number 16. This unique clustering is also encountered for other mammalian and avian chemokine genes identified so far suggesting that these genes may have arose by duplication (9).

All chemokines belonging to the same subfamily have the same gene structure. For CC members, they all exhibit a 3 exon/2 intron structure, where the first exon contains the 5' UTR and nucleotides coding for the leader peptide. The second exon encodes the signal peptide and half of the mature protein including the CC motif and the third cysteine. The third exon consists of the rest of the protein sequence which has the fourth (last) conserved cysteine and the 3' UTR (9). There is a highly conserved intron/exon junction among these genes: the position of the second intron in all of these molecules is precisely maintained splitting the codon of a conserved isoleucine residue. The CXC subfamily has a very similar structure to that of CC though they contain an additional intron separating the exon containing most of the 2<sup>nd</sup> half of the protein from an exon encoding a few carboxy terminal amino acids and the 3'UTR (9). The CC genes are termed Scya genes for small cytokines while the CXC genes are called IL-8 or IL-8 related genes (9).

### **Chemokine Receptors.**

The effects of chemokines on leukocytes are mediated by heptahelical receptors coupled to GTP-binding proteins called GPCRs for G protein coupled receptors. They are

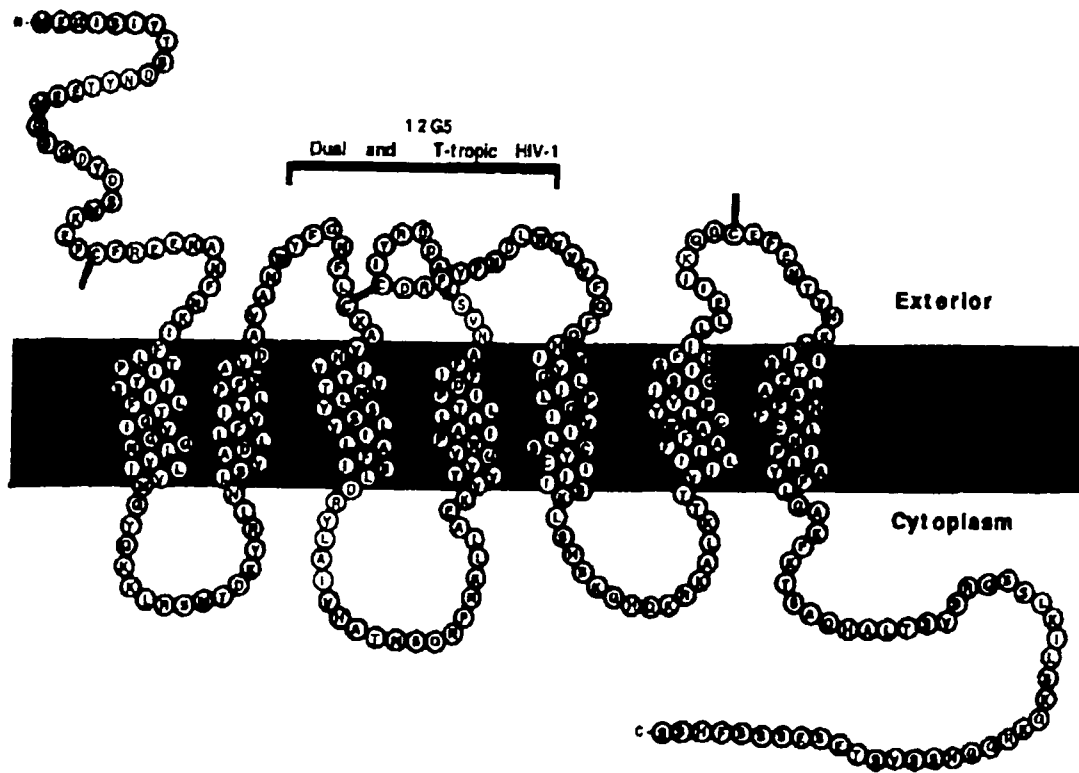


members of the extensive serpentine superfamily (10). This family is also named after the rhodopsin receptor that signals light sensitivity in the retina (10, 11). Chemokine receptors have seven hydrophobic transmembrane domains with an extracellular N terminus and an intracellular cytoplasmic C-terminal tail (figure 1). The hydrophobic regions of the receptor traverse the plasma membrane separating intra- and extra-cytoplasmic loops giving it a serpentine appearance and hence the name (10). The N terminus is quite variable between chemokine receptors and is believed to be involved in dictating ligand specificity. Both the N terminus and extracellular loops contain a number of conserved cysteine residues that form disulfide bonds important for conformational stability and rigidity. The seven transmembrane (TM) domains are highly conserved between chemokine receptors but one sequence: DRYLAI (forming a motif in the second intracellular loop adjacent to the third TM domain) is highly conserved and implicated in G-protein signalling (10). The cytoplasmic tail of these receptors is short usually with 40-30 amino acid residues. It contains a number of serine and threonine residues that can be substrates for phosphorylation by kinases and can thus mediate the signalling cascade. This tail has additional residues called arrestins that interact directly with G-protein (10).

Figure 1. Model of a chemokine receptor.

Representation of chemokine receptor CXCR4 integrated into cell membrane: Hydrophobic regions traverse the membrane while the amino terminus is exterior to the cell and the carboxy terminus is interior. The receptor is a co-receptor for HIV (Reference 11).

Figure 1.



### **Chemokine receptors: Expression and genomic localization.**

The chemokine receptors are classified according to their ligands' classification. For instance, there are CXCRs (CXC chemokine subfamily receptors) and CCRs (CC chemokine subfamily receptors) specific for CXC and CC chemokines respectively. The same receptor can bind to different chemokines within the same subfamily, however, and the same chemokine can bind to different receptors in the same receptor subfamily (11). For instance, RANTES can bind CCR1, CCR2 and CCR4 but MCP-1 and MIP-1 $\alpha$  can also bind to CCR2 (11). This overlapping relationship between chemokines and their receptors indicates a certain degree of redundancy which allows for a convergence of signal transduction. CC chemokine receptors have been mapped to chromosome number 6 in humans while IL-8 receptors (1 and 2) have been mapped to chromosome number 2 and studies are still trying to locate other CXC receptors (10). In fact, as the list of identified chemokines increases, more receptors are being hunted for.

The expression of chemokine receptors is completely dependent on the state of cellular differentiation and type of stimulus. Thus, naïve T cells express only CCR7 and CXCR4 whereas a more differentiated T cell expresses CCR1, CCR2, CXCR3 and CXCR5 (12). Some factors can modulate this expression like IL-5 which down-regulates expression of CCR2 and CCR1 in activated T cells or like IL-2 that can induce expression of those two same receptors (12).

### **Interactions between chemokines and their receptors.**

Binding of chemokines to their receptors triggers cascade of reactions that eventually lead to the attraction of leukocytes to inflammatory target sites. To define which a.a. residues are important for receptor binding, changes in amino acid sequences of MCP-1 introduced by site directed mutagenesis revealed that tyrosine at position 28 (highly conserved among all  $\beta$  chemokine subfamily members) is absolutely essential for receptor binding. Other residues like arginine, lysine and threonine are also important and are located within the first inter-cysteine loop (12). In  $\alpha$  chemokines, the tyrosine at position 28 is replaced by valine or leucine that are also highly conserved among the subfamily members. Furthermore, CXC members are structurally distinguishable by a conserved Glu-Leu-Arg (ELR) motif within the amino terminus region. This motif is an

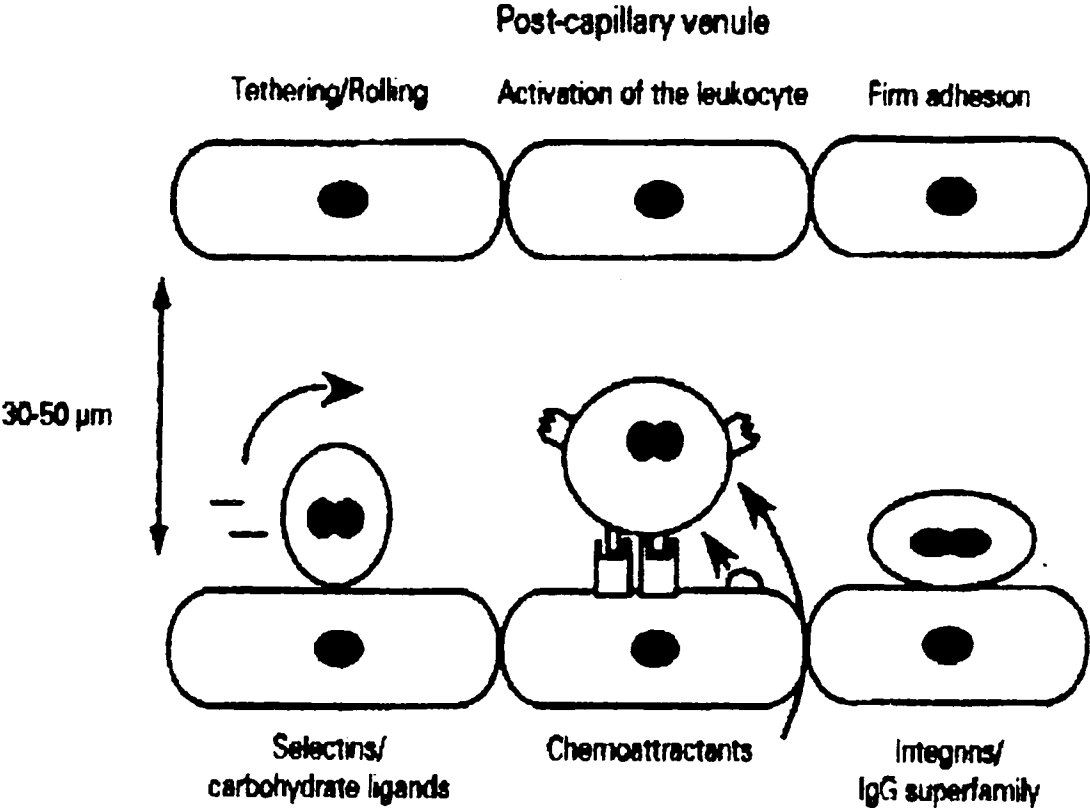
absolute requirement for chemotactic potency and signal transduction. Any change in this motif abolishes chemotactic activity without affecting the chemokine's ability to bind to its receptor (12,13). For CC chemokines, it is aspartate (D) within the amino terminus that mediates signal transduction. In fact, mutation studies have revealed that the amino terminus is essential for activity and that chemokines contact their receptors at multiple sites. The amino terminus of chemokine contacts the extracellular amino terminus of the receptor while the carboxy terminus of chemokine contacts regions within the receptor's external loops adjacent to TM domains (13).

The most impressive effect of chemokines on leukocytes is the shape change observed after addition of chemokines to a suspension of leukocytes. Polymerization and breakdown of actin leads to the formation and retardation of lamellipodia of migrating cells. Stimulation also induces the up-regulation and activation of integrins (extracellular membrane proteins which enable WBC adherence to endothelial wall) before migrating into tissue (figure 2). Other rapid and transient responses are characteristic of the activation of leukocytes by chemokines such as the increase in intracellular calcium concentration (13).

Figure2. Chemotaxis of leukocytes and migration from blood.

Three stages are involved: activation of cells by chemokines, integrin mediated diapedesis and finally passage through endothelial wall ( reference 13).

Figure 2.



Upon chemokine binding, receptor coupling with G-protein initiates a signalling cascade leading to the activation of phosphatidyl inositol specific phospholipase C, protein kinase C and small GTPases. Phospholipase C delivers two second messengers : Inositol 1,4,5 triphosphate (which releases Calcium from intracellular stores thus leading to a transient rise in the concentration of cytosolic calcium) and diacylglycerol which activates protein kinase C (11,14). It is the intracellular loops of chemokine receptors that interact with GDP-bound protein. Upon ligand binding, the receptor activates the G-protein that exchanges GDP for GTP. It is the GTP-bound form of G-protein that later activates effector enzymes, GTP is then hydrolyzed to GDP<sub>i</sub> and the GDP form of G-protein completes the circuit by reforming a complex with unoccupied receptors (11,14).

All chemokine receptors have been shown to be sensitive to PTX (pertussis toxin) and GTP- $\gamma$  S (guanosine-5'- $\gamma$ -thiotriphosphate). The sensitivity is actually due to G-protein coupling (11). PTX catalyzes the ADP-ribosylation of the 41 kDa G-protein with GTPase activity. Thus, the recycling between GTP and GDP is blocked and the signal transduction is interrupted. This abolishes the chemotactic potency of chemokines though its receptor binding is intact (11). Many scientists have used PTX to prove that their putative chemokines actually bind to G-protein coupled receptors as do all other identified chemokines. GTP- $\gamma$  S cannot provide a substrate for phosphorylation of GDP and thus has a blocking effect on chemokines and their receptors although it locks the G-protein in the activated state (11).

The activities of chemokines also overlap with activities of non-chemokine molecules like C5a the human complement protein (15) and fMLP the formylated peptide Met-Leu-Phe (16). FMLP is the prototype of bioactive formyl peptides that is actually a bacterial breakdown product potently chemotactic to neutrophils and monocytes. This molecule can also cause oxidative burst degranulation, leukotriene secretion and *de novo* synthesis of proteins by these cells (16). The pleiotropic effects of fMLP are mediated via a G-protein coupled receptor called the N-formyl peptide receptor NFPR. So far there have been reports about two different receptors for fMLP that belong to the same superfamily of chemokine receptors. Although chemokines and fMLP have no structural homology with each other, their receptors are the same and thus can exert the same chemotactic effect on leukocytes (16).



Other chemotactic agents whose receptors belong to the same superfamily as NFPR, include the C5a complement component. C5a is a 74 a.a long glycopeptide cleaved from the amino terminus of C5 complement during activation. The C5a fragment serves as a potent mediator of the acute inflammatory responses (anaphylatoxin) because it attracts neutrophils. Its ability to induce mast cell histamine release, neutrophil chemotaxis and lysosomal enzyme release is mediated through binding to its G-protein coupled receptor (15). Mutagenesis studies have revealed that residues Arg37, Arg40, Leu43, Arg46, Lys49, Glu53 and Arg62 of human C5a are important for receptor binding. It was recently found that C5a causes the release of IL-8 from bronchial epithelial cells suggesting a possible role in asthma (17). C5a can influence both humoral and cell mediated immune responses because it can augment antigen induced T cell proliferation and bind to macrophages stimulating the release of IL-1. All the effects of C5a are however abolished by anaphylatoxin inactivator (AI), which removes the carboxyl-terminal arginine from the molecule upon down regulation of inflammation (17).

### **Fish Chemokines.**

Recently, many immunologically important genes such as T cell receptors, major histocompatibility, and immunoglobulin genes have been successfully identified in many fish species. However, there is still much to be learned about fish immune systems and their functions including the presence of cytokines. Two different cytokines, TGF- $\beta$  (tumour growth factor) and IL-1  $\beta$  have been cloned from rainbow trout (*Oncorhynchus mykiss*) showing homology with mammalian and avian counterparts (18). Two different putative chemokine receptors CCR7 and CXCR4 have also been cloned in rainbow trout although there has been no attempt to clone their ligands the chemokines (19). Some researchers however, have succeeded in cloning chemokines from other fish species. For lamprey (*Lamperta fluvlatilis*) an IL-8 homolog was cloned (20) while for carp (*Cyprinus carpio*) a CC chemokine homolog and CXCR4 have been cloned (21). Although many reports have provided evidence for locomotive activity of fish neutrophils and other leukocytes and their ability to migrate in response to host-derived

factors (complement and eicosanoids) and pathogen-derived factors, the use of fish chemokines in such studies has never been attempted (22).

A previous experiment by Dixon *et. al.* succeeded in isolating an 824 bp long rainbow trout cDNA clone of which 508 bp are 3' UTR. The clone's open reading frame predicts a protein 73 a.a. long that has the characteristic cysteine motif identical to that of CC chemokines (23). This protein, called CK-1, had a 33-36 % a.a sequence similarity to the C6- $\beta$  subgroup within CC chemokines. Like all members of this subgroup, CK-1 had two additional cysteines besides the four conserved that align with the ones found in members of this subgroup and an aspartate preceding its first CC motif in the amino terminus (23). Although these characteristics are met by CK-1, its amino terminus is severely truncated because the only a.a after the signal sequence is actually an aspartate residue that precedes the first CC motif. Other CK-1 similarities to CC chemokines include the presence of 10 out of 11 amino acids conserved in all CC chemokines including the tyrosine that is critical for receptor binding. In addition, of three basic amino acids (either R or K) in a special motif responsible for binding to receptor, two (a.a 2 and a.a 4) are found within CK-1 (figure 3). Furthermore, the 3' UTR of CK-1 cDNA has a triple AUUUA motif characteristic of all cytokines, including chemokines, and other genes involved in the control of inflammatory reactions. These motifs are thought to be responsible for rapid post-transcriptional regulation of gene expression by affecting mRNA stability through degradation by RNAase E (23).

A genomic library made from a single rainbow trout designated trout J was screened by Dixon *et al.* with CK-1 cDNA probe to reveal the genomic organisation of CK-1 gene. CK-1 gene structure consists of 4 exons and 3 short introns. The first two exons encode sequences within the mature protein including the first CC motif and the third conserved cysteine plus one cysteine typical of the 2 additional cysteines found in the C6 group of CC chemokines. The third exon encodes additional sequences including the last conserved cysteine and the second cysteine typical of C6 group. The last exon consists of the entire carboxy terminus and the 3' UTR. The three introns are short and contain repetitive elements especially intron three which has 20 repeats of GT (23). Although CK-1 protein has structural features typical of CC chemokines, its gene organisation is not similar to that family. In fact it is similar to that of CXC chemokines,

which also have four exons (additional exon between exon 2 and 3) and three introns. It is worth mentioning that C6 group members are also exceptional in that they have 4 exons although their additional exon is between exon 1 and 2 of the common CC chemokine gene organisation (23). Thus, CK-1 and its gene share qualities with both mammalian  $\beta$  and  $\alpha$  chemokine genes implying that this gene may represent a primordial gene from

which modern mammalian chemokine genes have evolved (23).

Figure 3. Amino acid sequence of rainbow trout CK-1. The numbers indicate the amino acid positions in the consensus sequence derived from human CC chemokines MIP- $\beta$ , MIP- $\alpha$  and RANTES. Dashes (-) indicate identity with the consensus sequence, while stars (\*) indicate deletions. The dots (.) indicate the 11 amino acids that are conserved in CC chemokines while triangles indicate the position of the additional pair of cysteines and the aspartate which are common to CK-1 and C6- $\beta$  chemokines (reference 23).

Figure 3.

1            10            20            30            40            50            60            70

              Δ..            Δ            .            .            .            .            ..            .            .            Δ            .

*consensus* QPDSDTPTACCFSTARKIPRNFVADY·FE ··TSSKCSKPAVIFKTKRSKQVCADPSEKW·····VQEYMSDLFLNTQTPKIT  
*CK-1*        ········D - LKF·R·PVHCRWLKG-T·QDI ···· - DLN ····-QNL - N · F······ QDTKR ·· RCLRKRQEKKSQPKRV

## Objectives

Although CK-1 rainbow trout protein is predicted to have most features expected from a chemokine, no proof of chemokine function has been yet obtained.

Thus, the purpose of this study is to characterize the function of recombinant CK-1 expressed in prokaryotic expression vectors. For this purpose, tissue distribution and expression pattern of CK-1 mRNA will be investigated by northern blotting using RNA from different rainbow trout tissues like the spleen, head kidney, brain, liver and blood leukocytes at 0, 1 hour and 24 hour time intervals following fish mitogenic stimulation with PHA. In addition, to determine the CK-1 gene copy number in the trout genome, southern blotting of genomic DNA from trout using CK-1 genomic DNA as a probe has to be done. The ability of CK-1 protein to recruit rainbow trout leukocytes will be tested using chemotaxis assay, which is the most conventional method of checking for chemotactic activity.

Our interest in identifying fish chemokines resides in beneficial qualities of such molecules, which can be used as vaccine adjuvants against some fish infectious diseases and other veterinary purposes. We can study the possible effect of CK-1 as a vaccine adjuvant on the immune system of rainbow trout challenged with certain fish pathogens (for example haemorrhagic virus or certain bacteria like *Aeromonas salmonicida*). In addition, the discovery of divergent chemokine counterparts in other species is important from an evolutionary point of view and can prove that all mammalian and other lower vertebrate chemokines segregated from a common ancestral gene that was similar to a chemokine gene once found in the common ancestor of all vertebrates. CK-1 chemokine sequence can pave way for cloning other fish chemokines in different fish by using it as a probe.

## **II. MATERIALS AND METHODS**

### **I. A. CK-1 cDNA Expression Cloning in Bacterial Cells.**

The plasmid vector pRSET A (Invitrogen) with the CK-1 cDNA fragment cloned at the *BamH I* site (donated by B. Dixon) was checked by sequencing prior to use for protein production. The construct was transformed into *E. coli* strain BL21(DE3) (Novagen.) using the  $\text{CaCl}_2$  protocol (24) and grown on (50 ug/ml) LB+ampicillin plates. The presence of CK-1 cDNA insert in bacterial cells was then verified by picking a colony from the plate, doing plasmid extraction (phenol/chloroform protocol) and digestion with the restriction enzymes *Nde I*, and *Hind III*. The restriction digest was analysed on 1 % agarose gel (24). Bacterial cultures containing the insert were then induced with IPTG (1 mM) at 37 °C for 4 hours when bacterial broth showed an absorbance of 0.6 at 600 nm wavelength thus corresponding to the log phase of cell growth. To isolate recombinant fusion proteins formed as inclusion bodies, cultures were then centrifuged for 20 minutes at 4000x g. The supernatant was discarded and bacterial pellet was resuspended in lysis solution consisting of 25% sucrose, 50 mM Tris pH 8, 1 mM EDTA, 1 mM DTT, 1% Triton X-100 and 1 mg/ml lysozyme. DNase (30 ug/ml) was then added to the bacterial lysate and incubated for 30 min. at 37°C. After two successive cycles of freezing and thawing, the inclusion bodies were collected by spinning at 10,000 xg. The pellet was washed 5 times with wash solution consisting of 0.5 % Triton X-100, 50 mM Tris pH 8, 100 mM NaCl and 0.1 % azide followed by centrifugation at 10,000 xg for 20 minutes after each wash. After the last centrifugation, purified inclusion bodies were dissolved in urea solution (8 mM urea, 0.1 mM DTT and 1 M MES pH 6.5). The dissolved proteins were finally collected by spinning at 15,000 xg for 1 hour (24).

### **II. B. Purification of Inclusion Bodies.**

Recombinant proteins were purified from the urea solution under denaturing conditions on a nickel resin (ProBond, Invitrogen) that binds the vector-encoded histidine residues fused to recombinant CK-1. Excess bacterial cell proteins were eluted by the

addition of denaturing binding buffer (8 mM urea, 20 mM sodium phosphate, 500 mM sodium chloride) at pH 7.8. Host cell proteins with some inherent affinity for the ProBond resin were removed by washing with denaturing wash buffer, pH 6. The recombinant protein was eluted using pH 4 denaturing elution buffer and collecting five to six 1ml fractions. Protein concentration in each collected fraction was estimated by Bradford dye assay (Biorad) using Bovine serum albumin as a standard (35). Fractions containing the protein were then dialysed against 50 mM Tris buffer pH 7.5 and 0.1 mM DTT (24). Dialysis was gradual with the protein solution in 8 M urea being dialysed against 4 M urea solution for 4 hours then against 2 M urea solution for another 4 hours and finally against Tris buffer overnight (24). Recombinant CK-1 was then digested with enterokinase to remove the nickel binding domain (25,26). Digestion was carried out at 37 °C for 24 hours with a 1/40 enzyme:substrate ratio. Digested CK-1 was purified by passage over the nickel column and washing with denaturing binding buffer pH 7.8. Washes were collected and assessed for digested protein concentration this time. Undigested CK-1 still containing the nickel binding domain was eluted using elution buffer. To confirm removal of the nickel binding domain, digested CK-1 was analysed on a 17% SDS-PAGE stained with coomassie blue (24).

## **II.C Production and Purification of $\beta$ 2 Microglobulin.**

A plasmid vector pRSETA with  $\beta$ 2 microglobulin ( $\beta$ 2m) cDNA fragment from rainbow trout cloned at the *BamH I* site (donated by B. Dixon) was used for recombinant protein production of  $\beta$ 2m. This protein is a component of the major histocompatibility complex (MHC) class I and is important in antigenic presentation. The purpose behind the production of recombinant  $\beta$ 2m was to check for chemotactic activity in chemotaxis assay along with recombinant CK-1. This would check whether CK-1 chemotactic activity was due to protein itself or contamination with bacterial components due to the nature of expression method. All conditions for bacterial transformation and induction, protein purification and enterokinase digestion were exactly the same as those for CK-1 production. Recombinant  $\beta$ 2m was produced as a fusion protein 12 kDa in size and the digested mature protein was 8 kDa like the size of mature digested recombinant CK-1.



## **Chemotaxis Assay.**

### **II. D. Collection of Fish Blood Cells.**

Blood was collected from the caudal vessel of anaesthetised rainbow trout in heparinised syringes with 23 gauge needles. Fish leukocytes were separated by density gradient centrifugation using 54% Percoll (Sigma), assessed for viability by Trypan exclusion, counted on a hemocytometer and suspended in Minimal essential medium MEM at a final concentration of  $1 \times 10^7$  cells/ml (27).

### **II. E. Chemotaxis Assay.**

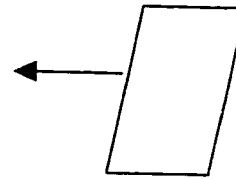
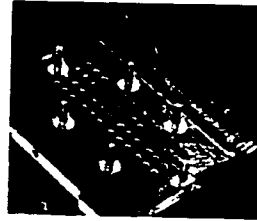
Chemotaxis was assayed in a microchemotaxis chamber (Neuroprobe Cabin John, Maryland). The lower chamber wells were loaded with 28.5 ul serial dilutions of recombinant CK-1 (100, 10 and 1 ug/ml). The upper chamber wells were assembled onto the lower chamber with a filter sheet containing 0.5 um pores in between (27,28) and then loaded with the prepared rainbow trout leukocyte suspension at a concentration of  $1 \times 10^7$  cells/ml (45 ul/well) (figure 4). The experiment was always duplicated for the positive control (29) (10 nM C5a complement component) and the negative control (MEM). After a 60 minute incubation period (5% CO<sub>2</sub> at 20°C), the chemotaxis chamber was disassembled, the filter scraped, then washed 3 times in PBS from the top side only to remove non-migrating cells and finally fixed in methanol. The filter was stained with Giemsa (Sigma) for 35 minutes, washed twice in water, mounted on a microscope slide, fixed with a histological mounting agent Permount (Fisher) and covered with a coverslip. The assay was scored by counting the number of cells which migrated through the filter in 5 different 40x fields per well and comparing results to those obtained with the positive and negative control (29). A total of seven different chemotaxis assays were done each using a different fish .

Figure 4. Schematic diagram of chemotaxis assay using the microchemotaxis chamber.

The leukocyte suspension is added onto upper plate wells while the chemokine or controls are added to lower plate. The filter fits between the two plate and is topped by a gasket to prevent leakage (reference 27)

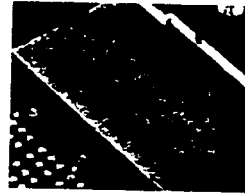
Figure 4.

**A.** Lower plate wells are loaded with chemokine or controls

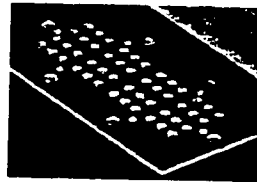


**B.** Transparent Porous filter is placed on top of lower chamber

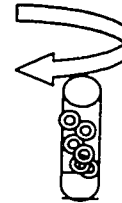
**C.** A gasket fits over the filter to fix filter on lower plate



**D.** An upper plate fits on top of the gasket and is screwed by bolts providing wells



**E.** Leukocyte suspension from buffy coat is pipetted into the holes of upper plate



## **II. F. Checkerboard Migration Assay of rCK-1 on trout Leukocytes.**

To determine if CK-1 induced chemotaxis (directed movement with a gradient attractant) or chemokinesis (random movement irrespective of a gradient) in trout leukocyte migration across filters, a checkerboard assay was performed (29). The chemokine was added to both upper and lower chambers of the apparatus using combinations of three different concentrations (1,10 and 100 ug/ml) such that the chamber had positive, negative or no concentration gradient between upper and lower wells. The incubation, washing and staining were the same as those described above for the typical chemotaxis assay.

## **II. G. Limulus Amebocyte Lysate (LAL) Test for Endotoxin.**

The use of a bacterial expression system in the production of recombinant CK-1 protein introduced the possibility of endotoxin contamination. The rCK-1 preparations were tested for contamination using E-Toxate Multiple Test Vial (Sigma) according to the manufacturer's instructions (30).

### **Southern Blotting.**

## **II. H. Isolation of Genomic DNA.**

One rainbow trout was anaesthetised and bled to death by cutting its caudal vessel. Its head kidney was homogenised using 1.5 ml fish extraction buffer (100 mM Tris pH 8, 10 mM EDTA, 259 mM NaCl and 1% SDS). Proteinase K (300 ug/ml final) was added and the homogenate was then incubated at 45°C overnight. The solution was extracted twice with an equal volume of phenol and twice with an equal volume of chloroform. The DNA was precipitated with equal volume of propanol at -80°C for an hour and centrifuged. The pellet was resuspended in 100 ul of 1 M Tris-EDTA (TE) pH 7.5 and precipitated with 10 ul of 3 M NaOAc and 250 ul of absolute ethanol. After being washed with 1 ml 70% ethanol, the pellet was finally dissolved in TE to a final concentration of 2.8 ug/ul (31,32).

## **II. I. Probe Construction for Southern Hybridization**

Based on the rainbow trout CK-1 genomic sequence, two primer pairs S5 5'TGGAGAGGGCTTTCATTACAG3' /AS 5'CCAAATACCCATTTTATTATC 3'and CKJ6f 5'AGGTGTAGAGTCTGTGTCCTGG3' CKJ6r5'TTTCCACGCTT ACATGCCTAC 3' were designed. The probe was split on purpose to avoid a highly repetitive region within rainbow trout genomic sequence. The first primer pair spanned a region of 490 bp along the trout CK-1 genomic sequence while the second pair spanned a region of 591 bp. PCR amplification was performed for 1 cycle of 5 minutes denaturation at 94°C, 35 cycles of : 30 seconds denaturation at 95 °C, 2 minutes annealing at (49 °C for S5/AS5) or (55 °C for CKJ6f/CKJ6r) and 30 seconds of extension at 72 °C. A final extension cycle was performed for 10 minutes at 72 °C. The reactions consisted of 1 ul *Taq* polymerase (Perkin Elmer), 4 ul of 10 pmole/ul forward and reverse primer, 10 ng genomic DNA template (from a genomic rainbow trout clone designated J6), 16 ul of 1.25 mM dNTP, 10 ul of 10X reaction buffer, 10 ul of 25 mM MgCl<sub>2</sub> and 25 ul pure distilled water. Total reaction volume was 50 ul per tube and all amplifications were done using a Gene Amp 2400 thermal cycler (Perkin Elmer). Amplified products were resolved on a 0.7% agarose gel stained with 10 ug/ul ethidium bromide run in 1x Tris borate EDTA (TBE) buffer (24,33).

## **II. J. Probe Labelling for Southern Hybridization.**

The probes amplified above were labelled using PCR DIG probe synthesis kit (Roche) (31). For labelling of the genomic PCR products (591 and 490 bp), 12.7 ul pure water, 2 ul PCR buffer with MgCl<sub>2</sub>, 2 ul PCR DIG probe synthesis mix (containing dATP, dCTP, dGTP, dTTP and DIG-11-dUTP), 0.3 ul Enzyme mix (3.5 *Taq* units/ul), 1 ul genomic clone J6 template and 2 ul of reverse and forward primers 10 pmole/ul each were mixed in a 20 ul reaction volume and PCR amplified. Amplification consisted of a 1 cycle of 2 minutes denaturation at 95°C and 35 cycles of denaturation for 30 seconds at 95 °C, annealing for 30 seconds at (55°C for CKJ6 fragment and 49°C for S5/AS5 product) and extension at 72°C for 1.5 minutes. The final extension was performed at the end of the 35 cycles for 7 minutes to ensure amplification (31,33). All products were run on 1.5% agarose gel stained with 10 ug/ul ethidium bromide using 1x TBE buffer and compared to unlabelled products for a shift in their molecular weights.

## **II. K. Southern Blotting.**

Genomic DNA was digested separately with 0.5 units of *Hind III* and *EcoR I* enzymes per 1 ul DNA (24). Samples (20 ug) were electrophoresed on 0.7% agarose gel using 1x TBE buffer overnight at 30 Volts and then transferred to a positively charged nylon membrane (Hybond, Pharmacia) by capillary action using 20x sodium chloride sodium citrate SSC as the transfer buffer (34). DNA was crosslinked onto the membrane by UV irradiation (150 mJ).

## **II. L. Hybridization and Stringency Washes.**

The blot was prehybridized at 42°C for 4 hours in 100 ml hybridization buffer consisting of 50% formamide, 5X SSC, 2% blocking solution (Roche), 0.5% SDS, 0.3% Sarkosyl and 5 mg/ml denatured bacterial DNA. Four ul denatured probe mix (boiled for 10 minutes and ice chilled prior to use) was then added to 20 ml hybridization buffer and allowed to hybridize with the membrane overnight at the same temperature. The membrane was later washed twice with 2X wash solution (2X SSC and 0.1% SDS) for 5 minutes at room temperature and twice with 0.1X wash solution for 30 minutes at 63 °C (34).

## **II. M. Immunological Detection.**

The blot was washed with DIG buffer1 (0.1 M Maleic acid and 0.15 M NaCl; pH 7.5) for 30 minutes at room temperature and then soaked in DIG buffer2 (1% blocking solution in DIG buffer1) for 30 minutes at room temperature and finally soaked in DIG buffer2 containing 1/10000 diluted anti-DIG alkaline phosphatase conjugate (Antibody solution/Roche) for 30 minutes at room temperature. After rinsing the membrane with DIG buffer3 (0.1 M NaCl, 0.1 M Tris-HCl pH 9.5), it was placed with DNA side facing up on a development folder and treated with 1 ml CSPD ready-to-use (Roche). The blot was covered immediately with a second sheet of folder to spread the substrate evenly and incubated for 15 minutes at 37 °C before X-ray film exposure. The signal is finally detected by photographic film development (24,34).

## **Tissue Distribution and Expression Pattern of CK-1 m-RNA.**

### **II. N. Preparation of trout Tissue Samples.**

Phytohemagglutinin (PHA, Sigma) 10 ug/g body weight was injected into the peritoneal cavity of two Rainbow trout (32). One fish was sacrificed after one hour of PHA treatment while the second one was sacrificed after 24 hours. Blood samples were withdrawn prior to dissection of these two fish. Their tissues (spleen, heart, brain, muscle, liver and head kidney) were removed and stored in RNAlater (Ambion) at - 20 °C. A third fish (non-treated) was sacrificed and had its tissues removed in the same way to serve as a negative control.

### **II. O. mRNA Extraction from trout Leukocytes.**

The three blood samples from the above mentioned fish were subjected to 54% Percoll fractionation to isolate leukocytes (32). Following separation and washing to remove the Percoll, mRNA was purified from the leukocytes using a Quick Prep kit (Pharmacia). Cells were centrifuged for 10 minutes at 15000 rpm and then suspended in 1.5 ml Extraction buffer and 3.5 ml Elution buffer. The extract was homogenized several times and passed through an Oligo (dT)-cellulose spun column. After three washes with High and Low salt buffer, mRNA was eluted, precipitated with 95% ethanol, dissolved and diluted to a concentration of 0.25 ug/ul (10). The three mRNA samples (3 ug/ml per lane) were fractionated at 45 volts for 5 hours on 1% agarose formaldehyde gel (2.2 M formaldehyde, 20 mM MOPS, 1 mM EDTA and 5 mM NaOAc pH 5) using 1x morpholinopropanesulfonic acid MOPS as a running buffer. Following electrophoresis, the gel was stained in ethidium bromide (20 ng/ml) for 10 minutes and then destained in DEPC-water for 90 minutes. Capillary transfer was performed using Hybond positively charged Nylon membrane (Pharmacia) and 20x SSC as transfer buffer (24,34).

### **II. P. Total RNA Extraction from trout Tissues.**

Total RNA for the rest of the tissues was extracted using Totally RNA kit (Ambion) according to manufacturer's instructions. Tissues were removed from RNAlater and homogenized in 1 ml Denaturation solution then treated with an equal volume of phenol:chloroform (24). Following centrifugation at 15000 rpm for 6 minutes,

the aqueous layer was mixed with 1/10 volume sodium acetate and an equal volume of acid:phenol:chloroform. The extract was again centrifuged. The aqueous phase was combined with an equal volume of isopropanol and stored at  $-20^{\circ}\text{C}$  for 30 minutes to precipitate RNA. Total RNA was purified with a 15 minute centrifugation at 15,000 rpm at  $4^{\circ}\text{C}$ . The pellet was washed with 1 ml of 70% ethanol and treated with RNase-free DNase (1 unit) at  $37^{\circ}\text{C}$  for 30 minutes. The DNase was later removed by extraction with an equal volume of phenol:chloroform 1:1 ratio. The aqueous layer was then treated with 1/10th volume of sodium acetate and 2.5 volumes of ethanol for 30 minutes at  $-80^{\circ}\text{C}$ . After centrifugation at 15,000 rpm for 15 minutes, the pelleted RNA was washed with 1 ml 70% ethanol. Finally, purified total RNA was suspended in 15  $\mu\text{l}$  DEPC-water and quantitated at  $A_{260}$ . The integrity of the purified RNA was verified on a denaturing formaldehyde gel. The RNA was blotted onto a positively charged Nylon membrane and UV crosslinked (33).

## **II. Q. Probe labelling for Northern Hybridization.**

Based on rainbow trout CK-1 cDNA sequence, two primers O14EPF 5'GGGGGGATCCTTCCAAATACCCATTTTATT3' and O14EPR 5'GGGGGGTACCGGCACGAGCTGAAGATTAGG 3' were designed. The forward and reverse primers amplified an 842 bp long region on the trout cDNA that was later used as a template for DIG labelling using the PCR DIG probe synthesis kit (Roche). PCR conditions consisted of a one cycle of denaturation at  $95^{\circ}\text{C}$  for 4 minutes and 35 cycles of: denaturation for 30 seconds at  $95^{\circ}\text{C}$ , annealing for 30 seconds at  $55^{\circ}\text{C}$  and extension for 2 minutes at  $72^{\circ}\text{C}$ . Amplified products were visualised on a 0.7% agarose gel in 1x TBE buffer and used as templates for another PCR amplification using DIG-dUTP (31).

## **II. R. Preparation of Control Probe for Northern Blotting.**

Two primers RPS15f and RPS15r were used to amplify a 396 bp region from rainbow trout ribosomal S15 gene. The DNA template (20  $\mu\text{g}$  of cDNA clone for S15) was PCR amplified for 30 cycles. Initial denaturation was carried at  $94^{\circ}\text{C}$  for 5 minutes and then cycled for 30 times as: denaturation at  $94^{\circ}\text{C}$  for 30 seconds, annealing at  $53^{\circ}\text{C}$



for 1 minute and extension at 72 °C for 30 seconds. A final extension for 7 minutes was done at 72 °C. Products were analysed on a 0.8 % agarose gel stained with ethidium bromide run in 1x TBE buffer (24) and were labelled with DIG-dUTP (24,33).

### **II. S. Northern Hybridization and Stringency Washes.**

The RNA blots were prehybridized and hybridized with hybridization buffer under the same conditions described for southern hybridization with the only difference that the probe was labelled CK-1 cDNA. For stringency washes, the membranes were washed twice with 2X wash solution (2X SSC and 0.1% SDS) for 5 minutes at room temperature and twice with 0.5X wash solution for 30 minutes at 55°C (34). Immunological detection was done as described before.

### **II. T. Control Northern Hybridization.**

The same RNA blots were stripped from labelled CK-1 cDNA and hybridized with labelled rRNA probe to control for the quantity and quality of RNA in each blot. The membranes were stripped by washing with 200 ml distilled water for 5 minutes at room temperature followed by washing with 500 ml of 0.1% SDS- 0.2 M sodium hydroxide solution at 37 °C for 15 minutes. The membranes were finally rinsed with 200 ml 2X SSC at room temperature (34). These blots were reprobbed with labelled rRNA cDNA under the same conditions mentioned above.

### III. RESULTS

#### III. A. Production of Recombinant CK-1 Protein and its Biological Activity.

To study the biological function of the candidate chemokine CK-1, a fusion protein with a 6x His tag attached to the N-terminal of the chemokine was made using the pRSET A expression vector (donated by B. Dixon). The presence of CK-1 cDNA insert in transformed bacterial colonies was verified by restriction analysis of extracted pRSET A with *Nde I* and *EcoR I*. The digest revealed an approximately 600 bp band and another 3.5 kb band on a 1% agarose gel. Because these two enzymes remove the entire multiple cloning site of the vector which is 136 bp long prior to CK-1 cDNA cloning, the 549 bp band verified the presence of the cDNA insert (549 bp long with the leader peptide sequence excluded). The 3.5 kb corresponded to the size of digested plasmid vector (figure 5).

After isolating successfully transformed bacteria, recombinant CK-1 fusion protein was produced as an approximately 12 kDa protein. Purification of recombinant CK-1 was performed using the nickel column by collecting the eluant protein in 6 different (1 ml) fractions where the maximum yield was always in the second fraction (Figure 6). Purified fractions revealed a 12 kDa band as expected when run on 17 % SDS polyacrylamide gel. (figure 7). An enterokinase recognition site before the chemokine protein allowed the cleavage of the His tag from the protein after purification. The optimal digestion time for enterokinase was determined as 24 hours after conducting pilot studies using different incubation times: 3, 6, 24 and 32 hours. The optimal incubation time was set at digestion of half of the protein because further incubation could lead to a degradation and complete loss of CK-1 protein (figure 8). The undigested fusion protein migrated as a 12 kDa single band, while the digested fraction showed 2 bands: one at 12 kDa representing remaining undigested CK-1 and another at 8 kDa representing the digested protein. When digested CK-1 was run over the nickel column, purified CK-1 lacking the His tag was collected from wash fractions. The remaining bound protein was undigested CK-1 which was eluted separately (figure 9). The yield recovery of purified and digested CK-1 was around 17% of the original undigested CK-1 as assessed by Bradford's method (36).

Figure 5. Restriction digest of extracted pRSET A from transformed bacteria with *Nde I* and *EcoR I* enzymes run on 1% agarose gel. Lane 1: GeneRuler 100 bp DNA ladder. Lane 2: undigested plasmid. Lane 3: blank. Lane 4: restricted plasmid.

Figure 5.

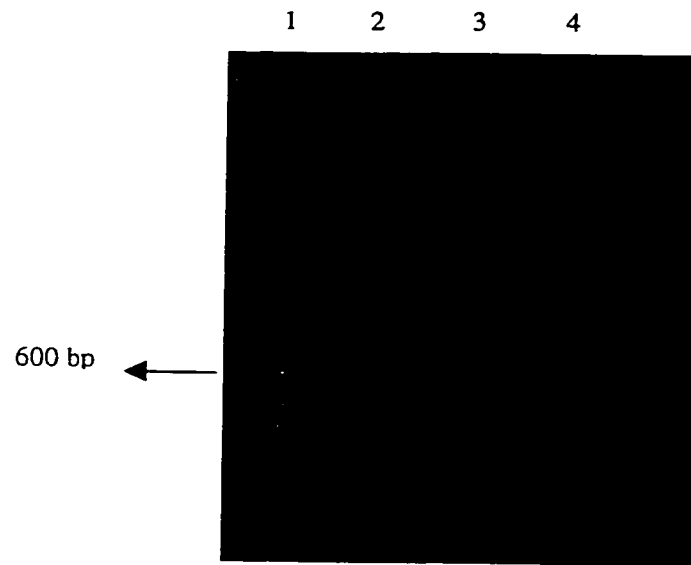


Figure 6. Recombinant CK-1 concentration (mg/ml) in the different eluant fractions upon purification using the nickel column.

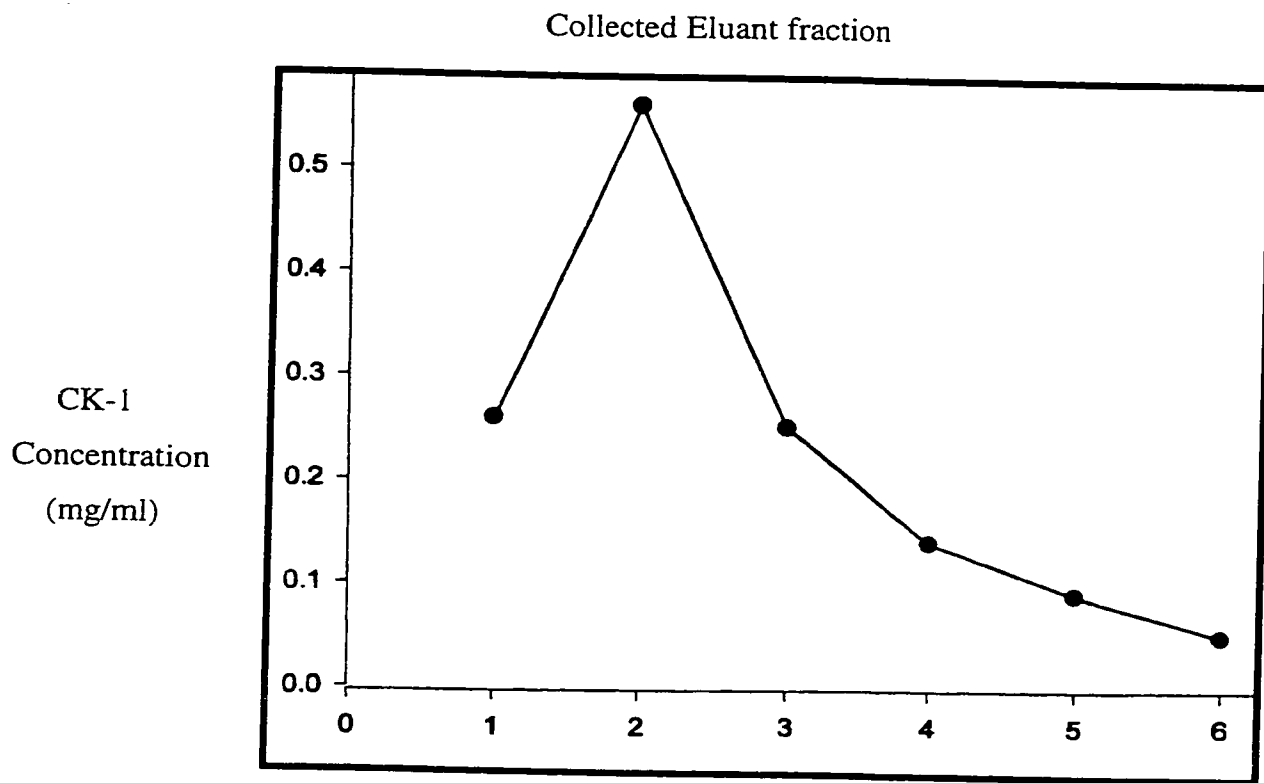


Figure 7. SDS-PAGE for recombinant CK-1 from purified fractions.

Lane 1: BioRad pre-stained low range molecular weight protein ladder.

Lane 2: sample from fraction 2. Lane 3: sample from fraction 4.

Each lane has 16 ul protein solution load prepared as a 1:4 loading dye:protein ratio.

Figure 7.

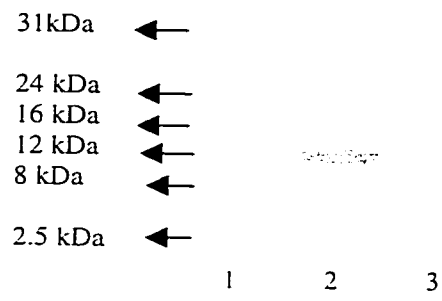


Figure 8. Pilot experiment of optimal CK-1 digestion with enterokinase.

Lane 1: CK-1 digestion for 32 hours. Lane 2: CK-1 digestion for 24 hours.  
Lane 3: Blank. Lane 4: CK-1 digestion for 6 hours. Lane 5: CK-1 digestion  
for 3 hours. All samples were from fraction 2 and each lane held 16 ul of the  
digest. Lane 6 : BioRad pre-stained low range protein molecular weight ladder.



Figure 8.

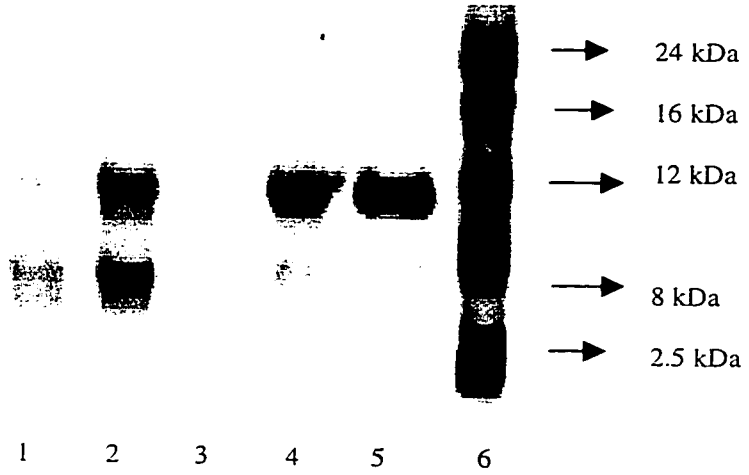
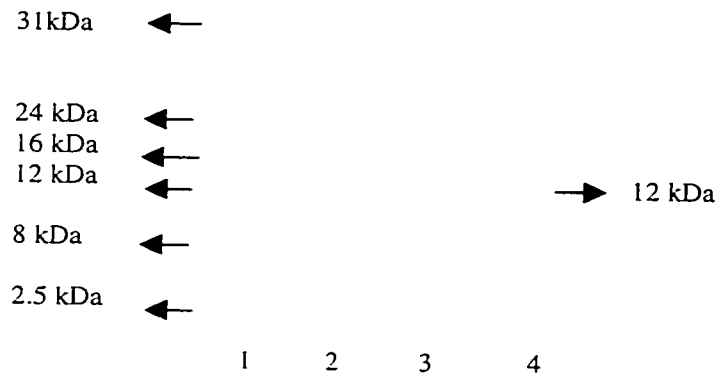


Figure 9. Purification of digested CK-1 protein from undigested proteins.

Lane 1: BioRad pre-stained low range molecular weight protein marker.

Lane 2: Uncut CK-1. Lane 3: wash collected representing completely digested CK-1. Lane 4: Eluant collected after CK-1 digestion and purification representing undigested CK-1 with the His tag.

Figure 9.



### III. B. Leukocyte migration assay.

Recombinant CK-1 was tested for its ability to induce rainbow trout leukocyte migration. Results from chemotaxis assays show that leukocytes were stimulated to migrate by CK-1 and the complement component C5a used as a positive control (figures 10-16). The optimal response for CK-1 was observed at 10 ug/ml while that of C5a was at 100 ug/ml. The chemotactic migration index (defined as the number of cells migrating towards the tested factor per the number of cells migrating in medium alone) for optimal CK-1 concentration was 2.7 while that of the positive control was 3 in averaged data.

To further characterize the type of trout WBC movement induced by CK-1, checkerboard analysis was performed. This assay allows the assessment of concentration gradient effect on cellular movement as CK-1 is added to both lower and upper chamber (table 1). The diagonal represents equivalent concentrations with a zero gradient using three different CK-1 concentrations: 1, 10 and 100 ug/ml. For CK-1, the chemotactic activity was reduced along the diagonal. However, there was a chemokinetic effect as well because migration was not inhibited completely suggesting that CK-1 could induce both a chemotactic and a chemokinetic effect on rainbow trout leukocytes.

The identity of migratory cell type in chemotaxis assay was obscured because of distortion of cells within the porous matrix of the filter used. Therefore, in one experiment the migrating cells were identified by staining cells that had migrated through the filter but were washed off and collected on a microscopic slide. The predominant cell type was lymphocytes as revealed by microscopic morphology. Lymphocytes represented almost 78 % (72 lymphocytes out of 92 total cells) of the migrating cells in ten different microscopic fields at 40X magnification (figure 17).

One chemotaxis assay experiment was done using recombinant CK-1 at 1, 10 and 100 ug/ml and  $\beta$ 2m at the same three different concentrations along with C5a at 100 ng/ml and MEM. CK-1 showed chemotactic activity like with the other experiments but  $\beta$ 2m failed to induce migration of rainbow trout leukocytes (figure 18). This indicated that CK-1 chemotactic activity was not due to contamination with bacterial components because otherwise  $\beta$ 2m should have shown chemotactic effect in chemotaxis assay.

Figure 10. Dose response analysis of rainbow trout leukocyte migration in response to recombinant CK-1 (experiment 1).

A. C5a: Human recombinant complement component five used at 100 ng/ml concentration as a positive control. CK-1: Recombinant CK-1 protein tested for 1, 10 and 100 ug/ml concentrations. MEM: Minimal essential medium used as negative control. Data represents the average of an experiment done in duplicate.

Error bars represent mean + or – standard error. Statistical significance was calculated by using the student's t-test :  $p < 0.005$  for the 5 columns on figure

B. Chemotaxis index representation for data in figure 10A.  $p < 0.004$  for the columns of C5a and CK-1 at 1 and 100 ug/ml on graph.

Figure 10A.

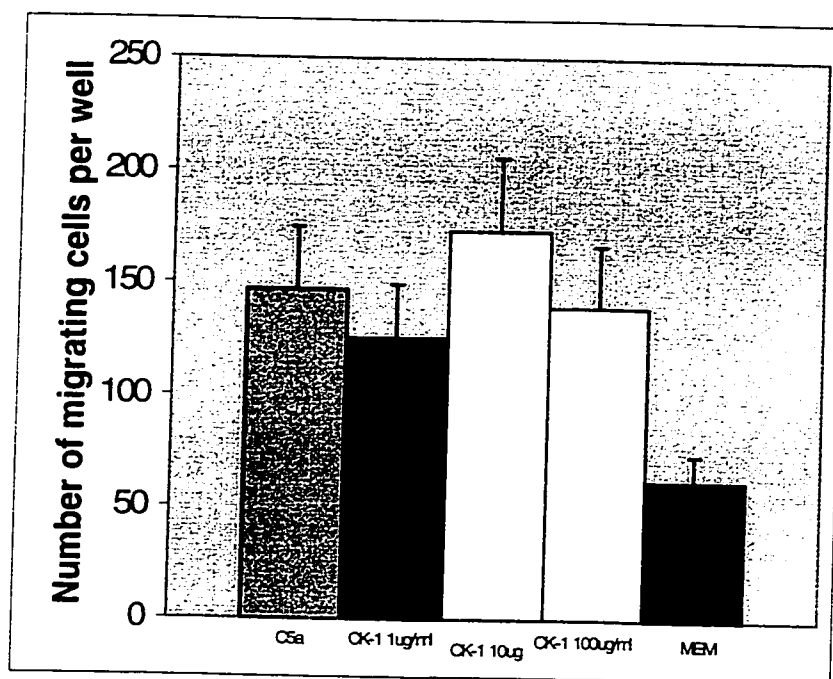


Figure 10B.

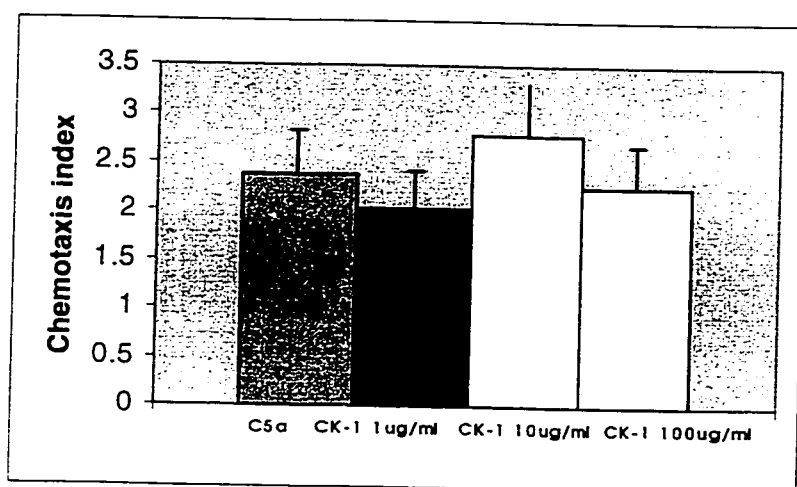


Figure 11. Chemoattraction of trout leukocytes by CK-1 (experiment 2).

- A. Dose response analysis of trout leukocyte migration in response to rCK-1. C5a: Human recombinant complement component five used at 100 ng/ml concentration as a positive control. CK-1: Recombinant CK-1 protein tested for 1, 10 and 100 ug/ml concentrations. MEM: Minimal essential medium used as negative control. Data represents the average of an experiment done in triplicate. Error bars represent mean + or - standard error. Statistical significance was calculated by using the student's t-test :  $p < 0.005$  for the columns of CK-1 (1ug/ml) and MEM on figure.
- B. Chemotaxis index representation for data in figure 11A.  $p < 0.005$  for all columns on graph.



Figure 11A.

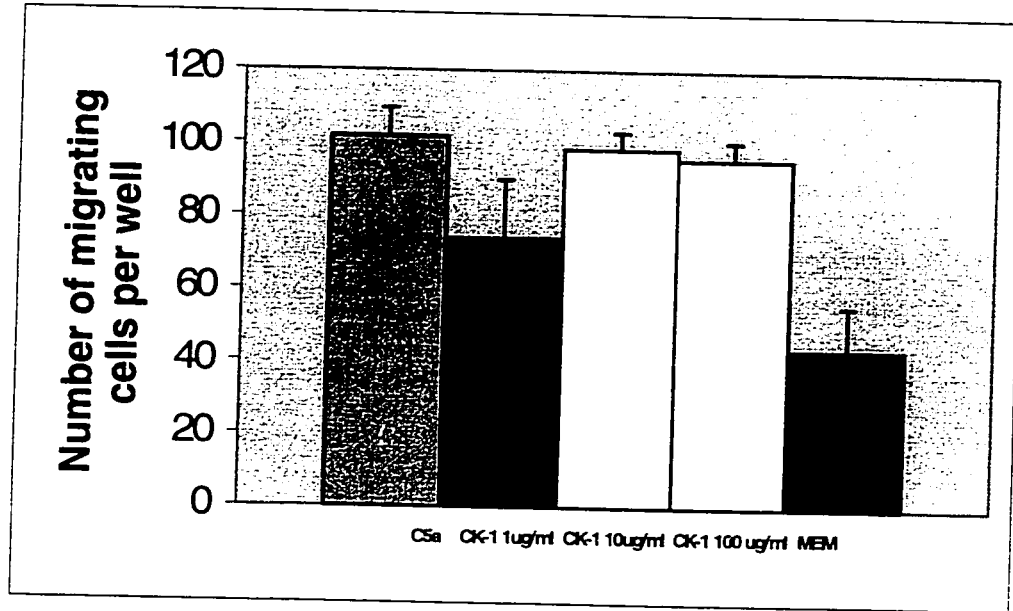


Figure 11B.

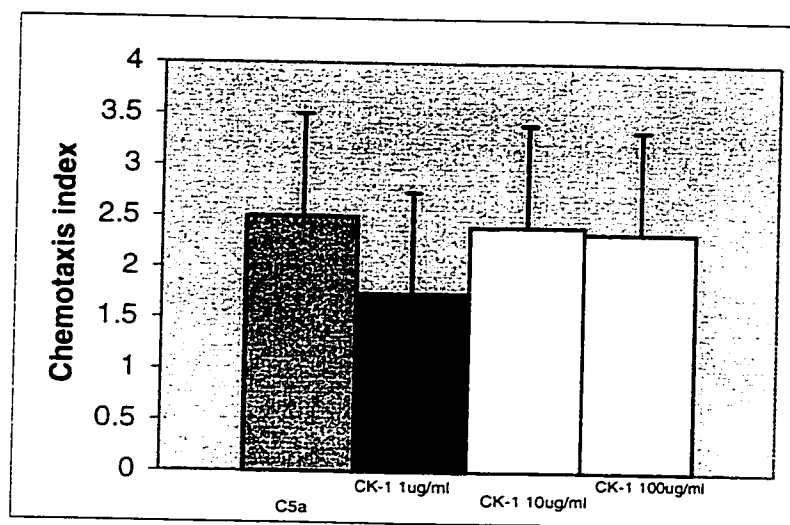


Figure 12. Chemoattraction of trout leukocytes by CK-1 (experiment 3).

A. Dose response analysis of trout leukocyte migration in response to CK-1. Data represents the average of an experiment done in duplicate,  $p < 0.005$  for C5a and MEM columns on graph.

B. Representation of chemotaxis index for data in figure 12A,  $p < 0.005$  for C5a and CK-1 (10 ug/ml) columns on graph.

Figure 12A.

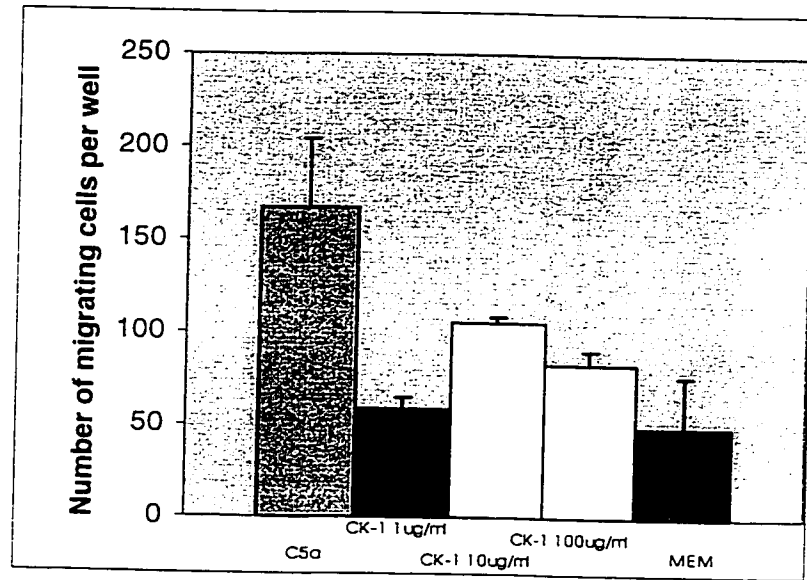


Figure 12B.

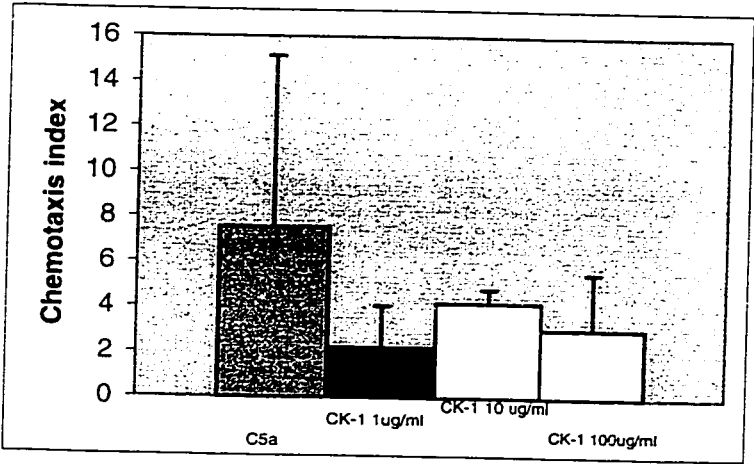


Figure 13. Chemoattraction of trout leukocytes by CK-1 (experiment 4).

- A. Dose response analysis of trout leukocyte migration in response to CK-1. Data represents the average of an experiment done in duplicate,  $p < 0.004$  for all columns on graph.
- B. Representation of chemotaxis index for data in figure 13A,  $p < 0.005$  for all columns on graph.

Figure 13A.

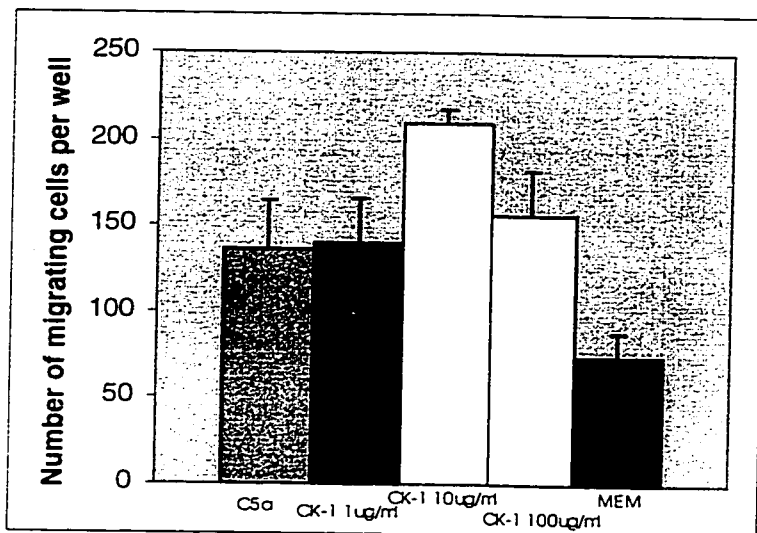


Figure 13B.

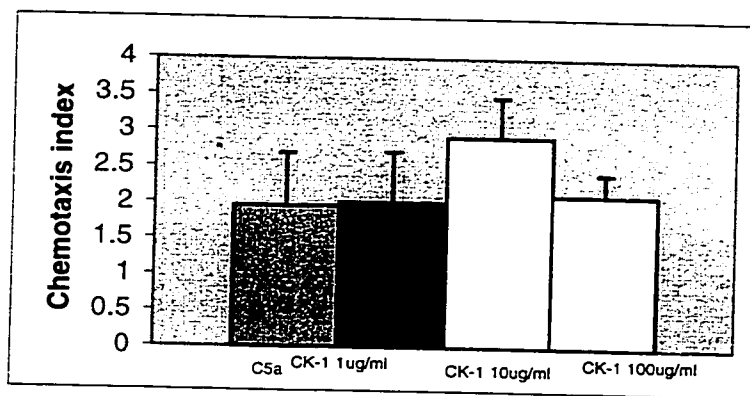




Figure 14. Chemoattraction of trout leukocytes by CK-1 (experiment 5).

A. Dose response analysis of trout leukocyte migration in response to CK-1. Data represents the average of an experiment done in triplicate,  $p < 0.005$  for C5a and CK-1 (100 ug/ml) columns on graph .

B. Representation of chemotaxis index for data in figure 14A,  $p < 0.005$  for C5a and CK-1 (100 ug/ml) columns on graph

Figure 14A.

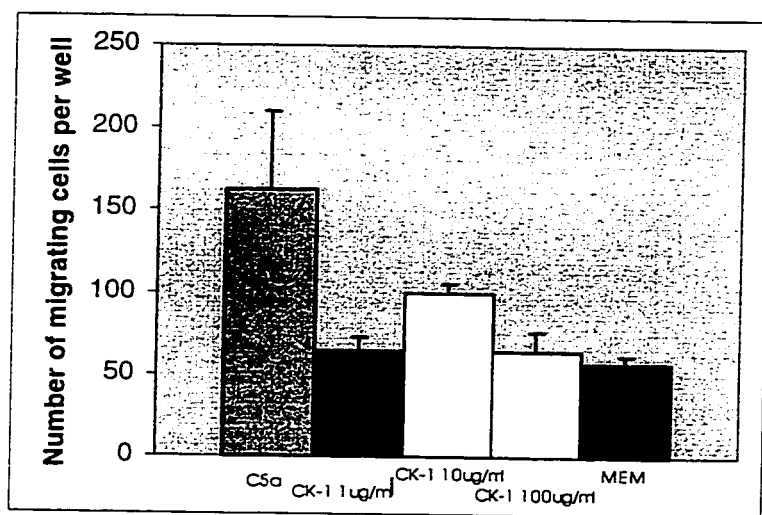


Figure 14B.

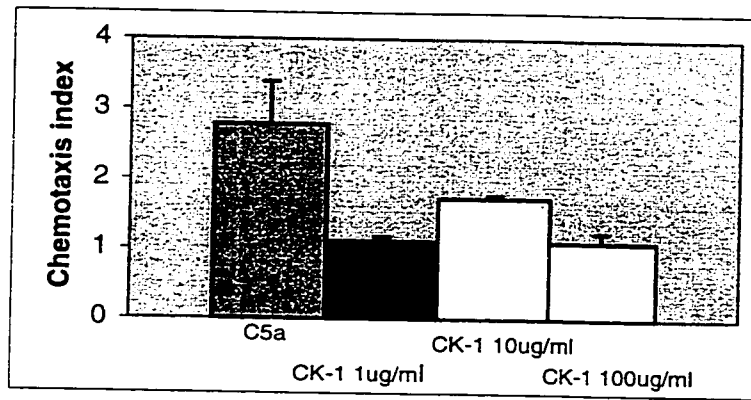


Figure 15. Chemoattraction of trout leukocytes by CK-1 (experiment 6).

A. Dose response analysis of trout leukocyte migration in response to CK-1. Data represents the average of an experiment done in duplicate,  $p < 0.005$  for all columns on graph.

B. Representation of chemotaxis index for data from figure 15A,  $p < 0.005$  for all columns on graph.

Figure 15A.

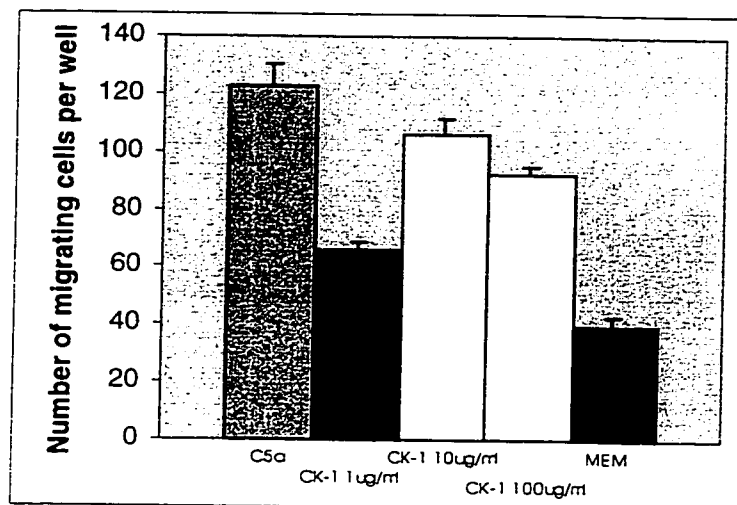


Figure 15B.

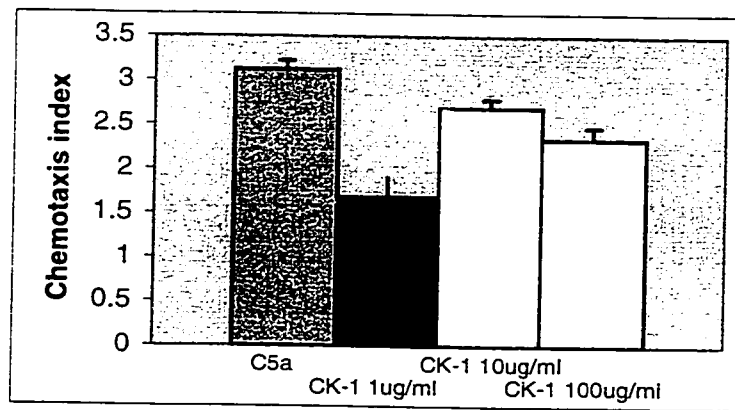


Figure 16. Chemoattraction of trout leukocytes by CK-1 (experiment 7).

A. Dose response analysis of trout leukocyte migration in response to CK-1. Data represents the average of an experiment done in duplicate,  $p < 0.005$  for all columns on graph.

B. Representation of chemotaxis index for data from figure 16A,  $p < 0.004$  for all columns on graph.

Figure 16A.

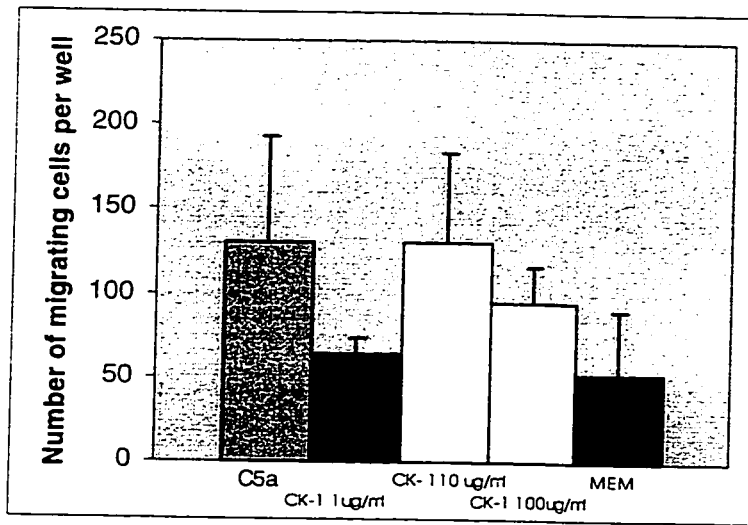




Figure 16B.

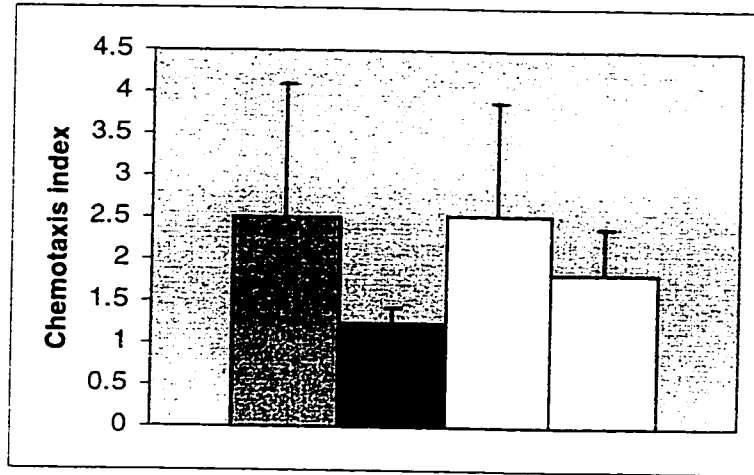


Table 1. Checkerboard migration assay of rCK-1 on trout leukocytes  
 Along the diagonal line, there is a zero gradient with the three  
 Different concentrations used for recombinant CK-1.

<b>[rCK-1] in upper plate ug/ml</b>				
<b>[rCK-1]/lower plate ug/ml</b>	0	1	10	100
0	14	32	46	28
1	82	40	90	120
10	121	112	75	76
100	20	75	77	35

Figure 17. Morphological Cell Typing of rainbow trout leukocytes from a chemotaxis experiment with CK-1. The dominant population of cells is lymphocytes with their characteristic round shaped nuclei. A PMN cell has a multilobed nucleus.

Figure 17.

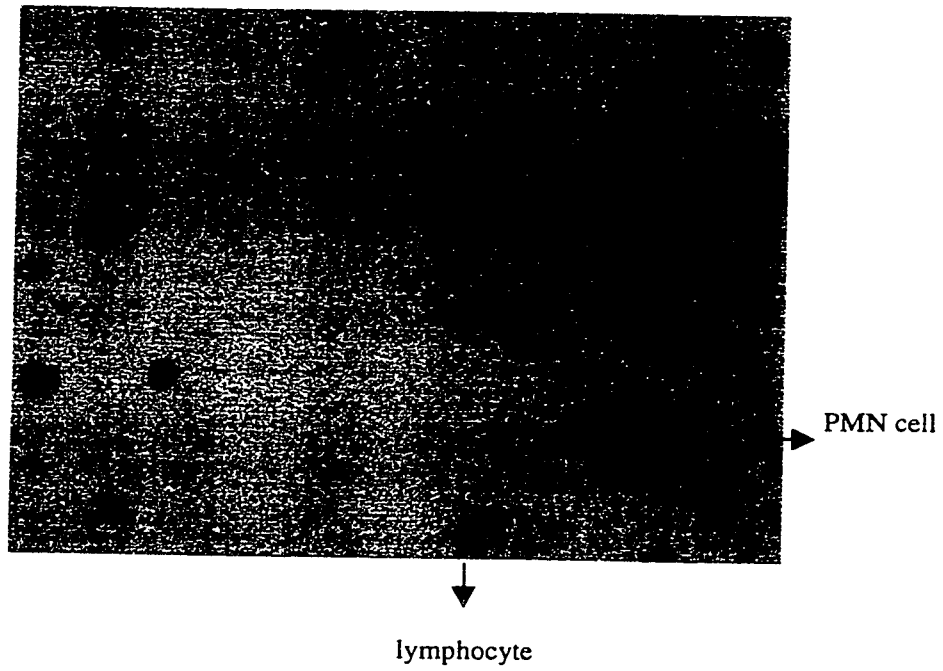
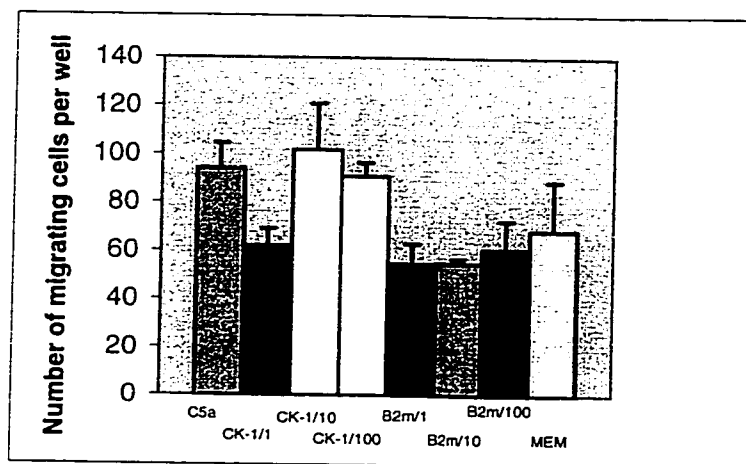


Figure 18. Dose response analysis of rainbow trout leukocyte migration in response to recombinant CK-1 and (2 m. C5a: Human recombinant complement component five used at 100 ng/ml concentration as a positive control. CK-1: Recombinant CK-1 protein tested for 1, 10 and 100 ug/ml concentrations.  $\beta$ 2m: Recombinant  $\beta$ 2m protein tested for 1, 10 and 100 ug/ml concentration. MEM: Minimal essential medium used as negative control. Data represents the average of an experiment done in triplicate. Error bars represent mean + or - standard error. Statistical significance was calculated by using the student's t-test :  $p < 0.005$  for all columns on graph except that of  $\beta$ 2m at 10 ug/ml.

Figure 18.



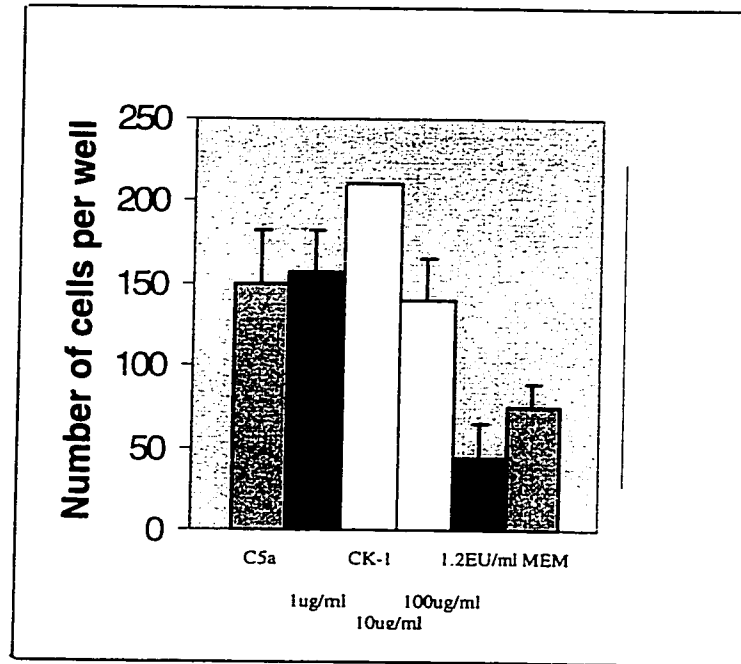
### **III. C. Endotoxin Assay.**

When recombinant CK-1 preparations were tested for endotoxin contamination, they were found to have 1.25 endotoxin units per ml. However, when a standard 1.25 EU/ml solution was prepared using standard endotoxin and water provided with E-Toxate kit, it showed no chemotactic effect in chemotaxis assay (figure 19).

Figure 19. Analysis of standard endotoxin chemotactic activity on trout leukocytes. The endotoxin was tested in six different wells while C5a the human complement component 100 ng/ml, CK-1 the recombinant protein used in 1, 10 and 100 ug/ml concentrations and MEM minimum essential medium were tested in duplicate,  $p < 0.004$  for all columns on graph.



Figure 19.



### **III. D . Probe Construction and Labelling for Southern Blot.**

PCR amplification for genomic rainbow trout clone J6 with primer pairs S5/AS5 and CKJ6FP/CKJ6RP gave 500 and 600 bp bands respectively. Both PCR products were labelled using DIG labelling kit and showed a shift in mobility when analyzed on 1.5% agarose gel (figure 20). The two probe fragments were used for hybridization of trout southern blot (figure 21).

### **III. E . Southern Blot Analysis.**

To determine the number of CK-1 gene copies in the trout genome, genomic DNA was digested with a number of restriction enzymes and electrophoresed on 1% agarose to reveal a smear of DNA (figure 21A). The hybridization experiment showed a single band for genomic DNA cut with *Hind III* and *EcoR I* suggesting that CK-1 is a single copy gene (figure 21B). The digested positive control J6 genomic clone showed two bands at 3.5 and 1 kb.

### **III. F . Probe Construction and Labelling for Northern Blot.**

The amplification of CK-1 cDNA with O14EFP and O14ERP primers gave an 842 bp fragment on a 1% agarose gel. This PCR product was used for DIG labelling that incorporates DIG-dUTP into the growing amplicon. The labelling was verified on a 1.5% agarose gel which revealed a shift in the molecular weight of the labelled product as compared to the unlabelled product. The shift resulted in the labelled product travelling higher (figure 22).

### **III. G . Probe Construction and Labelling for Control Northern Blot.**

Amplification of rainbow trout ribosomal S15 gene gave a 400 bp band as expected. The product was labelled with DIG (figure 23) and used as a probe for control northern hybridization of tissue and leukocyte blots after being stripped from O14 cDNA probe.

Figure 20. Analysis of DIG labelled genomic CK-1 probe on 1.5% agarose gel.

A. Analysis of S5/AS5 labelled PCR product on agarose gel.

Lane 1: 100 bp ladder, Lane 2: unlabelled product 500 bp long.

Lane 3: Labelled product.

B. Analysis of CKJ6FP/CKJ6RP labelled PCR product.

Lane 1: 100 bp ladder, Lane 3: unlabelled 600 bp long product.

Lane 4: labelled product.

Figure 20A.

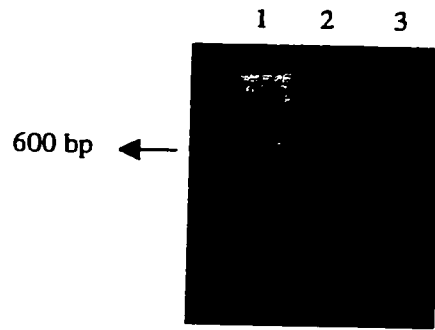


Figure 20B.

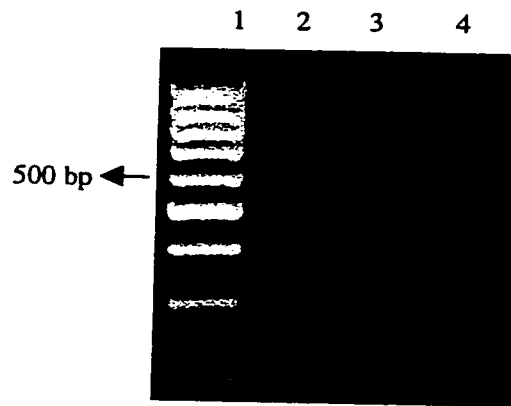


Figure 21 A. Analysis of rainbow trout genomic DNA digest on 0.7% agarose gel.

Genomic DNA was treated with restriction enzymes *Hind III* and *EcoR I* separately. Lane 1:  $\lambda$  DNA ladder. Lane 2: Positive control genomic clone J6 digested with enzyme *EcoR I*. Lane 3: Genomic DNA digested with *EcoR I*. Lane 4: Genomic DNA digested with *Hind III*.

B. Southern hybridization of genomic trout DNA with DIG labelled probe. Southern blot in figure 21 A was hybridized with genomic DNA probe.

Figure 21 A.

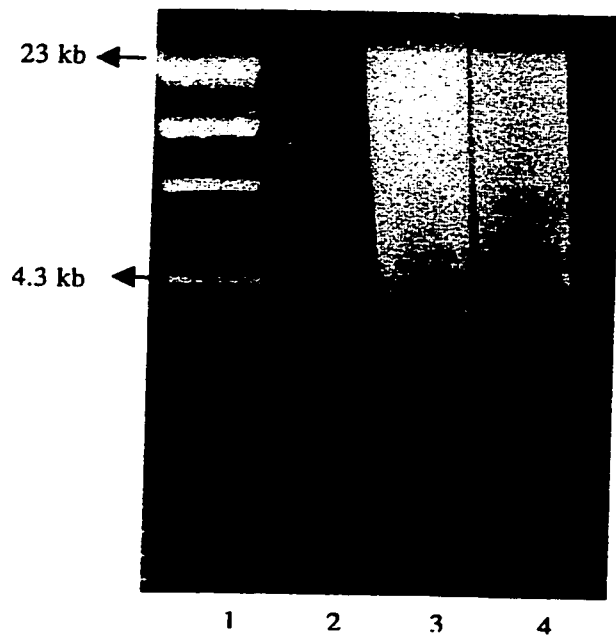


Figure 21 B.

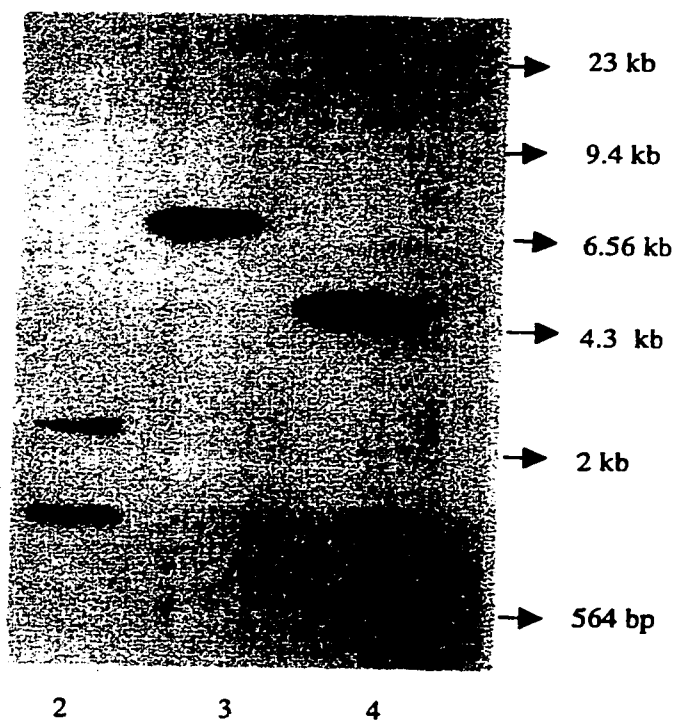


Figure 22. Analysis of DIG labelled CK-1 cDNA probe on 1.5% agarose.

Lane 1: DIG labelled product. Lane 2: unlabelled product.

Lane 3: Blank. Lane 4: 1 kb DNA ladder.

Figure 22.

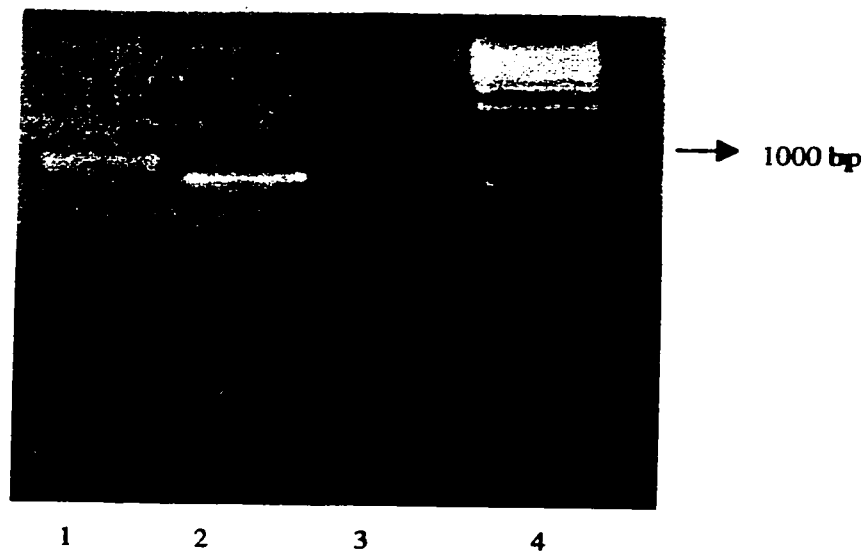
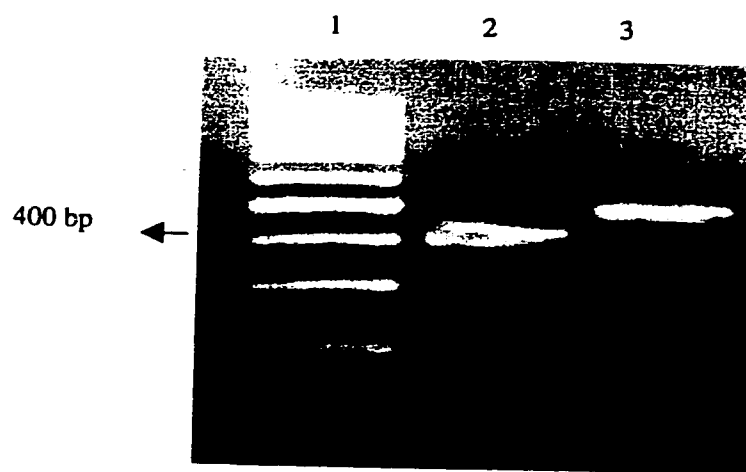




Figure 23. Analysis of DIG labelled rRNA S15 gene probe on 1.5% agarose gel.  
Lane1: 100 bp ladder. Lane2: unlabelled product. Lane 3: labelled probe.

Figure 23.



### **III. H . Northern Blotting of trout Tissue RNA.**

Because of the correlation between gene transcription and protein production, CK-1 transcription was assessed by Northern blotting. Total RNA extracted from leukocytes of three trout: an unstimulated fish and fish stimulated for 1 and 24 hours with PHA was electrophoresed on a 1% formaldehyde gel. The two bands that represent 28 S and 18 S rRNA ribosomal RNA were seen indicating that the RNA was intact (figure 24 A). Another formaldehyde gel with total RNA from head kidney, spleen, liver and brain of the same three fish was run and showed the integrity of each RNA sample again by the presence of the two rRNA bands (figure 25 A).

### **III. I. Northern Hybridization.**

Northern blot analysis of CK-1 mRNA in rainbow trout blood leukocytes using CK-1 cDNA DIG labelled probe showed a positive signal for 24 hour stimulated fish while the control and 1 hour stimulated fish did not reveal any signal upon hybridization (figure 24B). For tissue RNA, hybridization experiment detected CK-1 message in head kidney, liver and spleen of fish stimulated for 24 hour only (figure 25B).

### **III. J. Northern Hybridization with Control Probe.**

Control S15 probe labelled with DIG (figure 23) was used as a probe for a new hybridization of the tissue and leukocyte northern blots that were stripped from CK-1 cDNA probe to control for the quality and quantity of RNA (figure 24C and 25C).

Figure 24A.

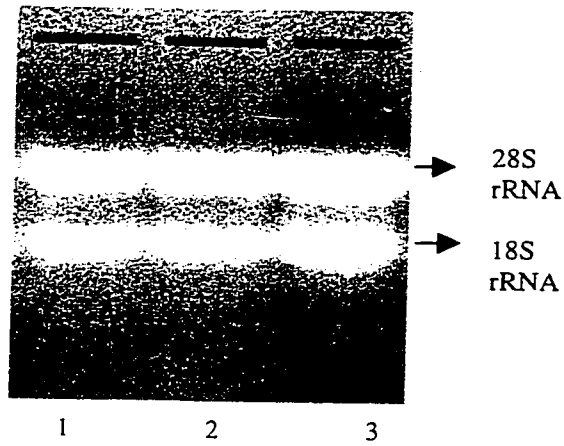


Figure 24B.

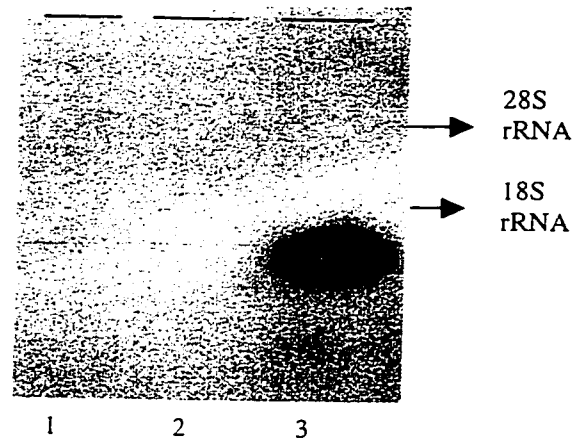
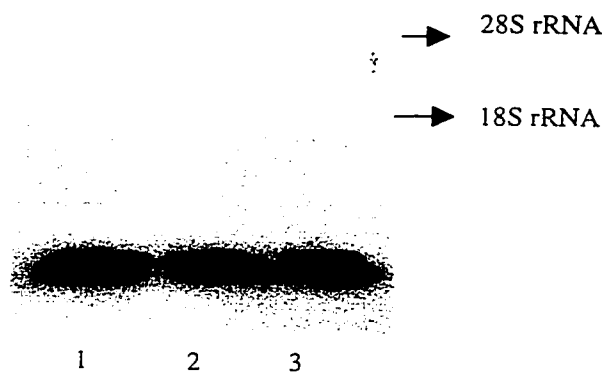


Figure 24C.



**Figure 25 A. Analysis of total RNA from trout tissue on 1% formaldehyde gel.**

Lanes 1-3 : RNA from head kidney of unstimulated fish, and fish stimulated for 1 and 24 hours respectively. Lanes 4-6: RNA from liver of unstimulated fish and fish stimulated for 1 and 24 hours respectively.

Lanes 7-9: RNA from spleen of unstimulated fish and fish stimulated for 1 and 24 hours respectively. Lanes 10-12: RNA from brain of unstimulated fish, and fish stimulated for 1 and 24 hours respectively.

**B. Northern blot analysis of rainbow trout total tissue RNA.**

Blot shown in figure 25A was hybridized with 800 bp CK-1 cDNA probe

**C. Control Northern blot analysis of rainbow trout total tissue RNA.**

Blot shown in figure 25A was hybridized with 396 bp S15 cDNA probe

Figure 25A

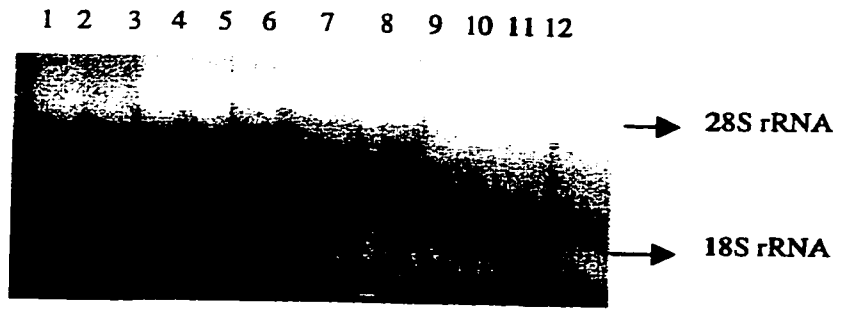


Figure 25B.

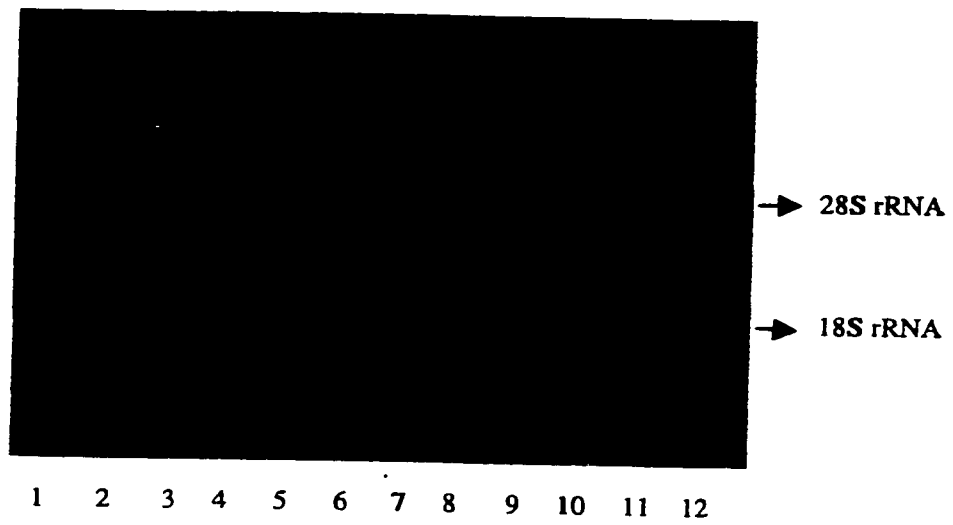
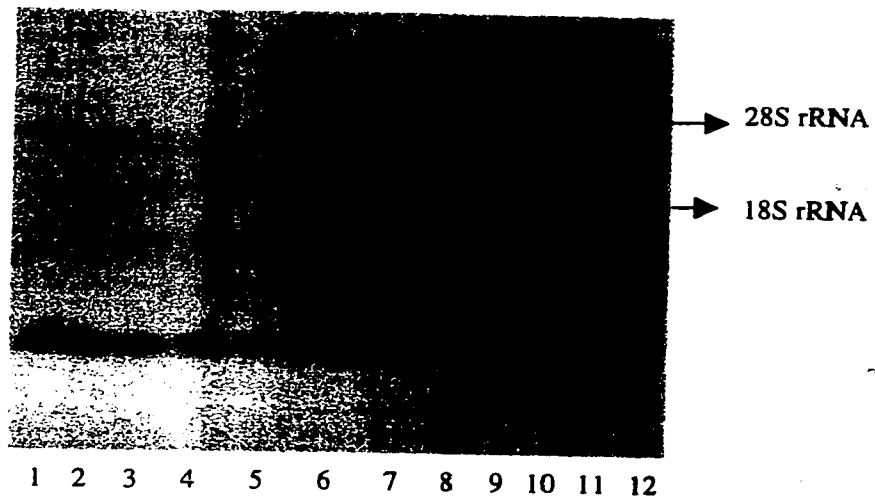


Figure 25C.



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#### IV. DISCUSSION

Only a few fish cytokine genes have been sequenced over the last year including IL-1 $\beta$  (36), and chemokines (20, 21, 23). However, evidence is still lacking about biological activity of chemokines in fish. In this study, we have expressed a previously cloned cDNA from rainbow trout as a prokaryotic recombinant protein designated CK-1 (23). The mature protein which had a molecular weight of 8kDa was purified under denaturing conditions using urea. However, it was dialysed against 50 mM Tris buffer to remove the urea and allow for protein refolding. This procedure lead to a reduction in original CK-1 protein yield because it lowered the solubility of the protein.

Many functional assays like the calcium flux assay, chemotaxis assay and *in vivo* chemotaxis assay have been used by scientists studying chemokines. The calcium flux assay can check for activation state triggered by chemokines but is laborious and requires the use of a fluorescent dye called fura-2 dye while the *in vivo* assay is very difficult to perform and requires an animal model although it measures chemotactic activity. The chemotaxis assay using chemotaxis chamber is the most conventional assay that can check for chemotactic activity and is so far the most commonly used because it is easier to perform than other assays. The use of a 48 well chamber in this assay is very convenient for repeating the experiment which can be run in triplicate or more (27).

In this study, we have used the chemotaxis assay to check for CK-1 chemotactic effect on rainbow trout leukocytes. The results of chemotaxis assays showed that both CK-1 and C5a exhibited migratory effect on rainbow trout leukocytes. The complement component was used as a positive control. Although the complement component we used was human recombinant protein, it was still able to induce WBC migration in trout. This is supported by a recent study which revealed that salmon macrophages and lymphocytes possess receptors that can bind human complement component C5a based on the chemotactic response obtained when rat serum activated with bacterial endotoxin was used on salmon immune cells (37). Because endotoxin (a potent stimulator of complement) leads to the generation of C5a, this study suggested that C5a complement component and its receptor are found in salmon with a significant analogy to the human counterparts (37). In addition, a study by *Hamdani et al.* (1998) used rainbow trout serum



as chemoattractant for trout WBCs (subtype not determined). Because the serum was not treated with any stimulants, it was concluded that proteolysis during clotting lead to generation of complement fragments thus causing a four fold increase in leukocyte migratory responses (38). Another study by *Griffin et al.* (1998) demonstrated that rainbow trout leukocytes were able to detect and migrate along a chemical gradient using trout serum activated with yeast zymosan. Zymosan is a polysaccharide obtained from yeast cell wall and can activate complement component C5 by cleaving it into C5a (22). Thus the role of C5a in rainbow trout is well established although the type of leukocytes attracted by complement has yet to be investigated. Complement components are proteins that are highly conserved throughout evolution with a significant similarity in many species, so it was of no surprise that human C5a actually worked on trout cells. This is supported by a study which succeeded in the purification of rainbow trout analogue of mammalian C5a from serum based on structural and functional similarity (39).

In one sense however, our use of C5a as a control was not justified because C5a is a chemoattractant for neutrophils in humans and other mammals (17,18) and CK-1 is actually similar to CC chemokines which do not act on neutrophils. However this has been established for mammals and not for fish, because evidence is still lacking concerning the particular subtype of leukocytes attracted by fish complement components (22,38).

#### **IV. A. Chemotaxis and Chemokinesis.**

Our studies showed that CK-1 was chemotactic to trout leukocytes (figures 10-16) with an optimal concentration of 10 ug/ml (1.25 nM). This concentration gave an average chemotaxis index from the seven different experiments of 2.7 which is comparable to that of C5a that had an average chemotaxis index of 3. *Schall et al.* (1998) in a study on RANTES, demonstrated that a migration index of greater or equal to 2 was significant (40) but another study by *Guo et al.* (1999) showed that a migration index of 1.77 for a chemokine called ScyA26 was significant (41). Thus, it can be concluded that CK-1 optimal concentration was indeed chemotactic to rainbow trout leukocytes. Although CK-1 used was produced in prokaryotic expression system, it was still biologically active. Many researchers have used recombinant chemokines produced in *E. coli*: *Hedrick and Zlotnik* (1997) expressed a 109 bp long cDNA sequence encoding a CC

chemokine called 6Ckine as a fusion protein whose biologic activity was confirmed using chemotaxis assay. The protein's optimal concentration was 100 ng/ml for monocyte chemotaxis and 1ug/ml for T cell chemotaxis (42). *Guan et al.* (1999) also used prokaryotic recombinant chemokine called MIP-4 and verified its activity using *in vivo* chemotaxis assays (43). Another study by *Nardelli et al.* (1999) used prokaryotic recombinant protein myeloid progenitor inhibitory factor MPIF-1 that was shown to be chemotactic to monocytes at an optimal concentration of 1 ug/ml (44). Most chemokines usually have an optimal concentration of 100 ng/ml if produced by eukaryotic recombinant expression vectors although some can display widely differing potencies. For example, the optimal concentrations of I-309, MCP-1 and TGF- $\beta$  for monocyte chemotaxis are 1000, 10 and 0.1 ng/ml respectively. Prokaryotic recombinant chemokines have been used by many researchers at higher concentrations with a 1 ug/ml optimal concentration most of the time (45). The optimal concentration for CK-1 that was used in our study was thus ten times higher than the conventional mammalian concentration. This might be attributed to improper folding of the protein during dialysis to remove urea following purification. The three dimensional structure of a chemokine is crucial for receptor recognition and binding and hence if less of the protein in solution is folded properly, the higher the concentration necessary for exerting chemotactic effect would be.

Our studies also showed that CK-1 exhibited both chemotactic and chemokinetic effects on trout leukocytes (Table 1 and figures 10-16). Chemotaxis is a unidirectional locomotion whereas chemotaxis is random. Studies by *Horikawa et al.* (1995) demonstrated that these two different activities could be exerted by a single factor on melanocytes by stem cell factor (SCF) (46). In addition, *Miller et al.* (1990) reported both chemotaxis and chemokinesis upon use of I-309 on human monocytes (47). In fish, there have been reports about leukocyte migration using both host derived and pathogen derived extracts. The host derived factors were chemotactic while the latter was chemokinetic. Fresh trout serum was the attractant when *Griffin et al.* demonstrated unidirectional migration of rainbow trout peripheral blood leukocytes (22). A study by *Sharp et al.* (1999) on rainbow trout reported a 20% increase in the rate of cellular migration in the presence of antigen-antibody complexes which generate C5a and a

chemokinetic effect responsible for enhanced leukocyte migration upon using extracts of plerococoids of cestode parasite. Thus both host and pathogen derived factors in the cell environment can influence either the direction (chemotaxis) or speed (chemokinesis) of cell locomotion (48).

It may be considered that chemokinesis induced by equivalent concentrations of CK-1 surrounding cells may reflect "turn on locomotion" signals; activating locomotive potency of rainbow trout leukocytes. On the other hand, chemotaxis with a gradient attractant may indicate that direction-oriented events will follow activation of locomotive potency. Chemokine stimulation is just one part of the mechanism of cell migration which involves a complex interplay between chemotactic factors, as well as cell-cell and cell-matrix interactions.

The chemotaxis assay experiments showed some variability in results (table 1 and figures 10-16). This might be due to the different states which leukocytes could be experiencing upon blood withdrawal from fish. Some leukocytes might have been preactivated before use in the assay and this would cause a desensitization effect, thus lowering chemotaxis index for this particular experiment.

The result of chemotaxis assay with CK-1 and  $\beta 2m$  showed that the latter had no chemotactic activity on rainbow trout leukocytes (figure 18). CK-1 was chemotactic as with other experiments but  $\beta 2m$  failed to induce leukocyte migration of rainbow trout at the same three different concentrations used for CK-1. Recombinant  $\beta 2m$  served as a control for the production of CK-1 because all conditions for the expression of both proteins in prokaryotic system and their purification were exactly identical. Using bacterial system for protein expression could lead to contamination especially during recombinant protein purification and could thus contribute to any chemotactic activity observed in chemotaxis assay using this protein. The absence of chemotactic responses with recombinant  $\beta 2m$  verified that CK-1 chemotactic activity was not due to contamination with *E. coli* components like the endotoxin but was in fact due to its identity as a chemokine.  $\beta 2m$  has no structural similarity with CK-1 or any other chemokine. It is a polypeptide that associates with a 45 kDa chain from MHC class I on the surface of most cells and is involved in presenting foreign antigens to cytotoxic T cells. It is essential for the immune system of rainbow trout and all other vertebrates. Its

role is to ensure that endogenous peptides are held in the cleft of MHC class I, then to dissociate following the loss of the endogenous peptide to prevent the class I molecule from obtaining any exogenous peptides (49).

Although CK-1 preparations had a 1.25 EU/ml as revealed by endotoxin assay, this amount of endotoxin was not contributing by any means to the chemotactic effect exerted by CK-1 on rainbow trout leukocytes. Rainbow trout leukocyte migration with an endotoxin standard was even less than the background migration observed with media (figure 19). Endotoxin contamination of CK-1 purified samples was due to the use of bacterial expression, however this contamination did not affect the results of our studies.

#### **IV. B. Cell Typing.**

Experiments done to reveal the identity of cells attracted by CK-1 showed that lymphocytes are the main target leukocytes (78% of total leukocytes attracted) (figure 17). This is in accordance with the type of CK-1 as a CC chemokine. Mammalian CC chemokines act on lymphocytes and monocytes but not neutrophils. However, rainbow trout lymphocytes account for approximately 59% of blood cell population and thus further experiments that can test for CK-1 chemotaxis on separated lymphocytes would verify this observation.

#### **IV. C. Southern Blotting.**

Southern blots with genomic labeled fragments revealed that CK-1 is present in only one copy per genome since the probe hybridized with a single *Hind III* fragment of about 5 kb and a single *EcoR I* fragment of about 8.5 kb (figure 21B). This finding is consistent with the evolutionary transition that salmonids are currently undergoing from tetraploidy to diploidy (50). The positive control plasmid digestion gave two bands in southern hybridization: one approximately 1 kb and another 3.5 kb. Complete digestion of the genomic clone plasmid should have produced only one band (1 kb) corresponding to the size of rainbow trout genomic clone sequence, however the presence of another band (3.5 kb) suggests that digestion with *EcoR I* was not complete and hence this band corresponds to the intact plasmid with the insert.

#### **IV. D. Northern Blotting.**

To investigate whether CK-1 expression was constitutive or inducible, RNA from stimulated and unstimulated rainbow trout was assessed. Northern blot analysis confirmed that the trout gene is inducible by typical inflammatory stimuli such as PHA. The gene is transcribed at high level after 24 hour stimulation but not after 1 hour stimulation or in the unstimulated fish (figures 24B and 25B). Thus, CK-1 has an inducible pattern of expression, a feature typical of all chemokines except RANTES. It is well known that mitogenic stimulation will change the transcription pattern of many cell specific genes resulting in the appearance of gene products not expressed before stimulation. The kinetics of expression for CK-1 has still to be studied using different time intervals following *in vitro* stimulation in culture. Whether 24 hour stimulation is the optimal condition for CK-1 mitogenic responsiveness needs to be investigated further by considering more time intervals between 1 and 24 hours and even after 24 hours.

Northern blot analysis has also revealed that CK-1 has a wide distribution pattern with the transcript being detected in blood leukocytes, head kidney, spleen and liver. The most significant source for chemokines is leukocytes so it was expected to detect CK-1 transcript in trout leukocytes (figure 24B). While leukocytes occur in all animal phyla and are known to be important defence mechanism, they are also a source of chemokines which cause their migratory stimulation to inflammatory site (51). Our detection of CK-1 message in the trout head kidney (figure 25B) is also consistent with the role of this organ. The foremost part of the kidney in rainbow trout, referred to as head kidney, is predominantly a hematopoietic organ with morphological similarities to bone marrow in higher vertebrates. It also serves as a lymph node analogue important in the induction and elaboration of immune responses because it hosts lymphocytes, granulocytes, macrophages and thrombocytes (51). Our studies also revealed that CK-1 is induced in the trout spleen. The spleen is another important lymphoid organ in fish that holds a diverse cell population of lymphocytes and macrophages which are important for trapping antigens and for producing antibodies. The liver is another site where CK-1 message was induced. This organ is rich in a certain type of immune cells called melanomacrophages which are rich in melanin. These cells are highly active in trapping immune complexes and retaining antigens. They are associated usually with a network of

immune cells (51). The brain tissue of rainbow trout did not show any signal in northern blot for CK-1 message. The brain belongs to the nervous system and is not rich in immune cells usually. Instead it is composed of a network of astrocytes, nerve cells and other supporting cells (51). Thus because the brain is not involved in immune responses in normal conditions, the absence of CK-1 message in stimulated brain total RNA is verified.

To control for both the quality and quantity of total RNA in each blot, the same northern blots for rainbow trout tissues and leukocytes total RNA were hybridized to ribosomal S15 gene probe. This is a house-keeping gene in rainbow trout whose expression is constitutive and not induced. This serves as a good control for gene induction studies in rainbow trout. The homogenous expression pattern as depicted by the presence of bands of comparable signal intensity after northern hybridization with S15 probe does indeed verify the quality and quantity of total RNA in blots (figure 24C and 25C).

## V. CONCLUSION

It is now becoming clear that teleosts, like the “higher” vertebrate possess a variety of leukocytes that mediate both innate and adaptive immune responses. They are mobile and will migrate in response to chemoattractants that are produced at inflammatory sites (22). Our studies extend this aspect by providing evidence for the existence of chemokine activity in rainbow trout. CK-1 was chemotactic to rainbow trout leukocytes in chemotaxis assay and is thus indeed a chemokine. This is the first trout chemokine whose biologic activity has been confirmed.

Our studies are important from an evolutionary point of view because they show that CK-1 is similar to a primordial gene from which all mammalian and avian chemokines evolved, supporting the idea that chemokines arose by gene duplication and divergence events with chromosomal dispersion (23). CK-1 protein will have many potential applications in the future especially for disease control in aquaculture because currently there is a large interest in the use of chemokines and cytokines as vaccine adjuvants.

Future studies should focus on the kinetics of CK-1 expression and on the production of anti-CK-1 antibodies. The antibodies can be used to neutralise CK-1 chemotactic activity in chemotaxis assay and will thus provide more evidence for its identity as a rainbow trout chemokine. Another important experiment would be the use of pertussis toxin in chemotaxis assay to check for inhibition of chemotaxis by CK-1. This study should prove that CK-1 receptor is coupled to a GTP protein like all other chemokine receptors. The production of CK-1 using eukaryotic expression vectors should also be attempted to check for the optimal chemotactic dose of CK-1 and whether it is comparable to that of mammalian chemokines produced in the same manner.

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