THE UNIVERSITY OF MANITOBA

THE ACUTE PHASE PROTEIN, C-REACTIVE PROTEIN, IN RAINBOW TROUT, *Oncorhynchus mykiss*

BY

MINGCHUAN LU

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THE ACUTE PHASE PROTEIN, C-REACTIVE PROTEIN, IN RAINBOW TROUT, *Oncorhynchus mykiss*

BY

MINGCHUAN LU

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

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ABSTRACT

During and after injury, infection and inflammation, physiological changes occur which prevent further tissue damage by destroying and removing infective organisms and/or activating the repair process. An acute phase reactant, C-reactive protein (CRP), rapidly increases its concentration in the serum during the early stages of inflammation of some mammals and fish. A rapid and reproducible method has been developed to purify rainbow trout CRP and a co-purifying molecule, serum amyloid protein (SAP), in which affinity columns (p-aminophenyl phosphorylcholine and CL-4B columns) were used. The purified CRP was characterized by SDS-PAGE using reduced and non-reduced conditions. Rainbow trout CRP appears to contain one subunit with molecular weight 23.9 KDa, non-covalently associated. Rabbit and mouse antibodies to rainbow trout CRP were raised and a capture ELISA detection system, sensitive to 3 ng of CRP, for rainbow trout CRP was established. Studies on the rainbow trout gene were initiated by first constructing cDNA (complementary DNA) and genomic libraries and then several methods were used to isolate the rainbow trout CRP gene. These included the polymerase chain reaction and antibody screening of the cDNA library. Based on screening of a cDNA library with rabbit antibody against rainbow trout CRP and confirmation by immunodetection methods, clones positive for CRP were isolated. The results of this thesis will allow the isolation of the gene and its product for potential therapeutic studies on fish and, for the first time, the regulation of CRP from a lower vertebrate can be studied.

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Thanks to my M.Sc committee members, Dr. L.C. Graham and Dr. K. Hayglass, for their comments and evaluation of the thesis.

This thesis is dedicated to my son, Austin.

LIST OF FIGURES

LIST OF ABBREVIATIONS

aa	amino acid
bp	base pairs
BSA	bovine serum albumin
C	complement
cDNA	complementary DNA
CPS	C polysaccharide
CRP	C-reactive protein
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DPPC	diazophenylphosphocholine
dpS IV	depyruvylated polysaccharide of pneumococcal type IV
E-CPS	sheep erythrocytes coated with CPS
E-CPS/CRP	erythrocytes coated with CPS/CRP
EDTA	ethylenediaminetetraacetate
ELISA	enzyme-linked immunoabsorbent assay
HRP	horseradish peroxidase
HT	hydroxylapatite
IL-1	interleukin-1
IL-6	interleukin-6
IPGT	isopropyl β-D-thiogalactoside
Kb	kilo bases (1000 bp)
KDa	kilo dalton
MOPS	3-[N-Morpholino] propanesulfonic acid

mRNA	messenger RNA
MW	molecular weight
PAF	platelet-activating factor
PBS	phosphate-buffered saline
PC column	p-aminophenyl phosphoryl choline
PC	phosphorycholine
PCR	polymerase chain reaction
pfu	plaque forming units
pI	isoelectric point
PMN	polymorphonuclear
Pn 27	*Streptococcus pneumonia* type 27
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
SAP	serum amyloid P component
SDS	sodium dodecyl sulphate
Spi	sensitivity to P2 interference
SPS	somatic polysaccharide extract of *Asperigillus*
SSC	3 M of sodium citrate and 0.3 M of sodium chloride, pH 7.0 (20X)
TBS	Tris-buffered saline
TBST	TBS+0.05% Tween 20
TCA	trichloroacetic acid
TE	10 mM Tris and 1 mM EDTA
TLL	tetra-L-Lysine
X g	gravity (unit for measurement of the relative

	centrifugal field)
X-gal	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside

GENERAL INTRODUCTION

After injury, trauma or infection of a tissue, wide ranging physiological changes occur which appear to prevent ongoing tissue damage by destroying and removing an infective organism and activating the repair process. The early and immediate reaction is an increase in the concentration of a number of specific plasma proteins (the acute phase proteins) and this is referred to the acute phase response. One of the first acute phase proteins discovered was called C-reactive protein (CRP) (Tillett and Francis, 1930) . This protein was obtained from patients who were acutely ill with an infectious disease and attracted a lot of attention since it had a precipitation reaction with somatic C-polysaccharide of pneumococci, a function similar to immunoglobulins. To date numerous studies have aided in the characterization of CRP and contributed to our understanding of the functions of this molecule. The major function of CRP is considered to be the removal of foreign pathogens and damaged cells by interacting with the humoral and cellular effector systems. The CRP gene from some species has been identified and it is becoming a good model to study acute phase protein gene regulation and expression. To date, The CRP gene of fish has been not been isolated. However, an understanding of fish CRP and its gene is essential to understand fish acute phase protein regulation, expression and function. The experiments presented in this thesis include the purification of rainbow trout CRP, the development of a detection system, and the identification of positive cDNA clones containing the rainbow trout CRP.

CHAPTER 1. LITERATURE REVIEW

Naming of C-reactive protein

Avery and Heidelberger (1923, 1925 as cited by Tillett and Francis, 1930) found that pneumococci contain two chemically and antigenically distinct components. A third component, derived from pneumococci, was reported by Tillett and Francis (1930) and was designated as Fraction C. Fraction C from pneumococci is a polysaccharide (Tillett *et al.*, 1930) and was designated as C polysaccharide (CPS). CPS is a heteropolymer containing N-acetyl-galactosamine-phosphate, murein (Liu and Gotschlich, 1963), ribitol phosphate (Brundish and Baddiley, 1968), choline (Tomasz, 1967), and diaminotrideoxyhexose (Distler *et al.*, 1966) . Tillett and Francis (1930) found that a substance in serum obtained from patients infected with pneumococcus induced a precipitation reaction with CPS. This precipitate was not limited to the sera from individuals infected with pneumococcus but occurred in some other cases. The compound in serum was a protein and was named C-reactive protein (CRP). It was later found that the precipitation of CRP and CPS only occurred in the presence of calcium ions (Abernethy and Avery, 1941).

Secretion of CRP

CRP is categorized as an acute phase protein since its concentration increases in the serum during tissue injury, infection and inflammation. CRP concentrations in humans have been reported to increase 1000-fold in response to an inflammatory stimulus (Claus *et al.* 1976). The concentration of CRP in the serum

of rainbow trout is normally 0.07 mg/ml but is known to reach a value of 1.06 mg/ml during acute chemical inflammation induced by an injection of turpentine or increase to 1.27 mg/ml following a shift in water temperature from 6 to 15°C (Winkelhake and Chang, 1982). CRP is secreted by hepatocytes (Hurlimann *et al.*, 1966; Kushner *et al.*, 1978), and the numbers of CRP-secreting hepatocytes increase following inflammation. More extensive tissue damage results in a longer period of secretion and consequently higher CRP serum levels. High CRP levels usually last for a relatively short period at the beginning of inflammation and then drop sharply.

The induction of the acute phase response in humans is mediated in part by cytokines produced by activated macrophages at the site of inflammation (Kushner, 1982 and Bendtzen, 1988). The role of interleukin-1 (IL-1) and Interleukin-6 (IL-6) has been evaluated in the regulation of CRP synthesis. Goldman and Liu (1987), Ganapathi *et al.*(1988), and Steel *et al.*(1991) reported that the synthesis of CRP was not induced by IL-1, but was affected by IL-6. Furthermore, IL-1 had an enhancing effect on cells pre-treated with IL-6 (Ganapathi *et al.*, 1988; Steel *et al.*, 1991). Other factors appear to be involved in CRP regulation as Yiangou *et al.* (1991) reported that heavy metals could also induce CRP synthesis at the level of transcription and the effect was independent from the inflammation response. Sex-hormones have also been reported to partially control the production of rat CRP (Nunomura *et al.*, 1990). In addition, a small number of normal human peripheral blood lymphocytes produce small amounts of CRP and

express it on their surface, but do not secrete it (Kuta and Baum, 1986).

Structure of CRP

Pentraxins constitute a family of plasma proteins consisting of single polypeptide chain subunits of 20-30 KDa, arranged in pentagonal cyclic symmetry, and having the capacity for calcium-dependent ligand binding. Pentraxins are classified into two groups in terms of binding properties: CRP has the ability to binding to PC (phosphorycholine); SAP (serum amyloid P component) has the ability to bind to agarose, in particular the pyruvate moiety (Maudsley *et al.*, 1987). The ability of CRP to bind to PC led to the development of a one-step method for purification of CRPs from many animals (Oliveira *et al.*, 1980). Serum was applied onto a PC affinity column, then CRP was eluted by PC-type buffer. CRPs from dogfish and plaice were eluted with an EDTA solution, which chelated divalent cations, other than PC (Pepys *et al.*, 1982 and Robey *et al.*, 1983) and further purification was done by simply running the elute through an agarose-based column to separate CRP from other proteins which bound to the column matrix other than PC.

Although the structure and ligand specificity of CRP is highly conserved, there are some differences among species with respect to presence of more than one type of subunit per molecule, number of subunits (five or more), and specificity of ligand binding. Human CRP consists of five identical and non-covalently bound subunits (Gotschlich and Edelman, 1967) with a molecular weight of 21,000

per subunit, arranged in cyclic pentameric symmetry (Osmand *et al.*, 1977) . The CRP molecule from *Limulus* consists of 12 subunits of A (M.W.18,000) and 12 subunits of B (M.W 24,000), combined non-covalently, to form a double stacked hexamer. Both dogfish CRP and *Xenopus* CRP consist of dimeric subunits. The molecular weight of dogfish CRP is 250,000, which dissociates into a 50,000 molecule unit under non-reducing condition and can be further dissociated into two identical subunits with a M.W of 25,000 under reducing conditions (Robey *et al.*, 1983). Each dimeric subunit of *Xenopus* has a molecular weight of 50,000$\pm$2,000, which dissociates into a monomer of 24,000$\pm$ 2000 under reducing conditions (Lin and Liu, 1993). A disulphide-linked dimer has also been reported from rat CRP (De Beer *et al.*, 1982). CRP from plaice consists of 10 non-covalently linked subunits of two different sizes, but with homologous amino acid sequences at the N-terminal (Pepys *et al.*, 1982). Pepys *et al.*, (1978) using electron microscopy showed that the subunits were comprised of two pentameric discs interacting face-to-face.

The study by Murai *et al.*, (1990) on CRP of rainbow trout (*Oncorhynchus mykiss*) suggested that trout CRP was a trimer containing one monomer subunit (MW 26,600) and one disulphide-linked dimer (MW 43,700). These conclusions on trout CRP were based on data which showed a single band under reduced conditions and two bands, at a molar ratio of 1:1, under non-reduced conditions. The size of trout CRP was estimated at 66 KDa from native gels and to be 81.4 KDa by a sedimentation equilibrium analysis. The exact

construction of trout CRP remains uncertain. One possibility would be a hexameric disc or a double-stacked hexamer, which was disrupted into two trimers or four trimers under the experimental conditions. The isoelectric point (pI) of trout CRP is 4.74 and the amino acid composition of trout CRP seems to resemble that of dogfish or plaice CRP.

While it appears that all CRPs studied to date have some common features, there are some anomalies. For example, CRP from goats does not bind to PC-sepharose column or precipitate with PC in immunodiffusion, but binds to agarose and this binding can be disrupted by PC or EDTA (Maudsley *et al.*, 1987). However, not all CRPs appear in tissue fluids as acute phase proteins. To date, only CRPs from humans, monkeys, rabbits, hamsters, horses, eels, and rainbow trout are considered to be acute phase proteins. CRPs from goat and mice are not acute phase protein nor is CRP from *Limulus* an acute phase protein, but it is maintained at high levels. CRP is absent in the serum of flounder (*Platichthys flesus L.*) (Baltz *et al.*, 1982).

Structure of CRP gene

The CRP genes from human (Lei *et al.*, 1985; Woo *et al.*, 1985), rabbit (Hu *et al.*, 1986), mouse (Ohnishi *et al.*, 1988; Whitehead *et al.*, 1990), syrian hamster (Dowton *et al.*, 1991), *Xenopus* (Lin and liu, 1993) and *Limulus* (Nguyen *et al.*, 1986) have been described. The typical model of the CRP gene in vertebrates contains two exons separated by an intron ranging from 217 bp (Hamster) to 1800 bp

(*Xenopus*). The exception occurs in *Xenopus* in which there is one extra intron (145 bp) before the translation start point and 3 exons. The first exon encodes 16-30 amino acids (aa) of a signal peptide and one or two amino acid(s) of the mature protein. A mature CRP contains 205 to 215 aa. There is no intron in the CRP gene of *Limulus* which encodes a 24 aa signal peptide and a 213 aa mature protein.

The human CRP gene is located on the proximal long arm of chromosome 1 (Whitehead *et al.*, 1983; Floyd-Smithet *et al.*, 1986). The mRNA cap site of human CRP is located 104 nucleotides from the start of the signal peptide. Human CRP gene has a typical promoter containing a TATA box and a CAAT box, located 29 and 81 bp, respectively, from the cap site. There is a 1.3 Kb non-coding region at the 3' end. The size of mRNA is 2.2 Kb in length. There is a CRP related pseudogene with a 50-80% region-specific homology located 7.7 Kb downstream from the end of CRP gene in humans (Goldman *et al.*, 1987; Ciliberto *et al.*, 1987a). There appears to be only one locus and one copy of the CRP gene per haploid chromosome in human, whereas multiple genes were found in *Limulus* (Nguyen *et al.*, 1986). The CRP gene from *Xenopus* shows 45.1, 41.9, 38.5, and 28.3% overall homology in amino acid sequence to human, rabbit, mouse, and *Limulus*, respectively. Four regions (amino acids residues 28-66, 95-114, 132-146, and 150-171 from *Xenopus*) with a identity equal to or more than 60% were found when compared to human CRP. The putative calcium-binding region has 87% identity to human CRP.

The CRP gene contains the sequence of HXCXS/TWXS, which appears in all pentraxin family proteins (Bairoch, 1991) and has also been reported for *Limulus* CRP (Nguyen *et al.*, 1986). The function of this sequence is unknown but it is suspected to be involved in formation of the overall structure of the protein molecule (Lin and Liu, 1993).

Studies on the expression of human CRP in transgenic mice found that cis-acting regulatory elements of the human CRP gene were responsible for both tissue specificity and acute phase inducibility of CRP expression and regulation is primarily at the transcription level (Ciliberto *et al.*, 1987b). Detailed studies of these elements were conducted by Arcone *et al.* (1988) and Li *et al.*, (1990). One element was located between -86 to -60 on the 5' flanking region of CRP gene and the second was found between -234 to -200. The two elements can function independently and contain a sequence, TG(G/A)AAAA, which was found on the rabbit CRP gene and discerned in the genes encoding the acute proteins, human haptoglobin and rat α1-acid glycoprotein. In addition, two constitutive enhancer-like elements are located distal to the promoter (from -332 to -292, -855 to -853) and a negative regulatory region is located between the two inducible elements, from -904 to -855. Other regulators may also be involved. Three areas homologous to the *Drosophila* heat shock consensus sequence were found at nucleotide -146 to -143, -130 to -119, and -95 to -86 of the human CRP gene (Lei *et al.*, 1985) but the functional significance of these areas remains unknown. CRP is also induced by

heavy metals (Hg, Cd, Pb, Cu, Ni, Zn) and a putative metal-response element was found in the promoter of the mouse CRP gene (Yiangou *et al.*, 1991). In addition, an IL-1-responsive sequence between -42 and +15 has been found in the human CRP gene and it is considered to act as a translational modulator (Ganter *et al.*, 1989) since IL-1 functions in translational regulation was demonstrated for the heavy and light chain mRNAs of ferritin (Rogers *et al.* 1990).

Recently, studies on the calcium binding response sequences showed that two distinct calcium binding sites were located in peptide 138-148 and 152-157 (Liu *et al.*, 1987, Mullenix and Mortensen, 1994).

Binding properties

There was little information on the binding site of CPS until appreciable amounts of choline phosphate in CPS was reported (Tomasz, 1967). Experiments using phosphorylcholine (PC) during the precipitation reaction between CRP and CPS found that PC was a major inhibitor and this suggested that phosphorylcholine was the major reacting site on the CPS (Volanakis and Kaplan, 1971). Serum which reacted with CPS to produce a precipitate did not react with CPS when the calcium ion was completely removed but became reactive after calcium was restored (Abernethy and Avery, 1941), suggesting that calcium ions were necessary for the binding of CRP with CPS. There are two calcium binding sites per CRP subunit (Gotschlich and Edelman, 1967, Kinoshita *et al.*, 1989, Mullenix and Mortensen, 1994) and the calcium concentration required for precipitation of

CRP with CPS is 0.001 mg/ml (Abernethy and Avery, 1941).

Conformational changes in CRP induced by calcium binding, essential for CRP to bind to PC, was first reported by Young and Williams (1978). This conformational change has been verified by using monoclonal antibodies to CRP (Kilpatric *et al.*, 1982). The three monoclonal antibodies (HB3-2, EA4-1 and FB2-1) reacted with CRP only in the presence of 2.5 mM Ca^{++}, but not in the presence of 1 mM EDTA. Further, this reaction was inhibited by 0.1 mM PC, which indicated that the antigenic determinant induced by calcium is located at or near PC-binding sites of CRP.

Immunoelectron microscopy studies revealed that all PC binding sites are on the same surface of the molecule and are nearly perpendicular to the plate of the molecule (Roux *et al.*, 1983). Each of the five, non-covalent subunits of CRP has one PC-binding site (Anderson *et al.*, 1978). A very interesting study used three derivatives of BSA to study the PC-binding site and concluded that there were two sites involved in the PC binding (Oliveira *et al.*, 1980). The primary site is responsible for Ca^{++} dependent binding of a phosphorylester moiety of PC while the second site binds to the cationic group of PC. This experiment also pointed out a major difference in the binding property between human CRP and rabbit CRP. Binding by two sites in human CRP is essential whereas only the binding of the phosphoryl group of PC in rabbit CRP is required.

CRP can bind nuclear chromatin (Robey *et al.*, 1984) and the U1 small nuclear ribonucleoprotein (DuClos, 1989) in a PC-inhibiting

manner. This provided more evidence that a potentially important role for CRP is in the clearance of exogenous infectious agents and endogenous damaged cells since the presence of PC is present within the cell walls of bacteria, fungi, and parasites, and as a component of eukaryotic cell membrane phosphilipids. CRP has also been reported to bind to the extracelluar matrix adhesion protein fibronnectin, laminin and lipoprotein in a calcium-dependent, PC-inhibiting manner (Salonen *et al.*, 1984; Tseng and Mortensen, 1989; Pepys *et al.*, 1985). These reactions may be involved in wound healing and repair.

CRP binds to certain polycations, including protamine sulphate, polymers of L-Lysine and leukocyte cationic proteins. This has been determined by investigating the interaction of CRP and protamine or synthetic polymers (DiCamelli *et al.*, 1980). A measurable aggregation reaction occurred between CRP and protamine irrespective of the presence of calcium and the reaction was enhanced in the chelate system. A small amount of TLL (Tetra-L-Lysine) inhibits the aggregation of CRP with the poly-L-arginine polymers (15,000 daltons) but a large amount of PC (0.01 M) did not inhibit the reaction. By contrast, the aggregation of CRP with CPS was only inhibited by a small amount of PC ($8X10^{-5}$ M) but not by a large amount of TLL (10^{-2} M). These results provide further evidence that there are two binding sites on the CRP molecule. The precipitation of CRP with polycationic polymers could be partially or completely inhibited by calcium (0.1 to 0.5 mM) (Potempa *et al.*, 1981), but the addition of PC reversed the calcium inhibition and

induced CRP-polycation precipitation. Perhaps PC serves as a modulator of the CRP-polycation interaction. The interaction of CRP with polycations was also affected by heparin (Potempa and Gewurz, 1983), which is to known to be a modulator of various reactions of coagulation, lipolysis, and vascular inflammation (Dougherty and Dolowitz, 1964; Engelberg, 1981). The data indicated that heparin induced a rapid and efficient dissociation of CRP-polycation precipitates once the amount of heparin reached equivalence with polycations.

Interestingly, CRP binds to liposomes but the reaction does not require calcium which makes it similar to the reaction of CRP with polycations. Liposomes are concentric lamellae of lipid bilayer composed of phospholipid such as lecithin or sphingomyelin, other lipids such as cholesterol, charged lipids, and glycolipids. To have significant binding the liposome must have more than 0.22 SA (stearylamine) which contains a cationic amino group. This binding was not inhibited by EDTA but could be inhibited by calcium and the high level of PC (22 mM) and TLL (33 mM) (Mold *et al.*, 1981). Increasing levels of cholesterol also enhanced the binding of CRP with liposomes but it was not essential for binding.

CRP also binds to metals, such as mercury at the free sulfhydryl groups of cysteine on CRP and is not inhibited by either the addition of cysteine or EDTA. Binding did not increase above a molar ratio of Hg/CRP=13.11. CRPs from rat and *Limulus* showed a similar pattern of binding to mercury and could indicate a role of CRP in detoxication processes (Agrawal *et al.*, 1989).

An interesting observation is the ability of CRP to bind to depyruvylated polysaccharide of pneumococcal type IV (dpS IV), which does not contain any PC or PC-like component. However, this binding requires calcium ions and the dpS IV reacted strongly with antisera to C-polysaccharide, but the native type of dpS IV did not react with the same antisera (Higginbotham *et al.*, 1970).

Since the initial study of Mortensen *et al.*, (1977) which showed the interaction of CRP with Fc receptor of mouse monocytes numerous studies on cellular receptors for CRP have been done (Muller *et al.*, 1986; Buchta *et al.*, 1987; Dobrinich and Spagnuolo, 1987; Zeller *et al.*, 1989; Ballou *et al.*, 1989; Zahedi *et al.*, 1989; Tebo and Mortensen, 1990). The exact function of the putative receptor is still not known. Two membrane proteins of 40 KDa and 58-60 KDa from U937, distinguishable from the FcrI receptor, react with CRP (Tebo and Mortensen, 1990). Interestingly, the immunoglobulin, IgG1, inhibited the cross-linking of CRP to the 40 KDa protein, but a monoclonal antibody (IV.3) which was known to inhibit binding of immune complexes to FcrII receptor had no effect. However, the receptor for CRP on phagocytic cells is still thought to be specific and functions in a reversible manner but more work is needed.

CRP function

CRP and complement system

Kaplan and Volanakis (1971) reported that CRP could activate the complement system which was later confirmed by determining the amount of complement consumed in the reaction of protamine sulphate

or CPS with human acute phase serum (Siegel *et al.*, 1974; Kaplan and Volanakis, 1974). This increased use of complement during the reaction of CRP with CPS was inhibited by PC. The complement system could also be activated by CRP with some homopolymers of poly-L-lysine and some natural occurring polycations (Siegel *et al.*, 1975), like leukocyte cationic proteins and histones which are available and may accumulate at sites of injury and tissue destruction. Further, the reaction of CRP with positively charged liposomes activated the complement system and resulted in the consumption of C1 to C9 and complement-dependent damage (Richards *et al.*, 1977). In the presence of CRP, there was no consumption of complement in serum deficient in C2. The results from the reaction of CRP with liposomes indicated that activation is through the classical complement pathway. This increased consumption of complement was observed when the membrane contained long chain unsaturated fatty acids and SA which was essential for the binding (Richards *et al.*, 1977).

Earlier experiments reported that 80% or more of C1, C4 and C2 and 42-66% of C3 to C9 in the human serum was consumed in the reaction of CRP with CPS and the consumption could be inhibited by PC (Kaplan and Volanakis, 1974). E-CPS (sheep erythrocytes coated with CPS) and sensitized by CRP was lysed by the human complement system. This lysis was similar to that produced by an anti-CPS antibody and partial lysis by C4 deficient pig complement system (Osmand *et al.*, 1975). These observations suggest that the alternative pathway for complement was also activated by the

reaction of CRP with CPS.

Variable effects were noted on the human complement system activated by CRP when protamine or a derivative of PC (DPPC, (diazophenylphosphocholine) was used in the reaction. Most of the utilization of complement was limited to C1, C4 and C2, with moderate amounts of C3 consumed, and no significant consumption of C5 to C9 (part of the membrane attacking complex)(Siegel *et al.*, 1974; Berman *et al.*, 1986). Other experiments using nucleated mammalian cells (HEp-2 cell line) with CRP also did not produce cell lysis when human complement system was activated by CRP with protamine and DPPC. On the other hand, the addition of antibodies induced lysis. Perhaps the increased binding of factor H to C3b on the cell surface inhibited C5 convertase activity of both the classical and alternative pathways (Mold *et al.*, 1984).

Similar to the reaction of antibodies with antigen, the aggregation of CRP with CPS could be solubilized by the complement system. This is considered a protective mechanism against the possible harmful effects of large immune aggregates. The solubilization of CRP/CPS occurred in normal serum but not in C2 deficient serum, which indicated that the classical pathway was required. The solubilization of CRP/CPS could be achieved by two steps: dissociation of the precipitate of CRP/CPS and formation of soluble complex. During the solubilization reaction C3 and C4 binds to CRP and CPS (Volanakis, 1982). In addition, complexes of CRP and the α' chain of C4 was also demonstrated (Volanakis and Narkates, 1983).

CRP binds chromatin (DNA with its natural proteins — histones) via the PC binding site of CRP but the complex of CRP/chromatin could be solubilized by the complement system. The amount of chromatin solubilized by the complement system increased with increasing amounts of CRP in serum and suggests CRP is involved in removal of chromatin released by damaged or dead cells at the site of inflammation (Robey *et al.*, 1984; Robey *et al.*, 1985).

The effect of CRP on rainbow trout complement system has also been studied (Nakanishi *et al.*, 1991). CRP increased the consumption of the trout complement when CPS was added. The growth of *Vibrio anguillarum* was suppressed when CRP/CPS complex was added in the presence of complement and the CRP/CPS complex enhanced the phagocytosis of *V. anguillarum* by glass-adherent cells. Nakanishi *et al.*, (1991) concluded that the function of CRP could be to activate the complement system, which in turn enhances phagocytosis or suppresses the growth of bacteria during the acute phase.

CRP as an opsonin

Opsonins, by definition, are substances occurring in serum which make bacteria susceptible to phagocytosis (Muller-Eberhard, 1965) . Experiments by (Lofstrom, 1943; Lofstrom, 1944) showed that human CRP caused the agglutination and capsular swelling of certain types of *Streptococcus pneumonia* and also enhanced the phagocytic activity of leukocytes to a wide variety of pathogenic bacteria (Ganrot and Kindmark, 1969; Kindmark, 1971). Additional support for

the opsonic effect of CRP was shown by using the E-CPS/CRP (erythrocytes coated with CPS/CRP) with lymphocytes and monocytes. When the complement system was activated by CRP/CPS, and CRP was present, the E-CPS/CRP adhered to the lymphocytes and B cell and phagocytosis occurred (Mortensen *et al.*, 1976). CRP bound to Pn 27 (*Streptococcus pneumonia* type 27) activates complement through the classical pathway and enhances opsonization of these organisms (Edwards, 1982). PMN (Polymorphonuclear leukocytes of human) could ingest CRP-opsonized sheep red blood cells with a mediator(s) released from stimulated peripheral blood mononuclear leukocytes and this interaction was independent of the presence of the complement system (Kilpatrick *et al.*, 1987). Human and rabbit CRP protected mice from *S. pneumonia* infections (Lofstrom, 1944; Mold *et al.*, 1981; Yother *et al.*, 1982) and optimal activity required a functioning complement system. Since the highest CRP peak occurred at the beginning of the infection and the CRP had an opsonic function, it appears to have a protective role by causing a delay in the development of pneumococci while anti-pneumococcal antibodies develop. Since both human and rabbit CRP protect against infections of *S. pneumonia* in mice it is not host species specifics (Horowitz *et al.*, 1987). In fish, the opsonic effect of rainbow trout CRP on phagocytosis of activated macrophages was considered instrumental in controlling the early stage of infection (Kodama *et al.*, 1989).

CRP with platelet and platelet-activating factor

Aggregated and ligand-complexed forms of CRP have been shown to initiate activation of platelets, which is like IgG, (Fiedel *et al.*, 1982a; Simpson *et al.*, 1982), whereas natural occurring CRP peptides inhibit platelet activation (Fiedel *et al.*, 1982b; Fiedel and Gewurz, 1986). Similar results were found with synthetic peptides, homologous with tuftsin (a bio-active immunoregulatory tetrapeptide). One peptide with an amino acid sequence of 109-116 on human CRP inhibited platelet aggregation and secretion while a second peptide (amino acid sequence of 199-206 on human CRP) initiated platelet activation (Fiedel, 1988). Fiedel (1988) suggested that the CRP modulating effect on platelet function may relate to the tuftsin homologue in CRP.

CRP reacts with platelet-activating factor (PAF), a PC-containing lipid (Hokama *et al.*, 1984). Detailed studies by Vigo (1985) reported that CRP inhibited PAF-induced platelet aggregation and it was mediated by CRP binding to PAF and to platelet membrane phospholipids. The binding of CRP to membranes may protect against the detergent-like effects of lysolipids and from the action of phospholipases by inhibiting the release of arachidonic acid, thereby blocking the production of inflammatory mediators which are potentially toxic materials. Thus, CRP may act as an early protective recognition mechanism at inflammation sites. CRP also inhibits PAF-induced degranulation and superoxide anion production by human neutrophils by preventing the binding of PAF to neutrophils (Kilpatrick and Virella, 1985; Tatsumi *et al.*, 1988;

Filep and Foldes-Filep, 1989). Collectively, the data suggests that CRP may play a important role in the control of the inflammatory response.

Function of CRP as an immunomodulator

Fragments in CRP which are homologous to a tetrapeptide, tuftsin (Thr-Lys-Pro-Arg), have been reported and the bioactivity of these fragments was documented. Tuftsin, found in the CH2 domain of the Fc segment of the immunoglobulin heavy chain residues 289-292, is considered to be a natural activator of phagocytic cells (Najjar and Nishioka, 1970). Furthermore, three tuftsin-like sequences (fragments) were reported from CRP (Osmand *et al.*, 1977). These fragments caused chemotaxis and production of superoxide by phagocytic leukocytes and induced mononuclear cells in vitro to produce interleukin 1 (IL-1) at concentrations similar to that required for tuftsin to function (Robey, 1987). However, native C-reactive protein did not induce the above changes. Robey (1987) speculated that CRP is degraded by phagocytic cells such as PMNs or macrophages and the biological fragments are released to boost the immune system during inflammation.

CRP, as a acute phase protein, appears during the early stages of injury, infection and inflammation at high concentrations and lasts for a short period. Based on studies to date the primary function of CRP may be to recognize foreign pathogens and damaged cells of host and then to initiate the activities of the humoral and cellular systems.

Much less is known about the structure and regulation of fish CRP than human CRP. Fish CRP is a major serum component at consistently high levels, e.g. about 400 ug/ml in dogfish and 55 ug/ml in plaice (Robey *et al.*, 1983; Pepys *et al.*, 1982). It is also considered to be an acute phase protein in rainbow trout (Winkelhake and Chang, 1982; Murai *et al.*, 1990). Methods for purifying CRP from fish are different from mammals and appear to give contradictory results in terms of its structure. Furthermore, there is no information on the CRP gene. This study was undertaken to,

1) Develop reproducible methods for purifying CRP and a co-co-purifying molecule, SAP.
2) Develop a detection system that was reliable and rapid and that could determine levels in fish serum.
3) Initiate studies to isolate CRP gene from rainbow trout.

CHAPTER 2. PURIFICATION AND CHARACTERIZATION OF RAINBOW TROUT C-REACTIVE PROTEIN

Introduction

Rainbow trout CRP is considered to be an acute phase protein because it is rapidly elevated by chemical and physical stress (Winkelhake and Chang, 1982) and a *Vibro anguillarum* infection (Murai *et al.*, 1990), with the highest CRP levels appearing 48 hours after induction. CRP has potential as an indicator of fish health due to its rapid elevation in serum. Furthermore, due to its association with bacterial and fungal infections it may have a therapeutic role, especially in the treatment of fish eggs against microorganisms like bacterial kidney disease (BKD).

Rainbow trout CRP was studied by Winkelhake and Chang (1982) and Murai *et al.* (1990) but knowledge at the molecular level is still lacking. Fish CRP, unlike mammalian CRP, does not have well defined methods for purification and identification. Since Osmand *et al.* (1975) first reported that human CRP was isolated by an affinity column coupled to CPS, CRPs have been purified from several animal species, based on the property of binding to PC in the presence of calcium.

The first purification of rainbow trout CRP used a PC-Sepharose affinity column (Winkelhake and Chang, 1982). Serum was applied to the affinity column and the bound protein was eluted by 0.1-0.5 M linear gradient of PC. Murai *et al.*(1990) purified trout CRP by a three step isolation procedure, which was based on the method of Volanakis *et al.* (1978) for purifying human CRP. After eluting from a CPS-Sepharose affinity column with an EDTA buffer,

the sample was loaded onto a DE-52 ion-exchange column and then eluted using a 0.09-0.5 M linear gradient of NaCl from the ion-exchange column. The sample which passed through the column was loaded onto a Sephacryl S-300 size selection column.

The first major objective of my studies on fish CRP was the development of methods to purify and detect CRP. This involved the induction of CRP, collection of large volumes of blood from rainbow trout, and the purification of trout CRP. Purification required a rapid and reproducible method to purify CRP and to separate it from a co-purifying molecule, serum amyloid protein (SAP). Following purification the development of a detection system which used antibodies was critical, especially if a rapid and easily used system such as the enzyme-linked immunoabsorbent assay (ELISA) was considered.

Materials and Methods

Induction of CRP in rainbow trout

Rainbow trout (*Mt. Lassen* strain) were fed according to food tables (Hilton and Slinger, 1981) and maintained at 12°C under ambient temperature and light. Prior to injection and bleeding rainbow trout were exposed to phenoxyethanol at a concentrations of 1 ml/litre in water. Rainbow trout weighing 200-500 gm were injected with turpentine at a 0.42 ml/kg of body weight. Forty-eight hours post-injection fish were bled from the caudal vein until no further blood could be removed. The blood samples were pooled, allowed to stand for two hours on ice or in a refrigerator at 4°C, and then using an applicator stick the clot was detached from the wall of the centrifuge tube. The blood samples were left at 4°C for a further 12 hours and then centrifuged at 800 X g for 15 min at 4°C and the supernatant (serum) removed, aliquoted into sterilized centrifuge tubes, and stored at -70°C.

CRP Purification and Concentration

Rainbow trout serum was thawed at 4°C, centrifuged at 12,000 X g twice, for 20 minutes each time. The serum was then dialysed in 4 litre of Tris-Ca^{++} buffer (0.02 M Tris, 0.15 M NaCl and 0.002 M $CaCl_2$, pH 8.0) (Murai *et al.*, 1990) for 24 hours at 4°C with three to four changes of the buffer. The dialysed serum was applied at a flow rate of one drop/ 10 seconds to a 5 ml p-aminophenyl phosphorylcholine (PC column, Pierce chemical Co., Rockford, IL)

previously equilibrated with Tris-Ca^{++} buffer. The column was washed with Tris-Ca^{++} buffer until the solution which passed through the column had a reading of less than 0.020 at 280 nm. All determinations of protein concentrations were done with a spectrophotometer (Spectronic 601, Milton Roy, Rochester, NY). The material bound to the column was then eluted with an EDTA buffer (Tris 0.02 M, NaCl 0.15 M and EDTA 0.01 M, pH 8.0) and collected in fractions of 1 ml/tube. The eluted protein peak was monitored at 280 nm. The tubes containing the eluted proteins were pooled and dialysed for 24 hours at 4°C, with three to four changes of buffer. The samples were then loaded onto a 5 ml CL-4B column equilibrated with Tris-Ca^{++} buffer. The void volume containing the highest protein peak was saved since it contained CRP (see section on Immunological Diffusion, page 28). The column was then washed with Tris-Ca^{++} buffer and EDTA buffer was used to elute the bound protein. The protein peaks were also monitored at 280 nm. The samples from the two peaks recovered from the CL-4B column following elution with the EDTA buffer from CL-4B column were concentrated by lyophilizing, desalting on a 10 ml Sephadex-25 column and dissolving in Tris-Ca^{++} buffer.

Ion-exchange chromatography

After eluting from the PC column by 0.1 to 0.5 M PC gradient and dialysed overnight in the buffer containing 0.05 M Tris and 0.1 M NaCl, pH 8.0, the samples were loaded onto a column (20X260 mm) packed with DEAE Sephadex A-80 (Pharmacia, Uppsala, Sweden). After

the column was washed with Tris buffer until the solution which passed through the column had a constant reading at 280 nm, the bound samples were eluted with a linear gradient of 0.1 to 0.75 M NaCl in the Tris buffer.

Immunological Diffusion

Human CRP was purchased from Sigma (Sigma Chemical Co., St. Louis, MO). Somatic polysaccharide (SPS) was obtained from mycelia of pathogenic fungus, *Aspergillus flavus*, which was cultured in Czapek Dox (Sigma) culture medium at 27°C. SPS was extracted from the mycelia using the hot phenol-water method (Westphal *et al.*, 1952) and then lyophilized.

Three mls of 1% agarose in the gel buffer (Tris 0.02 M / $CaCl_2$ 0.001 M/ NaCl 0.05 M, pH 7.0, Oliveira *et al.*, 1980) was poured into a Petri dish (50X9 mm). After cooling, a large well (0.5 cm in diameter) was cut from the gel in the centre using a gel punch and 6 smaller peripheral wells (0.4 cm in diameter) were cut from the gel and arranged around the larger well (Fig. 6). A total volume of 20 ul of sample was loaded into the middle well and a 10 ul each of samples (concentration 1-2 ug/ul) was loaded into each of the smaller peripheral wells. After the samples were loaded, the Petri dish was placed in a moist container (paper towels were placed at the bottom of the container and saturated with water) and allowed to incubate at 4°C for 24 hours to allow a precipitate to form. The diffusion plate was photographed using a single lens camera with a No.12 Wratten filter (Kodak, Rochester, NY).

SDS PAGE

The method for SDS PAGE was based on that described by Laemmli (1970). A protein sample, treated with 4% (w/v) sodium dodecyl sulphate (SDS) and 10% (v/v) 2-mercaptoethanol or only 4% SDS, was electrophoresed on a 5%-15% gradient polyacrylamide gel containing 0.1% SDS (w/v) by using Protean™ II (Bio-Rad Laboratories, Richmand, CA) at a constant voltage until the marker dye (blue) ran off the gel. Proteins on the gel were visualized by staining with a solution containing 25% isopropyl alcohol, 10% acetic acid and 0.2% coomassie brilliant blue R-250 (Kodak) for 2 hours. Destaining was carried out in the same solution without coomassie brilliant blue until the background was clear.

Mouse Immunization

An emulsion (200-300 ul/mouse) was prepared by combining equal volumes of PBS (phosphate-buffered saline) containing 15 ug of CRP and complete Freund's adjuvant and mixing with a 18G needle attached to a glass syringe until a stable emulsion was produced. Mice were handled according to the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals. Injections were done intraperitoneally with a 25G needle. Booster injections were given on days 7 and 21, after the initial injection, by intraperitoneally injections of an emulsion of equal volumes PBS containing 15 ug CRP and incomplete Freund's adjuvant (total volume of 200-300 ul/mouse).

Mouse blood was collected four days after the third injection

from the tail by first swabbing the tail with 70% ethanol, and then nicking the tail vein with a sterile scalpel blade. A heat lamp was used to facilitate the collection of blood by dilating the blood vessels. Blood collected in 500 ul centrifuge tubes was allowed to clot for one hour, the clot then separated from the wall of the tube, left overnight at 4°C, centrifuged for 3000 X g, and the supernatant (serum) was removed and stored at -70°C.

Rabbit Immunization

An emulsion (one ml in total) was prepared by combining equal volumes of PBS containing 90 ug of CRP and complete Freund's adjuvant and mixing with a one ml glass syringe using a 18G needle until a stable emulsion was produced. Injection of a New Zealand white rabbit (about 2.5 Kg), at four sites, was carried out subcutaneously with a 25G needle. Booster injections were given at 2-weekly intervals by injecting an emulsion of equal volumes PBS containing 90 ug CRP and incomplete Freund's adjuvant. Rabbit antiserum to CRP was obtained from the rabbit ear vein by a 21G1 needle 10 days after the last injection. The bleeding was carried out 10 to 20 minutes after the rabbit was injected with 0.1 to 0.2 mg of acepromazine (Ayerst Co., USA) for sedation and vasodilation. Each time a volume of 5 to 20 ml blood was collected. Rabbit serum was treated as described above for mouse serum.

Antibody Purification

A 10 ml column was packed with Bio-Gel HT (Hydroxylapatite, Bio-Rad). The column was equilibrated with phosphate buffer (0.01 M sodium phosphate, pH=6.8). Serum (400-500 ul) was diluted 1:10 in the phosphate buffer and centrifuged at 4000 X g for 15 min. The column was washed with phosphate buffer after the sample was loaded until the O.D. reading (280 nm) of the elute was zero. The bound protein was eluted by applying 10 to 300 mM of phosphate buffer along a linear gradient. The eluted sample was collected in 1 ml fractions per microcentrifuge tube. The location of IgG was determined by ELISA (see method below, this chapter). The tubes containing antibodies were pooled. Further purification and concentration was done by stepwise ammonium sulphate precipitation: the proteins precipitated when ammonium sulphate concentration reached 25% were discarded but the supernatant was treated with increasing concentrations of ammonium sulphate (25% to 50%) and the precipitated protein was collected following centrifugation at 3000 X g. The precipitated protein was dissolved in 200 ul of PBS buffer (pH 7.4) and dialysed overnight against PBS buffer (pH 7.4). The protein concentration was determined by the method of Lowry *et al.*, (1951).

ELISA

Locating antibodies from HT column was done as follows; an ELISA plate was coated by adding 50 ul/well (0.25 ug/50 ul) of purified trout CRP which was diluted in 0.05 M carbonate buffer (pH

9.6) and the contents of the plate incubated overnight at 4°C. The wells were then rinsed three times with PBS-washing buffer (PBS+0.05% Tween 20), 200 ul of blocking buffer (PBS+3% BSA) were added to each well and the contents of the microtiter plate allowed to incubate at room temperature for 2 hours. Following three more washes with PBS-washing buffer, 0.626 to 0.156 ug of eluted sample (rabbit or mouse anti rainbow trout CRP) from the HT column diluted in 50 ul of blocking buffer was added to each well and the microtiter plate was left for 1 hour at room temperature. After washing 5 times with PBS washing buffer, 50 ul HRP (Horseradish peroxidase) conjugated goat Ab to rabbit IgG or mouse IgG (Sigma) was added to each well and the plate was incubated at room temperature for one hour. After washing the microtiter plate, 5 times with PBS washing buffer, 50 ul of substrate buffer, containing 0.72 gm of Na_2HPO_4, 0.71 gm of Na citrate, pH 5.0, 0.04 mg o-phenylenediamine and 0.04 ml of H_2O_2 (30% solution)/per 100 ml, was added to each well and then incubated for 30 minutes. The colour reaction was stopped by adding 50 ul of stop buffer (7.4% HCl) and the absorbance of each well was read at 490 nm wave length using a Biotech EL308 microplate reader (Mandel Scientific, Rockwood, ON).

Capture ELISA

An ELISA plate was coated by adding 50 ul/well (1 ug/50 ul) of rabbit Ab to trout CRP which was diluted in 0.5 M carbonate buffer, pH 9.6 and the contents of the plate were incubated overnight at

4°C. The wells were then rinsed three times with PBS-washing buffer (PBS+0.05% Tween 20), 200 ul of blocking buffer (PBS+3% BSA) were added to each well and the contents of the plate allowed to incubate at room temperature for 2 hours. Following three more washes of 50 ul of 1 to 10 ng purified trout CRP diluted in blocking buffer was added to each well and the microtitre plate was left for 1 hour at room temperature. Following 3 more washes with PBS-washing buffer, 50 ul of mouse Ab to trout CRP (1 ug /50 ul) was added to each well and the plate was left at room temperature for one hour. After washing 3 times with PBS washing buffer, 50 ul HRP conjugated goat Ab to mouse IgG (Sigma, diluted 1:10,000) was added to each well and the plate was incubated at room temperature for one hour. The remaining procedures used in capture ELISA are the same as those reported for the ELISA (see page 31). Test procedures using rainbow trout serum were the same as those reported above.

RESULTS

Elution profiles of a PC column with EDTA and a CL-4B column are given in Figs. 1 & 2. The profile of proteins from the ion-exchange column eluted with NaCl is shown in Fig. 3. Figures 4 & 5 illustrate the patterns of samples from CL-4B column and from the ion-exchange column run on SDS-PAGE.

When a 0.1 to 0.5 M PC gradient was used to elute the PC column the sample contained many proteins with the largest about 110 KDa (verified by using a reduced SDS gel). When this protein mixture was applied onto a DEAE Sephadex A-80 ion-exchange column, the void volume contained the 110 KDa protein and the samples eluted by NaCl contained 5 major bands, 75, 68, 54, 34, 13 KDa on SDS PAGE in a reduced condition (Fig. 5). There were no precipitates formed between samples eluted with NaCl and SPS when they were examined by diffusion.

Two major proteins were eluted from the PC-column with 0.01 M EDTA (Fig. 4). The sizes of the proteins were 30.2 KDa and 23.9 KDa, respectively, as determined on SDS gel in a reduced condition. When this mixture passed through the CL-4B column, the 23.9 KDa protein was part of the void volume and the 30.2 KDa protein bound to the CL-4B column and was eluted from the column with 0.01 M EDTA. The 23.9 KDa protein, shown as a 21 KDa band in a non-reduced condition (Fig. 4), reacted with SPS during the double diffusion experiment in the absence of EDTA but there was no reaction with SPS when 0.1 M EDTA was present. There was no reaction between the

30.2 KDa protein with SPS in the double immunodiffusion, whether EDTA was present or absent (Fig. 6).

A capture ELISA which used purified polyclonal antibodies developed in mouse and rabbit to trout CRP (23.9 KDa band) and purified trout CRP detected CRP at concentrations as low as 3 ng (Fig.7). This capture ELISA system when applied to rainbow trout serum also able to detect CRP but non-specific binding (background) was high.

Figure 1. Elution profile of rainbow trout serum (90 ml) from the PC column. 5 ml/fractions for void volume and 1 ml/fractions for eluted volume were collected, respectively. The EDTA elute peak contains CRP and SAP.

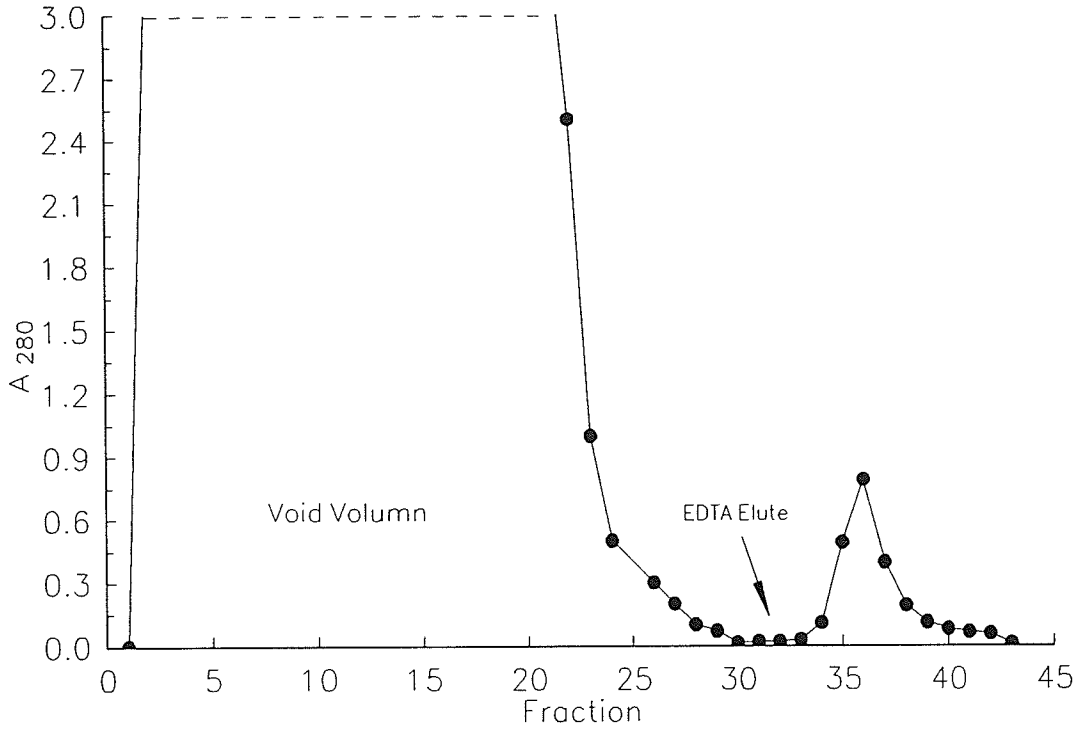

3.0
2.7
2.4
2.1
1.8
1.5
1.2
0.9
0.6
0.3
0.0
A 280
Void Volumn
EDTA Elute
0
5
10
15
20
25
30
35
40
45
Fraction

Figure 2. Elution profile showing the separation of trout CRP and SAP on a CL-4B column. 1 ml/fractions. The void volume contains CRP and the EDTA elute peak contains SAP.

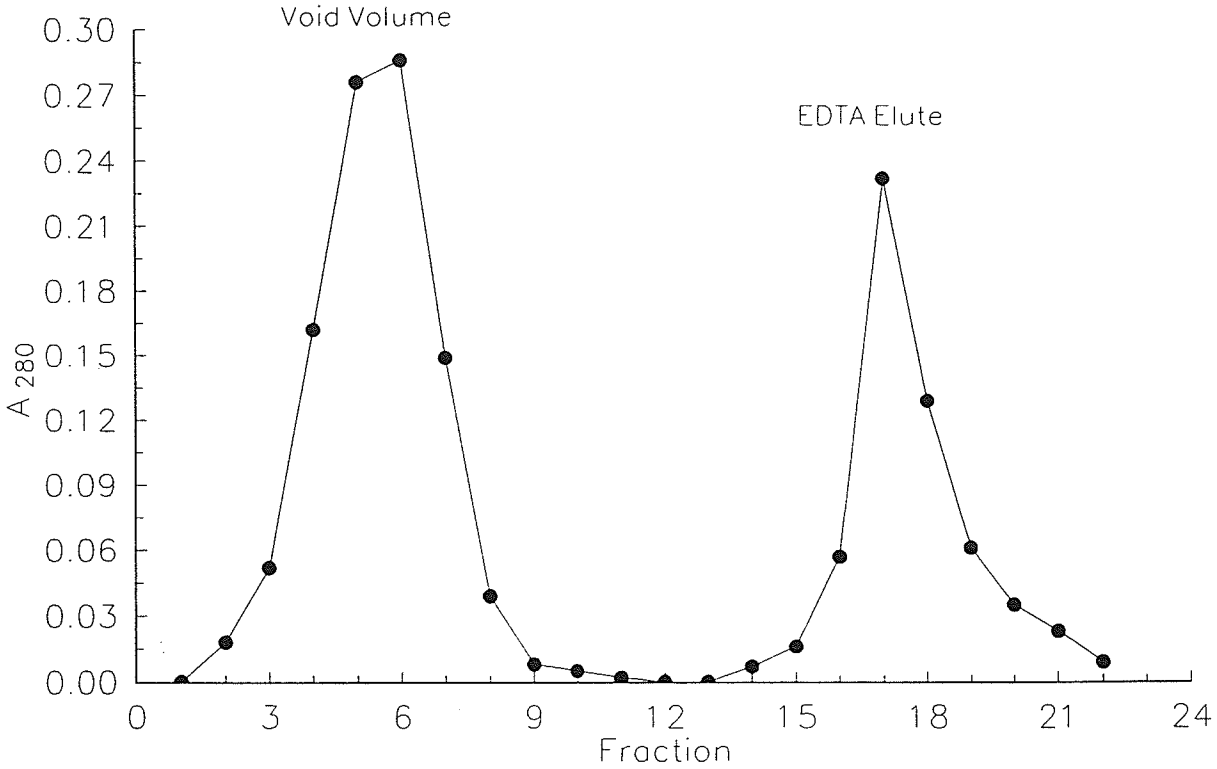

Void Volume
EDTA Elute
0.30
0.27
0.24
0.21
0.18
0.15
0.12
0.09
0.06
0.03
0.00
A 280
0
3
6
9
12
15
18
21
24
Fraction

Figure 3. Elution Profile of ion-exchange column. 100 ml of 0.1 to 0.75 M NaCl was used and 1 ml/fractions were collected. CRP was expected to appear between fraction 100 to 200.

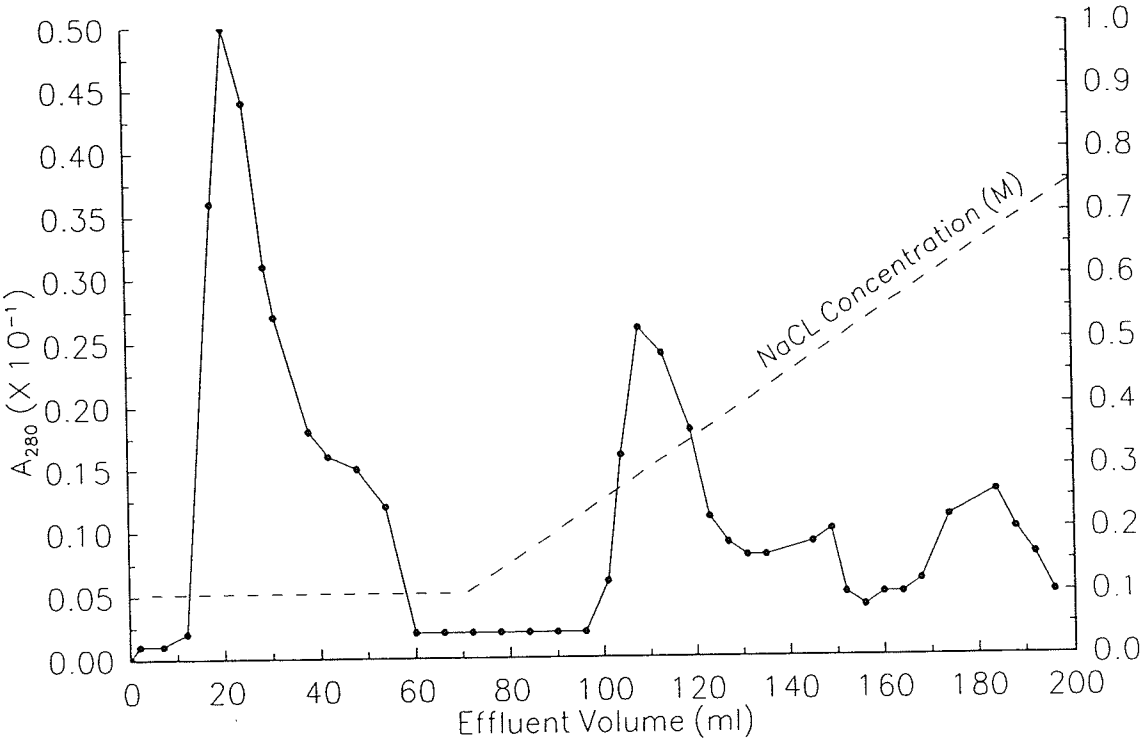

0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
A_{280} (X 10^{-1})
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
NaCL Concentration (M)
0
20
40
60
80
100
120
140
160
180
200
Effluent Volume (ml)

Figure 4. Sodium dodecyl sulphate-polyacrylamide gel of the proteins from CL-4B column, stained with coomassie blue. A, SAP eluted from CL-4B column with EDTA in reduced condition. B, CRP from the void volume of CL-4B column in the non-reduced condition. C, CRP from the void volume of CL-4B column in the reduced condition. Note the faint bands at 21.5 KDa.

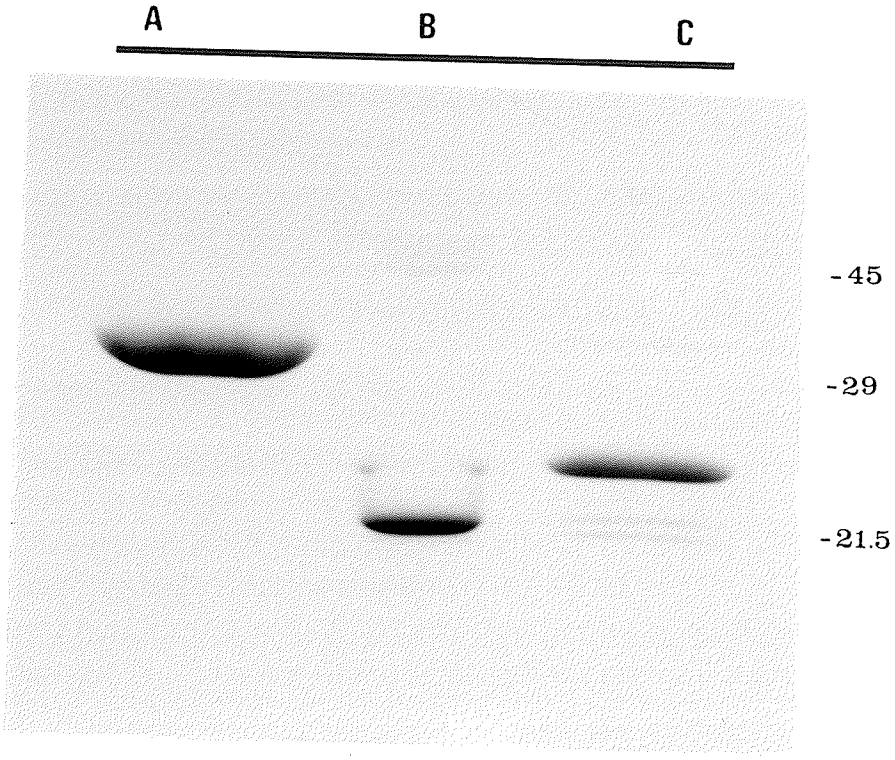

A
B
C
-45
-29
-21.5

Figure 5. Sodium dodecyl sulphate-polyacrylamide gel of the proteins from a ion-exchange column, stained with coomassie blue. A, proteins in the void volume. B, proteins eluted with NaCl.

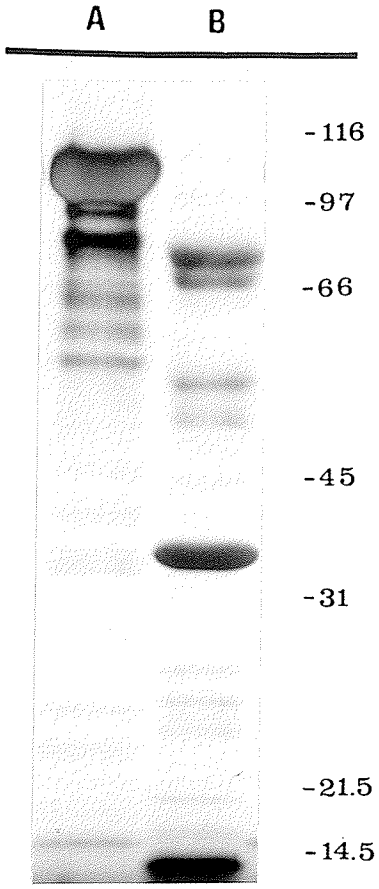

A
B
-116
-97
-66
-45
-31
-21.5
-14.5

Figure 6. Immunodiffusion of SPS to samples from PC and CL-4B column. A, Human CRP; B, sample eluted from PC column eluted with EDTA; C, trout SAP; D, trout CRP. Centre well contains SPS.

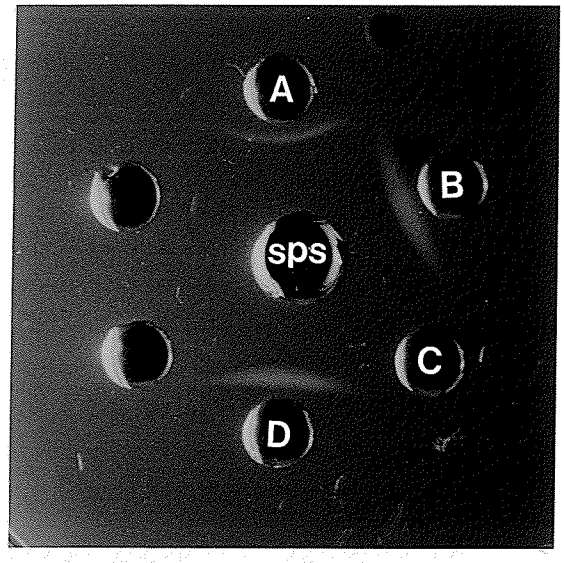
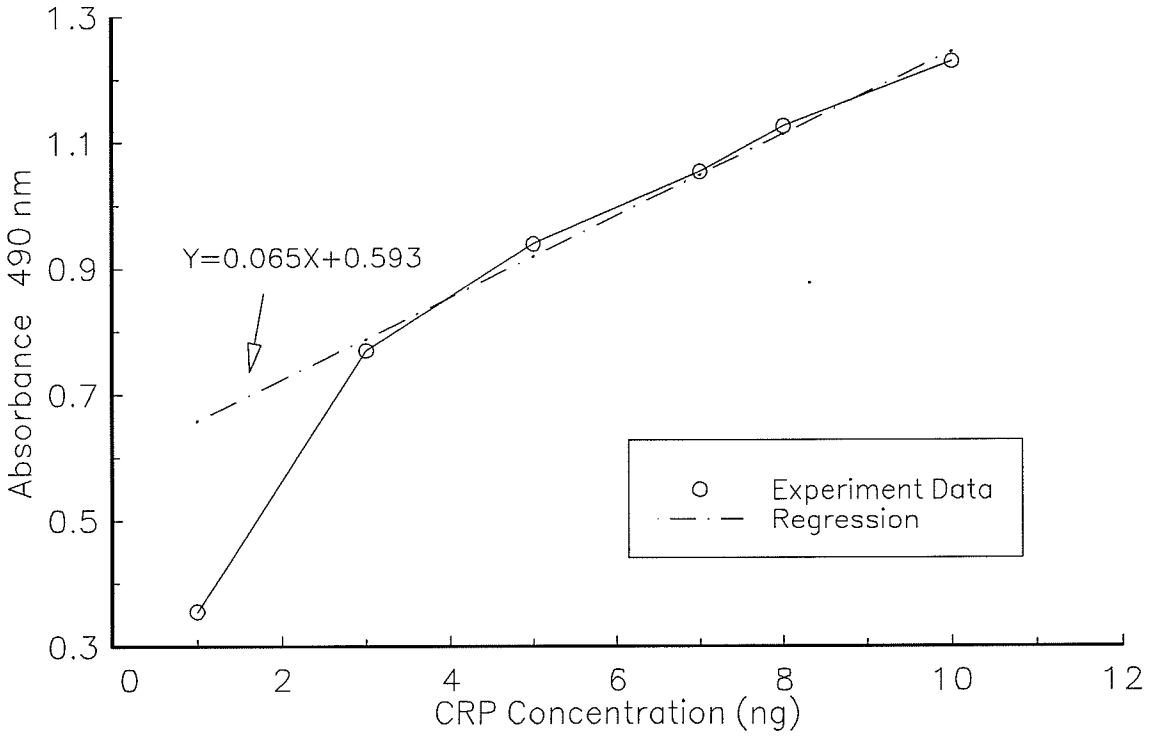

A
B
sps
C
D

Figure 7. ELISA data showing relation of CRP concentration and absorbance. Regression line was calculated without O.D. reading at 1 ng.


1.3
1.1
0.9
0.7
0.5
0.3
Absorbance 490 nm
Y=0.065X+0.593
Experiment Data
Regression
0
2
4
6
8
10
12
CRP Concentration (ng)

Discussion

Several methods were used to purify CRP and separate it from the co-purifying serum amyloid protein (SAP). The first purification experiments used the method developed for human CRP. Briefly, the method was as follows; the sample was first eluted from the PC column by the PC buffer (Oliveira *et al.*, 1980; Robey and Liu, 1981; Winkelhake and Chang, 1982) and subsequently loaded onto the ion-exchange column. According to the properties of CRP, rainbow trout CRP should be in the sample eluted from the PC column by the PC buffer. Since there was no reaction in the double immunodiffusion using the sample from the PC elute, the large number of other proteins in this sample and the inability to detect CRP from this sample when eluted with NaCl from the ion-exchange column, this approach was discontinued. The complex mixture of proteins in the PC elute may be due to the high negative charge of the PC residue (De Bee and Pepys, 1982) and complicates further purification. This observation corroborates other work where proteins eluted from a CPS affinity column with EDTA caused a problem in purifying horse CRP; in this study a continuous gradient of NaCl was replaced by a stepwise gradient in the anion-exchange chromatography (Takiguchi *et al.*, 1990). The different properties of DEAE-80 and DE-52 used by Volanakis *et al.* (1978) and Murai *et al.* (1990) could be another reason that CRP did not bind to the DEAE-80 ion-exchange column. My results indicate that PC is not the best buffer for eluting fish CRP from the PC column due to

contamination (i.e. large amount of other proteins present in the elute).

The method which was eventually used and where reproducible results were obtained was a modification of that described by Robey *et al.* (1983) for purification of dog fish CRP. It is a two step procedure including a PC-Sepharose column and a Sepharose CL-4B column. It was reported that only two proteins, CRP and SAP, were eluted by EDTA from PC-Sepharose column in the case of dog fish (Robey *et al.*, 1983). When samples were applied to the sepharose CL-4B column the two proteins were separated because only SAP binds to agarose while CRP passed through in the void volume. The principle of this method was applied in the purification of plaice CRP, in which a agarose-based column, Ultrogel AcA44, was used to remove plaice SAP (Pepys *et al.*, 1982). The results from dogfish (Robey *et al.*, 1983) and the rainbow trout CRP purification (this thesis) are similar. First, two proteins were eluted from the PC column by EDTA. The sizes of two proteins are 23.9 and 30.2 KDa in rainbow trout. Second, one protein does not bind to agarose and one protein does bind to agarose when the mixture of two proteins was passed through a CL-4B column. The 23.9 KDa protein in rainbow trout appeared in the void volume from the CL-4B column, which indicates it does not bind to agarose. The only difference between a PC column and CL-4B column is the PC column contains PC while the CL-4B does not. This indicates that the 23.9 KDa protein binds to PC and not agarose and this binding is calcium dependent, a property similar to human CRP. The 30.2 KDa protein which remained

in the column could be eluted with EDTA and indicates this protein reacted with agarose in a calcium dependent manner (a property of SAP). The behaviour of the two proteins indicates that the 23.9 KDa protein which reacted with PC is likely rainbow trout CRP and the 30.2 KDa protein reacting with agarose, is likely SAP. Further confirmation was clearly shown in the double immunodiffusion (CRP/SPS). The 23.9 KDa protein in rainbow trout strongly reacted with SPS as did human CRP sample while 30.2 KDa did not (Fig.6). This precipitation reaction was not a reaction between immunoglobulin and antigens since there was no precipitation reaction in the presence of EDTA. All of the previous studies with human CRP used C-polysaccharide to produce a precipitation band in immunodiffusion experiments. Furthermore, C substance -like activity has been detected in extracts from fungi, parasites, bacteria, and some vegetable materials (Longbottom and Pepys, 1964 and Capron *et al.*, 1964 as cited by Baldo *et al.*, 1977; Potter, 1972; Pepys and Longbottom, 1971). However, since SPS forms a precipitate with human CRP it has a C substance -like activity (Baldo *et al.*, 1977). Consequently, verification of CRP by its ability to form a precipitate with SPS supports this earlier work and my results clearly show the similarity of human CRP and trout CRP in this reaction.

There is still some uncertainty about the structure of the native rainbow trout CRP protein. Trout CRP was characterized by Winkelhake and Chang (1982) and Murai *et al.* (1990). A PC column was used and the bound sample was eluted with a 0.1 to 0.5 M linear

gradient of PC (Winkelhake and Chang, 1982). Winkelhake and Chang (1982) concluded that the predominant size of the eluted protein molecules was a 110 KDa with minor peaks at 60 KDa and 20 KDa (verified by molecular sieve column) and that the rainbow trout protein exists naturally in oligomeric and monomeric forms corresponding to a basic subunit molecular weight of about 20 KDa. My first attempts at purifying rainbow trout CRP used the method of Winkelhake and Chang (1982) but the major proteins eluted from the PC were not CRP. Murai *et al.*(1990) reported that the non-reduced CRP appeared as two subunits with molecular weights of 43.7 and 26.6 KDa and the reduced CRP appeared as a double band with a size of 26.6 KDa. Murai *et al.*(1990) also concluded that rainbow trout CRP was a trimer comprised of one monomer subunit and one disulphide-linked dimer. On the other hand, the molecule weight of the rainbow trout monomer was reported to be about 20 KDa (Winkelhake and Chang, 1982), which differs from the 26.6 KDa reported by Murai *et al.*(1990). The size of trout CRP (23.9 Kda), from my study, is larger than 20 KDa and slightly lower than 26.6 KDa. The larger sizes can be explained by the different methods used for verification (molecular sieving column versus SDS gel). The difference between my results and Murai *et al.* (1990) may be the result of a calculation difference and/or a gel effect. For example, the molecular weight of human CRP was calculated at 24 KDa by Volanakis *et al.* (1978), 23.3 KDa by Osmand *et al.* (1975) and 21 KDa by Oliveria *et al.* (1977). It is worth noting from the work of Murai *et al.* (1990), where human CRP and rainbow trout CRP were run

side by side on a SDS gel under reduced conditions, that trout CRP was calculated at 26.6 KDa but it migrated further than human CRP (min. 21 and max. 24 KDa). Nevertheless, two major differences are obvious when my results are compared with those of Murai *et al.* (1990) on rainbow trout CRP. First, there was only one band of 21.5 KDa on the SDS gel in the non-reduced condition (Fig. 4), suggesting there were no disulphide bonds between the trout CRP subunits. This is not surprising since CRP from plaice, a teleost like rainbow trout, did not contain disulphide bonds (Pepys *et al.*, 1982) and is similar to CRP from eels (Nunomura, 1991). Furthermore, disulphide bonds were not required for precipitating activity of the dogfish CRP (Robey *et al.*, 1983). Second, rainbow trout CRP appeared as a single band on the SDS gel in the reduced condition. My results indicate that trout CRP has one type of subunit with a molecular weight of 23.9 KDa, non-covalently associated.

Protein contaminants were seen in the sample profiles from SDS gel (45 ug/lane). They appeared as two bands around 21.5 KDa. Volanakis *et al.*(1978) reported large amounts of other proteins in the EDTA elute from PC-agarose column and they considered this contamination was due to non-specific Ca^{++} - dependent binding and/or C-reactive protein-mediated binding. The DE-52 ion-exchange column separated most of these proteins from CRP, with one exception complement (C1s). However, CRP purified by the method of Volanakis *et al.*(1978) still contained some contaminants (James, 1980 in De Beer and Pepys, 1982; Fiedel, *et al.*, 1982). In the case

of dogfish CRP and rainbow trout in my study only two major proteins were collected from the PC-column. Perhaps the differences in the amount of contamination in human CRP preparation and none or minor amounts in dogfish CRP and in my samples of rainbow trout CRP are related to the species. Regardless, the procedure and buffer system I used was much simpler than the three-step purification procedure and the sample purity is far higher than samples recovered by eluting with PC.

When antibody concentrations were 1 ug/50 ul, ELISA results had an $O.D._{490}$ reading which was linear, increasing with the increasing concentration of trout CRP except values of 1 ng CRP which were too low to be detected (Fig. 7). The ELISA system can be used to detect concentrations of CRP as low as 3 ng CRP and it can detect CRP in fish serum. Its usefulness in detecting differences in serum levels in fish requires further refinements since there is still some interference due to high background readings. It is clear, however, due to the purity of the initial CRP, that the rabbit polyclonal antibody has considerable value in the screening cDNA libraries (see Chapter 5, Screening cDNA Library with Antibody, page 80).

CHAPTER 3. THE USE OF THE POLYMERASE CHAIN REACTION TO ISOLATE THE RAINBOW TROUT CRP GENE

Introduction

There are numerous ways to isolate a gene (Sambrook *et al.*, 1989). The polymerase chain reaction (PCR) is widely used in molecular biology because it is very quick and depending on the sequence homology may be highly efficient (Saiki *et al.*, 1988).

Gene maps and partial sequences of CRP genes are shown in Figs. 8 and 9, respectively. Two highly conserved regions in human CRP and *Limulus* CRP protein sequences have been reported by Nguyen (1986). In region 1 residues 51-66 in human CRP and 52-67 in *Limulus* were considered to be involved in the binding of phosphorylcholine ligand (Liu *et al.*, 1982). In region 2 residues 133-144 in human CRP and residues 139-153 in *Limulus* were considered to be a part of the Ca^{++} binding site because it was similar to the consensus sequence found in calmodulin and related molecules (Dang *et al.*, 1985).

The objective of these experiments was to assess the human sequence and from this information determine primer sequences which could be used in the isolation of the CRP gene from fish.

Previous work done in this laboratory using Taq DNA polymerase on RNA isolated from the pituitary of rainbow trout has shown a successful isolation of the rainbow trout growth hormone gene without reverse transcriptase (unpublished data). This activity of Taq DNA polymerase on mRNA has been described by Jones and Foulkes (1989).

Figure 8. Gene maps of human, rabbit, mouse, and *Limulus* CRP. A, AvaI; B, BamHI; H, HindIII; K, KpnI; M, MstII; N, NcoI; P, PvuII; S, SphI; St, StuI.

Note: CRP in mice is not an acute phase protein.

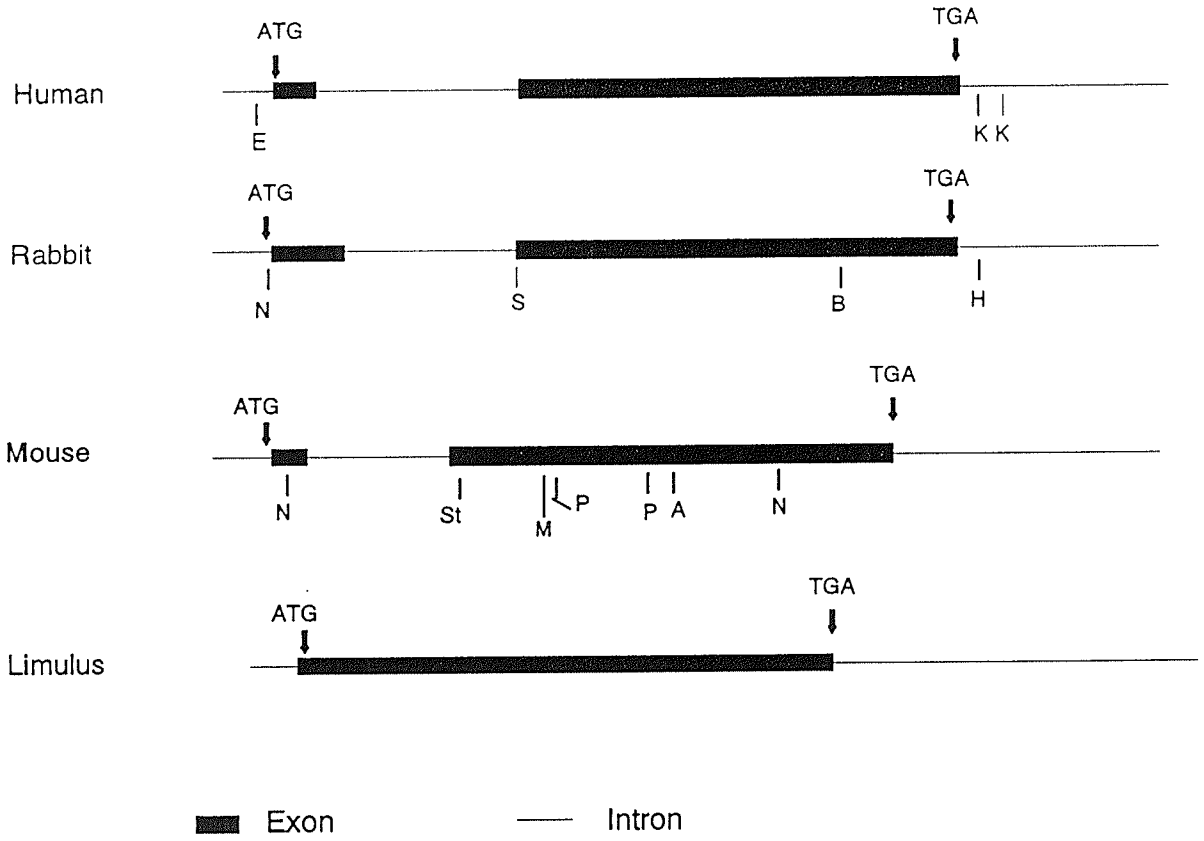

Human
ATG
TGA
E
K K
Rabbit
ATG
TGA
N
S
B
H
Mouse
ATG
TGA
N
St
M
P
P A
N
Limulus
ATG
TGA
Exon
Intron

Figure 9. Nucleotide sequences of human, rabbit, mouse, and *Limulus* CRP in the two highly conserved regions and region for primer CRP-start.

1. TWO HIGHLY CONSERVED REGIONS:
(proposed by Nguyen *et al.* 1986)

Region One:

```
L  ATATTTTCGTACAATACAGCTAAAAACGACAATGAGCTTCTGACATCT 52-67 aa
H  ATTTTCTCGTATGCCACCAAGAGACAAGACAATGAGATTCTCATATTT 51-66 aa
R  ATTTTCTCCTATGCCACCAGGAGACAATTTAACGAGATCCTCCTGTTT 50-65 aa
M  GTCTTCTCTTATGCTACCAAGAAGAACTCTAACGACATTCTCATATTT 50-65 aa
```

```
CRP-ECOR      5'GGAATTCTAATGAGATTCTCATATTT
               EcoR I  C  C         C   C
```

Region Two:

```
L  GTTGTTCTTGGACAAGAGCAGGACAGTGTCGGTGGTGAGTATGAT 139-153 aa
H  ATCATCTTGGGGCAGGAGCAGGATTCCTTCGGTGGGAACTTTGAA 133-147 aa
R  ATTATTCTGGGGCAGGATCAGGATTCGTTTGGTGGAAGCTTTGAG 132-146 aa
M  ATCATCTTGGGGCAGGAGCAGGACTCGTATGGCGGTGACTTTGAT 132-146 aa
```

```
5' GGGCAAGAGCAGGACACAAGCTTCC 3'
     A  G  T     TT
```

2. REGION FOR CRP-START PRIMER:

```
L    -24 to -12 aa  ATGAAAACATTTCACGGGCCGACTTTCGGGACTGCTGTG
H    -18 to -6 aa   ATGGAGAAGCTGTTGTGTTTCTTGGTCTTGACCAGCCTC
R    -20 to -8 aa   ATGGAGAAGCTGCTGTGGTGTTTCCTGACTTTGGTCAGC
M    -19 to -7 aa   ATGGAGAAGCTACTCTGGTGCCTTCTGATCATGATCAGC
```

```
CRP-START GGGAATTCATGGAGAAGCTGTTGTG
          EcoR I              AC C
```

Note: R = Rabbit; H = Human; M = mouse; L = *Limulus*.

Materials and Methods

Primer selection

Based on two conserved regions reported and comparisons with the sequences of rabbit and mouse CRP, two primers were synthesized; CRP-EcoR (Residues 59-67) and CRP-Hind (Residues 135-147). The sequence of the third primer was based on comparisons of the regions after the translation start point in human, mouse, rabbit and *Limulus* CRP. The orientation of primers is shown in Figure 10.

Primer CRP-Start 5' **GGGAATTC**ATGGAGAAGCTGTTGTG -3'
EcoRI AC C

Primer CRP-EcoR 5' **GGAATTC**TAATGAGATTCTCATATTT -3'
EcoRI C C C C

Primer CRP-Hind 5' **GGAAGCT**TGTGTCCTGCTCTTGCCC -3'
HindIII AA A C T

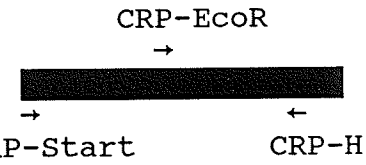


Figure 10. Positions and orientation of CRP primers.

Preparation of tissue samples for RNA extraction

In order to increase the proportion of CRP mRNA, rainbow trout were injected with turpentine (0.3 ml/600-800 gm fish, see Chapter 2, Purification and Characterization of Rainbow Trout C-Reactive Protein, page 23). Procedures for handling fish are also given in

chapter 2. After forty-eight hours the turpentine injected fish were anaesthetized with phenoxyethanol and livers were quickly removed (using dissection tools which were autoclaved), then divided into samples of 1 gm and stored in liquid nitrogen.

Extraction of total RNA

The extraction method was that of Davis *et al.*(1986). The steps were as follows: one gram of trout liver frozen in liquid nitrogen was placed in 4 ml of ice cold GIT buffer (Guanidine thiocyanate buffer containing 4 M of guanidine thiocyanate, 5 mM sodium citrate, 1 mM EDTA, 0.5% N-lauroylsarcosine, 0.1% anti-foam A and 12 mM 2-mercaptoethanol), then immediately homogenized with a polytron (Brinkmann Instruments, Rexdale. Ontario) for 1 minute on ice. All buffers and ultracentrifuge tubes were treated with 0.1% DEPC (diethyl pyrocarbonate) at 37°C overnight and then autoclaved. Glassware was baked at 180°C overnight. The sample was transferred to a 10 ml centrifuge tube and centrifuged at 7000 rpm (revolution per minute) for 10 minutes using a JA17 rotor on a J2-21 model centrifuge (Beckman Instruments Inc., Palo Alto, CA) to remove any insoluble particles. The supernatant was transferred to a polyallomer ultracentrifuge tube (Beckman) containing 4 ml of CsCl solution (5.7 M CsCl and 0.14 M sodium acetate, pH 6.0). Light paraffin oil (Fisher Scientific Limited, Nepean, Ontario) was used to top up and balance (by weight) the tubes. The ultracentrifugation was done with a Beckman SW41 rotor on a model L8-55 ultracentrifuge at 32000 rpm (174,000 X g) for 24 hours at

25°C. The supernatant was removed with a glass pipette. A surgical scalpel blade was used to cut the tube 0.5 cm from the bottom. Any liquid adhering to the wall of the short piece of centrifuge tube was removed with kimwipe but the clear precipitation on the bottom, which was primarily RNA, was left intact. This precipitate was washed twice with 100 ul of 70% ethanol, the ethanol allowed to evaporate and then 100 ul of H_2O was added to dissolve the RNA. The RNA concentration and purity was measured with a spectrophotometer at wave lengths of 260 and 280 nm. Three ug of total RNA sample were loaded onto a formaldehyde gel. The formaldehyde gel was made according to Lehrach *et al.*(1977). Briefly, two hundred ml of gel were made from 2 gm of agarose, 10.8 ml of formaldehyde (37%), 15 ul of ethidium bromide (10 mg/ml), 20 ml of 10 X MOPS buffer (0.2 M of 3-[N-Morpholino] propanesulfonic acid, 0.05 M of Na acetate and 0.01 M EDTA, pH 7.0). RNA samples were treated at 55°C for 15 minutes in 20 ul of buffer containing 2 ul of 10X MOPS buffer, 10 ul of formamide, and 3.5 ul of formaldehyde and then loaded onto the gel before mixing with 3 ul of RNA gel loading buffer (50% of glycerol, 0.4% of xylene cyanole FF and bromophenol blue and 1mM of EDTA). The gel was run in 1 X MOPS buffer at 20 V overnight and at 30 V on the next day until RNA samples were well separated. The RNA gel was exposed to ultraviolet light (transilluminator model TL-33, Ultraviolet Products, Inc.), photographed using a fotodyne camera (Bio/Can, Ontario) with coaterless instant pack film (Polaroid, Cambridge, MA).

PCR reaction

The rainbow trout template RNA was used at a level of 4 ug/reaction. The reaction buffer contained 0.5 unit Taq/100 ul (*Thermus aquaticus* strain YT1, Promega, Madison, WI), 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X100, and 0.2 mM each of dNTPs. The reaction was carried out at 94°C for 1.5 minutes, 47°C for 2 minutes and 72°C for 3 minutes for a total of 45 cycles on a PHC-2 thermal cycler (Techne, Cambridge, U.K.). Ramp time (the time of changing from one temperature to another) was set at 4. The mineral oil was placed in tube holder on the thermal cycler to enhance heat exchange. The annealing temperature, 47°C, in the CRP was calculated using the formula (Annealing temperature = 2°C X $Number_{(A+T)}$ + 4°C X $Number_{(C+G)}$ - 5°C), reported by Itakura *et al.*(1984). At the completion of the cycle samples were held at 10°C until stored or used. Twenty ul of each reaction (DNA from the PCR of the RNA) were loaded onto a 0.8% agarose gel and run at 45 V. At the same time 0.1 ug of cDNA arms (lambda ZAPII) was loaded onto gel as a reference. The DNA on the gel was, photographed, denatured in 0.5 M NaOH/1.5 M NaCl and neutralized in 1.5 M NaCl/0.5 M Tris (pH 7.2) and transferred to a membrane (Hybond-N, Amersham Canada, Oakville, Ontario), according to the method of Southern (1975).

Purification DNA from agarose gel

The method was based on that described by Seth (1984). After 10 ug of PBr322 DNA containing *Xenopus* CRP gene were digested with

EcoR I at 37°C for 2 hours, the vector and insert DNA were separated on a 1% agarose gel. The plasmid (PBr322) with *Xenopus* CRP cDNA insert(about 940 bp) was kindly donated by Dr.Lin (Food and Drug Administration, Bethesda, Maryland, USA). The gel band containing the insert DNA was excised and transferred to a 1.5 ml microcentrifuge tube. The excised piece of gel was weighed and crushed in the tube and 0.1 ml of phenol/0.1 gm gel added to the tube. This mixture was placed in liquid N_2 for 10 minutes, then centrifuged at 13,000 rpm on a Biofuge A microcentrifuge (Baxer, Ontario). The supernatant was transferred to a new tube and extracted with phenol, phenol/chloroform, chloroform, consecutively with the aqueous layer saved each time (Davis *et al.*, 1986). The final aqueous layer, containing the DNA, was precipitated by adding 0.1 volume of 3 M Na acetate (pH 5.2) and 2 volume of 95% ethanol at -20°C. This material was centrifuged at 13,000 rpm, the ethanol decanted and the pellet air-dried. The pellet was re-suspended in TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0).

Hybridization

Xenopus CRP gene (see section Purification DNA from Agarose Gel, page 56) was labelled by using the Primer-A-Gene DNA labelling system (Promega). Free nucleotides and labelled DNA fragments were separated in a Chroma Spin-100 column (ClonTech, Palo Alto, CA) by centrifugation at 400 X g in a swinging bucket rotor.

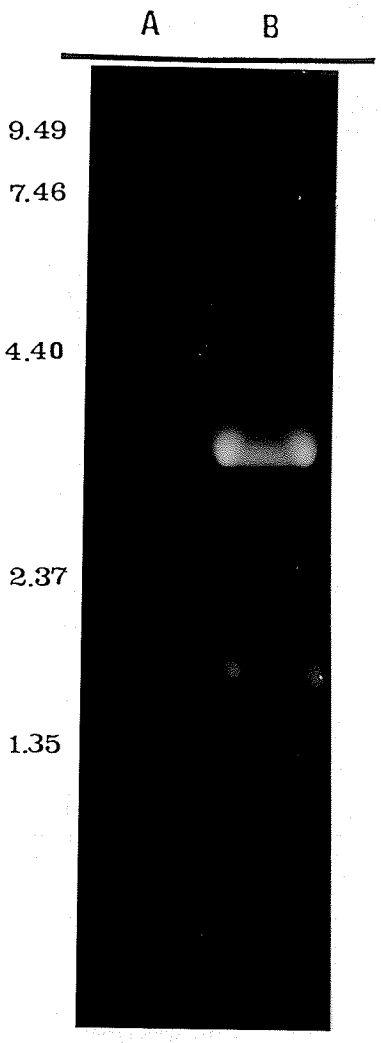
The membrane containing the PCR products (see section PCR Reaction, page 56) was pre-hybridized in the buffer containing 1 M

NaCl and 1% SDS for at least 4 hours and the DNA probe (*Xenopus* gene), denatured by boiling for 5 minutes, was added to the mixture and hybridization was done overnight at 50°C. The membrane was washed in washing buffer 1 (2 X SSC and 0.5% SDS) for 10 minutes at room temperature, followed by washing buffer 2 (0.1% SSC and 0.1% SDS) at 50°C for 30 minutes. Autoradiography was done by placing the membrane and X-ray film (Kodak) in a cassette with two intensifying screens (Kodak) at -80°C overnight.

Results

RNA extracted from rainbow trout is shown in Fig. 11. When the reaction mixture containing primer CRP-start and CRP-Hind was run on an agarose gel a single band of about 400 bp appeared. No band appeared on the gel from the reaction containing primer CRP-EcoR and CRP-Hind.

Figure 11. Formaldehyde gel of total RNA from trout liver. A, RNA standard, size, 9.49, 7.46, 4.4, 2.37, 1.35 Kb. B, rainbow trout total RNA.


A
B
9.49
7.46
4.40
2.37
1.35

Discussion

The bands of ribosome RNA in the total rainbow trout RNA shown in Fig. 11 are very sharp and the density of the higher band is about twice that of the lower band, which indicate a high quality RNA has been obtained (Sambrook *et al.*, 1989). Since RNA is easily degraded, the quality of RNA is important for a PCR reaction and specially for construction of cDNA library.

The PCR method while extremely rapid can sometimes give misleading information (Innis and Gelfand, 1990). Initially it looked as if the CRP gene of rainbow trout had been isolated, based on a size of about 400 bp. This is supported by my calculation of the DNA fragment produced by PCR using between primer CRP-start and CRP-Hind, would be 484 bp for humans, 487 bp for rabbits and mice and 520 bp for *Limulus*, based on published sequence data. Although the size of the DNA fragment was slightly smaller the results still looked promising. However, the organisms from which information on the CRP was available were phylogenetically quite far removed from fish. It was clear from the published information that the amount of sequence homology for CRP genes was not high, e.g., *Xenopus* CRP gene is 45.1, 41.9, 38.5, and 28.3% homology in amino acid sequence to human, rabbit, mouse, and *Limulus*, respectively (Lin and Liu, 1993). The recent report on the nucleotide sequence of CRP from *Xenopus* was significant for studies on fish as *Xenopus* is much closer phyllogenetically to fish.

I reasoned that if the homology of the *Xenopus* CRP gene was

high with the fish gene it could be used to verify the presence of rainbow trout CRP. Interestingly there was no hybridization between the 400 bp DNA fragment from PCR and the *Xenopus* CRP at 50°C, which suggests low homology between these two DNA fragments. Perhaps hybridization would occur at lower temperatures but reactions at 50°C are considered to have relatively low stringency. One could conclude from this that the PCR product was the wrong fragment, the probe was not what it appeared to be or there is very low homology between *Xenopus* CRP and rainbow trout CRP.

The first experiment was to test the uniqueness of the *Xenopus* gene. Surprisingly, the *Xenopus* CRP DNA hybridized with lambda ZAPII at 50°C, a vector for constructing our cDNA library. Clearly whatever was cloned into PBr322 (*Xenopus*) is not homologous with any PCR product from rainbow trout and the 400 bp DNA may not be trout CRP. Furthermore, the 400 bp PCR fragment was later shown not to hybridize to a trout cDNA positive clone selected by immunological screening (see Chapter 5, Screening cDNA Library with Antibody). It appears that the 400 bp DNA fragment from the PCR reaction in my experiments is a product of the two primers and an unknown fragment of template DNA.

Considering the ubiquitous nature of CRP throughout the animal kingdom it was surprising that rapid molecular biological methods did not work. It is well known that the calcium binding site (36 bp) and PC binding site are common for all CRPs studied to date, but the significance of the nucleotide sequence in the region of PC binding is not clear since conformational changes seem to be more

important (Young and Williams, 1978). Furthermore, while it is relatively easy to construct a highly homologous primer from the calcium binding site it is very difficult to find another homologous region. Consequently, more traditional methods of isolating the gene are likely required, especially from phyllogenetically distant organisms.

CHAPTER 4. CONSTRUCTION OF cDNA AND GENOMIC LIBRARIES

Introduction

The purpose of cDNA (complementary DNA) library construction is to convert mRNA into double strand cDNA and clone it into a vector for screening with either nucleic acid probes or antibodies. Construction of a cDNA library from mRNA is an essential step for many studies in molecular biology since the message (product of the gene) is transcribed from the genomic sequence. Once a cDNA clone is isolated it can be used to screen genomic libraries and when the gene is isolated experiments can be designed to study the location of the gene, its' structure, expression and regulation. Other reasons for constructing rainbow trout libraries for screening C-reactive protein are, (1) there was no information on fish CRP gene but fish are considered to be a key evolutionary link between the horseshoe crab (*Limulus*) and mammals and (2) the current method of collecting CRP is costly and time consuming due to the large number of fish required to obtain serum.

Material and Methods

mRNA purification

The methods of tissue preparation and the RNA extraction were the same as described in Chapter 3. mRNA was purified by using Poly(A) quit™ mRNA purification kit purchased from Stratagene (La Jolla, CA). The major steps were as follows; up to 500 ug of RNA was heated at 68°C for 5 minutes, 10X sample buffer (10 mM Tris.Cl, pH7.5, 1 mM EDTA and 5M NaCl) was added to the RNA sample to bring the buffer concentration to 1X, and the sample placed on ice. A prepacked oligo(dT) cellulose column was conditioned with 400 ul of high salt buffer (10 mM Tris.Cl, pH 7.5, 1 mM EDTA and 0.5 M NaCl), the entire RNA sample was applied onto the column and the void volume was re-applied to ensure that any mRNA which passed through the column was recovered. The column was washed with 400 ul of high salt buffer and 600 ul of low salt buffer (10 mM Tris.Cl, pH 7.5, 1 mM EDTA and 0.1 M NaCl) prior to eluting the bound mRNA with 800 ul of elution buffer (10 mM Tris.Cl, pH 7.5, 1 mM EDTA) at 65°C. The concentration and purity of RNA was determined using a spectrophotometer set at 260 nm and 280 nm.

Construction of cDNA library

Numerous methods have been used to construct cDNA libraries. The method used in these experiments is that of Sambrook *et al.*(1989). Briefly, first strand cDNA synthesis used Oligo(dT) primers and reverse transcriptase. Second strand synthesis was

initiated by combining the mixture from the first strand synthesis with RNase H and *E. coli* DNA polymerase, in which RNA from cDNA:RNA hybrid is replace by newly synthesized DNA. Gaps in the double stranded cDNA were repaired by adding *E. coli* DNA ligase and the ends of cDNA fragments were phosphorylated by bacteriophage T4 DNA polynucleotide kinase. After methylation of the cDNA, the ends of cDNA fragments were repaired by bacteriophage T4 DNA polymerase, then ligated to EcoRI linkers. These fragments of DNA were digested with EcoR I and following size selection (see section on Ligation to EcoR I linkers, EcoR I digestion and size selection, page 70) the cDNA was ready to ligate into a bacteriophage vector which in turn was used to infect bacteria cells (see section on Ligation to Bacteriophage arm DNA (ZAPII), packaging and plating, page 71).

First strand cDNA synthesis

This reaction was carried out in 50 ul of a mixture containing 10 ug of mRNA, 10 ug of oligo$(dT)_{18}$, 50 mM of Tris (pH7.6), 70 mM KCl, 10 mM $MgCl_2$, 1 mM of each dNTP, 4 mM of dithiothreitol, 25 units of RNAgard (RNase inhibitor), and 400 units of murine reverse transcriptase. In order to monitor the reaction, a small scale parallel reaction was set up by removing an aliquot of 2.5 ul of the main reaction mixture and combining this with 0.1 ul of [α-^{32}P]-dCTP (10 uCi/ul). The main reaction and parallel reaction were incubated at 37°C for 1 hour.

The yield from the first strand synthesis was determined by the TCA (Trichloroacetic acid) precipitation method. A small volume

of radioactive labelled sample (0.5 to 2 ul) was added to each of two glass fibre filters and dried. One filter was placed in 300 ml of washing buffer and then gently shaken with the aid of forceps for two minutes. This washing was repeated two more times. Finally, it was briefly rinsed in 70% ethanol and dried under light. Each of the two filters were placed individually in scintillation vials containing 5 ml scintillation fluid (Sigma) and counted. Counts of the washed and unwashed filters were used to calculate the efficiency of the synthesis.

Second strand cDNA synthesis

Two reactions were involved in this procedure. Reaction 1 was combined with 50 ul from the first strand synthesis mixture with 70 ul of 10 mM $MgCl_2$, 5 ul of 2 M Tris (pH7.4), 10 ul of [α-^{32}P]-dCTP(3000 Ci/mmole;10 uCi/ul), 1.5 ul of 1 M $(NH_4)_2SO_4$, 1 unit of RNase H, and 45 units of *E. coli* DNA polymerase I, for a total volume of 140 ul. This mixture was then incubated at 16°C for 4 hours. Reaction 2 was prepared by adding 1 ul of 50 mM NAD, 100 unit of *E. coli* DNA ligase and 3 units of bacteriophage T4 DNA polynucleotide kinase and then incubated at room temperature for 15 minutes. Five ul of 0.5 M EDTA was added to stop the reaction and a small aliquot (3 ul) of the mixture was taken out to determine the yield using TCA precipitation. The remainder of the mixture was precipitated with a 0.1 volume of 3 M Na acetate (pH 5.2) and 2 volumes of 95% ethanol at -20°C, overnight.

The size of the cDNA fragments from the first strand and

second strand synthesis were checked by alkaline denatured gel electrophoresis.

Methylation of EcoRI sites

Methylation was done using chemicals from Promega (Madison, WI). The cDNA from reaction 2 was precipitated, (see section on Second Strand cDNA Synthesis, page 68) by first spinning at 13,000 rpm in a microcentrifuge, then the ethanol was evaporated and the pellet resuspended in 74 ul of TE buffer (pH 7.6). The methylation reaction was prepared by combining the cDNA in TE and 10 ul of 10X methylation buffer (1 M Tris, pH 8.0 and 100 mM EDTA), 2 ul of 10 mM S-adenosyl-L-methionine and 10 ul of BSA (1 mg/ml). During the methylation experiment two aliquots of 3 ul each were removed from the reaction mixture; one prior to adding methylase (4 ul containing 40 units) and one after the methylase was added. Both 3 ul-reaction mixtures contained 0.1 ug of air-dried Pst I digested pBR322 DNA. The reactions were done at 37°C for a hour and stopped by heating at 68°C for 15 minutes. The two 3 ul reactions were analyzed by EcoRI digestion at 37°C for 2 hours in 20 ul of the buffer containing 10 units of EcoRI, 5 mM Tris, pH 8.0, 10 mM NaCl and 0.1 M $MgCl_2$ and then electrophoresed on a 1% agarose gel. The cDNA from the main reaction mixture was precipitated after extracting once with an equal volume of phenol:chloroform (1:1) and once with chloroform.

Ligation to EcoRI linkers, EcoRI digestion and size selection

The cDNA from the methylation reaction was recovered using methods previously described (Methylation of EcoRI sites, page 69). The ends of cDNA fragments were repaired in a 50 ul reaction mixture containing 18 mM of $(NH_4)SO_4$, 66 mM of Tris.Cl (pH8.3), 6.6 mM of $MgCl_2$, 10 mM of β-mercaptoethanol and 2 units of bacteriophage T4 DNA polymerase at 37°C for 15 minutes. The reaction was stopped by adding 1 ul of 0.5 M EDTA (pH 8.0). The sample was extracted with phenol:chloroform (1:1) once, then applied onto S-300 spin column (Pharmacia) and centrifuged using a swing bucket rotor at 400 X g for 2 minutes. The void volume was collected and the cDNA was precipitated with ethanol, recovered and resuspended in TE buffer.

Ligation of cDNA to EcoRI linkers was done at 16°C for 12 hours in 20 ul of a reaction containing cDNA, 1 ug of phosphorylated EcoRI linkers (10 mer), 200 units of T4 DNA ligase, and 4 ul of 5X ligation buffer (330 mM Tris, pH 7.5, 25 mM $MgCl_2$, 5 mM dithioerythritol, 5 mM ATP). The sampled was heated at 68°C for 15 minutes to inactive the enzyme and an aliquot (0.5 ul) was removed for further analysis. The volume of the remaining sample was increased to 200 ul by the addition of EcoRI (200 units), 20 ul of 10X restriction enzyme buffer, and water. A second aliquot of 2 ul from the 200 ul reaction mixture was removed and combined with 0.1 ug of air-dried and Pst I digested pBR322 DNA as a positive control. The digestion reactions (main reaction mixture and two

aliquots) were incubated at 37°C for 3 hours. The two aliquots were analyzed on a 1% agarose gel to determine the success of the reaction. The main sample was extracted once with phenol:chloroform (1:1) and loaded onto a S-400 size selection spin column (Pharmacia). The material in the void volume (cDNA) was precipitated, recovered by centrifugation and resuspended in 10 mM of Tris.Cl buffer. The concentration of DNA was determined by using ethidium bromide agarose plate which contained 1% agarose and 4 ul of ethidium bromide solution (10mg/ml) to which was applied the sample and various concentrations of DNA and the gel was viewed with UV light.

Ligation to bacteriophage arm DNA (ZAPII), packaging and plating

The bacteriophage vector, Lambda ZAPII (Stratagene), was pre-digested with EcoRI and dephosphorylated. The concentration of vector and insert DNA in the ligation mixture was based on an equal molar ratio. The volume of the ligation reaction was 5 ul and contained 66 mM Tris, pH 7.5, 5 mM $MgCl_2$, 1 mM dithioerythritol, 1 mM ATP, cDNA, bacteriophage vector, and 2 units of T4 DNA ligase. Four test ligation reactions (0.25 ug lambda DNA/reaction with different amounts of cDNA) were done prior to a large scale ligation reaction (1 ug lambda DNA/reaction). Following ligation all mixtures were exposed to the Gigapack™ II plus packaging extract (Stratagene). Briefly, the ligation mixture was first added to the freeze/thaw extract and then 15 ul of sonic extract was

added. The mixture was stirred, followed by a brief centrifugation, and incubated at room temperature for 2 hours. Five hundred ul of SM buffer (5.8 gm of NaCl, 2 gm of $MgSO_4$, 50 ml of 1 M Tris, pH 7.5, 5 ml of 2% gelatin per 1 litre) and 20 ul of chloroform were added. After mixing gently the mixture was centrifuged and the supernatant containing the bacteriophage was stored and ready be titered. Titering was done by combining the supernatant (bacteriophage) ranging from 1 to 10 ul, 200 ul of *E.coli* XL1-Blue cell ($O.D_{600}$=0.5), 3 ml of 48°C top agar (0.75% agar in NZY medium: 5 gm NaCl, 2 gm of $MgSO_4.7H_20$, 5 gm of Yeast Extract, 10 gm NZ Amine per litre, pH 7.5), 15 ul of 0.5 M IPGT (Isopropyl β-D-thiogalactoside) and 50 ul of 250 mg/ml X-gal (5-Bromo-4-chloro-3-indolyl β-D-Galactopyranoside) onto NZY plates (1.5% agar in NZY medium). After incubation at 37°C for 8 hours the ligation reaction was assessed by counting the number of blue and white plaques. Based on those number, the total number of plaques in the library was determined. The average insert size of cDNA was determined by randomly picking 12 individual clones, these clones were amplified and the DNA of each clone extracted, digested with EcoRI and electrophoresed on 0.8% agarose gel (Klickstein, 1993).

Construction of Genomic library

Extraction of genomic DNA

Rainbow trout livers were removed from fish using sterile procedures and frozen in liquid nitrogen. Liver (0.2-1 gm) was ground to a fine powder on dry ice with a pestle and mortar and

then suspended in 1.2 ml digestion buffer (100 mM NaCl, 10 mM Tris, pH 8.0, 25 mM EDTA, 0.5% SDS and 0.1 mg/ml proteinase K) per 100 mg tissue in a tightly capped 50 ml tube. The tube was incubated with shaking at 50°C for 12-18 hours. The sample was extracted, three times, with an equal volume of phenol and centrifuged at 1700 X g. The aqueous layer was transferred to a new tube, the DNA precipitated by the addition of 0.5 volume of 7.5 M ammonium acetate and 2 volumes of 95% ethanol. The precipitated DNA was transferred with a glass loop to a new centrifuge tube containing 70% ethanol. The DNA pellet recovered by centrifugation at 1700 X g for 5 min was washed one more time with 70% ethanol. The DNA pellet was air-dried and resuspended in TE buffer by gently shaking overnight at room temperature.

Preparation of genomic DNA fragments

Genomic DNA was first reduced in size by partial digestion with Sau3A, an enzyme which cuts DNA frequently and without any bias regarding the Sau3A restriction site. Following digestion and size selection (see below) the fragments were inserted into the lambda vector for a future screening. Lambda EMBL-3 (Stratagene) vector was used for construction of genomic library. This vector accepts fragments of DNA with sizes ranging from 9 to 23 Kb and combined with the host bacteria, XL1-blue MRA(P2) strain utilizes Spi (Sensitivity to P2 interference) selection to prevent recombining of stuffer fragments into EMBL3 (Zissler *et al.*, 1971). Consequently, only recombinant phages can grow.

Partial restriction digestion

After high molecular weight genomic DNA was isolated it was necessary to reduce the size range to construct a genomic library (size range 15-23 Kb). Since the basis of partial digestion depends on the amount of restriction enzyme and incubation time of the reaction, a test reaction was run before a large scale digestion reaction. Conditions for the restriction digests were optimized in the small scale reaction and consisted of a few microcentrifuge tubes containing 3 ug of DNA, concentrations of Sau3A enzyme ranging from 0.031 to 2 units in the same buffer condition, and incubation of 45 minutes at 37°C. The restriction enzyme concentration for the large scale preparation was determined by analyzing each sample on 0.4% agarose gel after restriction digestion for a DNA size range of 15-23 Kb. The concentration of reagents and conditions for large scale digestion (100 ug DNA in 500 ul per reaction) were identical to the small scale reaction with the optimal enzyme concentration being 2.09 units/500 ul. An aliquot of 20 ul of DNA from the digestion was analyzed on a 0.4% agarose gel to determine the size range of the digestion products. The digested DNA was extracted with 1 volume of phenol/chloroform (1:1) and centrifuged at 12000 X g for 10 minutes. The upper aqueous phase was transferred to a fresh tube and extracted with phenol/chloroform (1:1). The DNA pellet was resuspended in TE buffer and stored at 4°C until used.

Size fractionation by Sucrose gradient

A 10-40% continuous sucrose (RNase, DNase, Proteinase free; Mallicroft) density gradient was prepared by layering volumes of 3 ml of 40% sucrose, 2.4 ml of 30% sucrose and 2 ml each of 20% and 10% sucrose into a Beckman SW41 polyallomer tube. The buffer containing 20 mM Tris.Cl, pH 7.4, 1 M NaCl, 10 mM EDTA, and H20 was autoclaved and then sucrose was added. The tube was left overnight at room temperature to allow the formation of a continuous gradient.

About 150-200 ug/80 ul of the DNA from the large scale digestion sample was heated for 10 minutes at 68°C, cooled to 20°C, and then loaded onto the top of the sucrose gradient. Ultracentrifugation at 28,000 rpm (135,000 X g) for 16 hours at 20°C in a SW41 rotor was followed by the collection of 0.35 ml fractions per microcentrifuge tube. The size of the DNA in each fraction was determined on a 0.5% agarose gel. Following electrophoresis, the fractions containing DNA fragments within the 15-23 Kb size range were pooled. These pooled samples were diluted twice with TE buffer (pH 8.0) and the DNA precipitated. The DNA recovered by centrifugation at 12,000 x g for 15 minutes was resuspended in TE buffer.

Ligation and Packaging

This procedure is the same as that described in the construction of a cDNA library (see section Ligation to Bacteriophage Arm DNA (ZAPII), Packaging and Plating, page 71). The

DNA was ligated to EMBL3 genomic cloning vector and bacteriophages were packaged with the Gigapack™ II plus packaging extract. The ligation result was titered by plating bacteriophage with LE392 bacteria cells onto NZY plates. Plaques were counted to determine the quality of the library.

Results

The first strand synthesis reaction, from 10 ug of extracted mRNA, produced 2.06 ug of the first strand synthesized and the total yield was 160 ng of cDNA with fragment size greater than 500 bp. During synthesis of the cDNA, sizes of the first strand and second strand products were also checked by alkaline denaturing gel and the two strands were found to be almost identical in length. Initial efforts to obtain methylated products did not work and were eventually traced to inactive enzyme and/or substrate. A similar problem was encountered with the ligation experiments where the selection for blue and white plaques gave high blue background (more than 1:10). This was traced to inadequate dephosphorylation of the arms of the vector and they were replaced by the manufacturer. Eventually methylation, ligation cDNA to EcoRI linkers, and EcoR I digestion worked, based on the small scale reaction or a control reaction. After cDNA was ligated to bacteriophage vector, about $8.6X10^4$ recombinant with average insert size about 700 bp were obtained.

The procedures obtaining SAU3A digested DNA fragments 15-23 Kb in length for genomic library worked well. After the fragments were ligated to EMBL-3 an aliquot was plated and about $1.8X10^6$ of clones were estimated.

Discussion

The final yield of the cDNA (160 ng of cDNA/10 ug of mRNA, size larger than 500 bp) was less than the anticipated yield of 250 to 400 ng per 10 ug of mRNA. This could be due to the first strand synthesis reaction where the yield was 20.6% of mRNA compared to normal yield close to 50% of mRNA. Perhaps the first strand synthesis reaction yield was low due to a less active reverse transcriptase. The similar lengths of the two strands of cDNA indicated that an insignificant proportion of the second-strand molecules had been generated by self-priming rather than by replacement synthesis (Sambrook *et al.*, 1989). The total number of cDNA ($8.6X10^4$ of recombinant) lies within the range 10^4 to 10^5 for a good cDNA library (Williams, 1981). Since the CRP mRNA in the total mRNA was enriched by induction with turpentine in the present experiment, the size of library is less important than the number of plaques. Copies of CRP mRNA are usually high following induction since CRP production is directly correlated with the number of hepatocytes activated in rabbit (Kushner *et al.*, 1978). The average insert size was slightly smaller than anticipated 1 Kb or more. This could be due to the low yield of the first strand synthesis reaction, which generated relatively more small sized cDNAs. The inserts are on the low side for the isolation of the entire CRP gene but since antibodies recognized CRP expressed by clones a substantial component of the gene is present. With a substantial portion of the sequence determined it is then possible to use the

entire sequence as a probe or to select unique sequences as primers for the PCR.

Calculation of the size of rainbow trout genomic library reveals that the number of independent clones ($1.8X10^6$) is larger than $2.3X10^5$ estimated by the equation of Clarke and Carbon (1976). I used 10^9 bp for fish genome size, $2X10^4$ bp for average size of cloned DNA fragments and 99% for probability are used.

This construction of the cDNA and genomic library is an initial step in isolation of the CRP gene as these libraries are not available commercially.

CHAPTER 5. SCREENING cDNA LIBRARY WITH ANTIBODY

Introduction

There are numerous methods to screen cDNA or genomic libraries (Grunstein and Hognes, 1975; Wallace *et al.*, 1979; Skalka and Shapiro, 1976). One of the most widely used but requiring a substantial investment in time is the use of an antibody specific to the product of a gene (Skalka and Shapiro, 1976). A limitation of this method is that it only detects proteins. The antibody screening method works best if the protein to be detected has high purity and this protein is then used to induce either polyclonal or monoclonal antibodies. There is an extensive literature on the pros and cons of monoclonal versus polyclonal antibodies in detection systems (Kimmel, 1987) but a polyclonal system was chosen since I wanted a detection system which would recognize the broadest range of antigenic determinants on the CRP molecule. Rabbits were used since screening of cDNA libraries requires substantial quantities of antibodies.

The purpose of a cDNA library is to obtain copies of genes without introns and to have those genes express their products in some circumstances. This expression can be enhanced provided a suitable combination of host bacteria and vector are chosen. Complimentary DNA encoded determinants is expressed as a fusion protein which is recognized by specific antibodies. The ZAP II and XL1-blue were chosen because there is colour selection to distinguish recombinant from non-recombinant, and there is a multicloning site for adapting different restriction enzyme

digested DNA fragments and two promoters, T3 and T7, which can be used to easily access the insert DNA. It can also be converted to a plasmid without subcloning, and the expression of foreign DNA can be controlled by the lac repressor (Mieredorf *et al.*, 1987). ZAPII/XL1-Blue system contains LacZ gene encoding β-galactosidase and lac repressor. The foreign DNA is inserted into the multicloning site located inside lacZ gene when the library is constructed. When the lac repressor is inactivated by IPGT transcription from the lacZ promoter is initiated and the fusion protein is expressed. After the fusion protein is immobilized on a membrane immunodetection is carried out (Skalka and Shapiro, 1976). A major problem with this method is antibodies to proteins from the host bacteria, if not removed, will give a false positive reaction in immunodetection systems (Kimmel, 1987).

Once specific clones are identified the bacteriophage containing the gene of interest is converted to a plasmid to remove the arms of the bacteriophage and to make the size of the clone more manageable for further analysis.

The objectives of these experiments were to screen a cDNA library using antibodies generated in rabbits to rainbow trout CRP and identify and isolate positive clones.

Material and Methods

Preparation of primary antibody to CRP

The procedures for obtaining antibodies to purified CRP are given in Materials and Methods of Chapter 2 (see page 26). Since there was the possibility that rabbits used to produce a specific antibody to CRP have the antibodies to *E.coli* proteins these antibodies must be removed to reduce any interference prior to screening. Consequently, antiserum from rabbits was treated with proteins extracted from *E.coli*. The proteins were extracted from *E.coli* using the method of Sambrook *et al.*(1989). Briefly, the method consisted of growing a one litre culture of bacteria XL1-Blue, lysing the bacteria cells with lysozyme at room temperature, and then degrading bacterial DNA with DNase. After removing the cell debris by centrifugation the supernatant containing bacteria proteins was used to absorb rabbit antibodies to E. *coli* proteins. Four pieces of nitrocellulose membrane (5X10 cm, Nucleic acid and protein transfer membrane, Schleicher & Schuell, Keen, NH) were immersed in the *E.coli* extract for 30 minutes at room temperature and air-dried. After washing three times in TBS (10 mM Tris /HCl, pH 8.0, 150 mM NaCl) the membranes were air- dried and blocked with 1% BSA in TBS for 30 minute. Each membrane was washed three times in TBS and then incubated with the primary antibody (rabbit anti-CRP) which was diluted 1:5 in TBST (TBS+0.05% Tween 20) for 10 minutes at 37°C, with gentle shaking. The membranes were discarded and primary antibody was collected for screening.

Optimization of primary antibody concentration

Before immunoscreening the primary antibody concentration was optimized by using a dot blot. Known concentrations of antigen (25, 2.5, 0.25, 0.050, 0.025 ng) were applied to a nitrocellulose membrane as five separate replicates and allowed to air-dry. After blocking with 2% BSA in TBS for 1 hour, the membranes were washed 3 to 5 times in TBST. This washing step was also done between each of the following treatments of the membrane. Each of the five replicated antigen concentrations from the BSA-blocked membranes were incubated in one of five different antibody dilutions (1/100, 1/500, 1/1000, 1/5000, and 1/10000 in 2% BSA solution) for 1 hour. The membranes were washed (see above) and to each was added the alkaline phosphatase conjugated secondary antibody (monoclonal antibody against rabbit immunoglobulin, diluted 1:8000 in 2% BSA) and then incubated for 1 hour. The membranes were washed again and incubated in the substrate solution which contained 100 mM Tris, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$, 0.3 mg/ml of NBT and 0.15 mg/ml of BCIP, until a positive reaction was clearly visible. The colour reaction was stopped by washing the membranes with a buffer containing 20 mM Tris, pH 2.9, and 1 mM EDTA or several changes of deionized water. All the membrane strips were evaluated and the optimum concentration of the rabbit anti-CRP was determined based on a combination of colour intensity to the test sample and a minimum background (non specific colour reaction).

Screening

A total of $3X10^4$ pfu (plaque forming units) of bacteriophages (cDNA in lambda ZAPII) with 0.2 ml of XL1-Blue strain bacteria ($O.D_{600}$= 0.5) was applied to a 150 mm NZY plate and the plates were incubated at 42°C for 3.5 hours. A hybond-C extra membrane (Amersham), 137 mm in diameter, was soaked with 10 mM of IPGT for 1 minute, and air-dried. These membranes were overlaid onto each plate which were incubated for 4 hours or more at 37°C and then placed at 4°C for at least two hours. The membranes were removed and rinsed briefly with TBST to remove any remaining agar and given three 15 minute washes with TBST. All procedures outlined below involved gentle shaking. Each membrane was placed in an individual Petri dish, blocked with 2% of BSA in TBST buffer for 1 hour, then incubated for 1 hour with a 1:500 dilution of primary antibody (rabbit immunoglobulin against rainbow trout CRP) in TBST buffer. Procedures involving the secondary antibody and substrate are the same as those given above for optimization of the primary antibody. Positive clones were picked with a glass pipette and stored individually at 4°C in a microcentrifuge tube containing 500 ul SM buffer and 20 ul chloroform.

Excision of positive clones in Lambda ZAPII

An advantage of the ZAPII/XL1-Blue is that pBluescript phagemid can be directly excised from the ZAPII vector in the presence of helper phage without subcloning. After excision the insert DNA is much easy to work with since the large sized lambda

phage arms in plasmid have been removed.

This procedure was carried out using the ExAssist/SOLR system (Stratagene). One hundred ul of the mixture of a positive clone and the SM buffer was mixed with 1 ul of ExAssis helper phage (>1X10^6 pfu) and 200 ul of XL1-Blue cells (OD_{600}=1) and allowed to incubate at 37°C for 15 minutes. Following incubation 3 ml of 2X YT medium was added and the mixture incubated at 37°C for a further 2 hours, with shaking. The supernatant, containing the rescued phagemid, was heated at 70°C for 20 minutes, centrifuged for 15 minutes at 4000 X g, transferred to a 15 ml centrifuge tube, and stored at 4°C. The phagemid, in the 15 ml tube, was mixed with 200 ul of SOLR cells and plated onto a LB-ampicillin plate (50 ug/ml). After incubation at 37°C overnight, colonies appearing on the plate contained the pBluescript SK(-) double-stranded plasmid with the cloned DNA insert. The plasmid DNA with cloned DNA insert was extracted by alkaline lysis method (Birnboim, H.C., 1983) and the length of insert DNA was analyzed on a 0.8% agarose gel after digesting with EcoRI at 37°C for one hour.

Isolation of the fusion protein

Bacteria containing the plasmid (pBluescript SK-) were grown to mid-log phase ($O.D_{600}$=0.2) at 37°C, IPGT was added to a final concentration of 10 mM and the culture was allowed to grow until reaching the stationary phase ($O.D_{600}$=1). The bacteria were pelleted at 1600 X g for 15 minutes and resuspended in lysis buffer 1:4 (W/V) (50 mM Tris, pH 8.0, 1 mM EDTA, 1 mM PMSF and 10% sucrose).

Lysozyme was added (1 mg/ml) to the lysate, which was then incubated for 10 minutes on ice, after which triton X-100 was added to 0.1% final concentration and the entire mixture incubated on ice for an additional 10 minutes. The sample was centrifuged at 20,000 rpm for 1 hour and the supernatant removed for immunoblotting and immunodetection. the protein concentration of the supernatant was determined by the Lowry method.

Immunoblotting and immunodetection

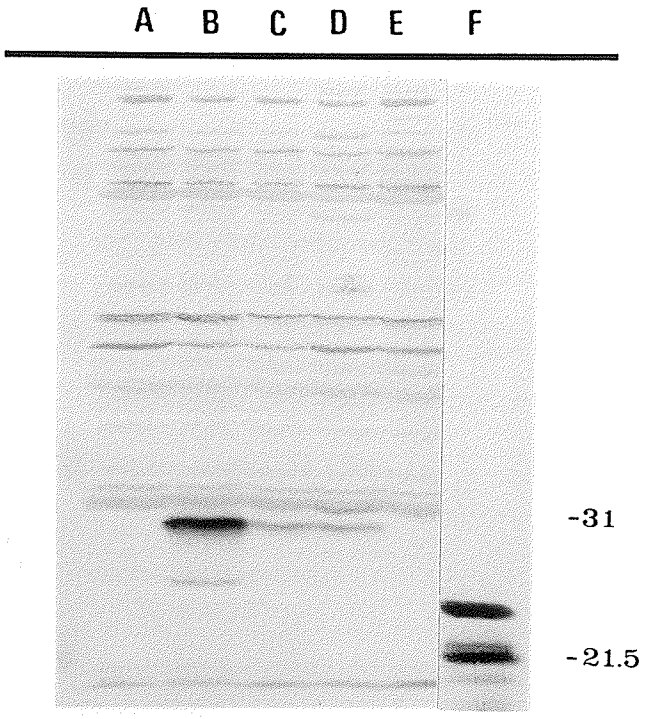
The mixture containing the fusion protein (see section isolation of the fusion protein) was applied (30 ul/well) to SDS-PAGE (see Chapter 2, page 29). Following electrophoresis the proteins, separated by the gel, were transferred to nitrocellulose membrane (Schleicher and Schuell) using a Trans-Blot Cell (Bio-Rad) at constant voltage (70V) for 6 hours in Towbin transfer buffer (Tris 25 mM, Glycine 192 mM and 20% Methanol, pH about 8.3). After the membrane was air-dried immunodetection was carried according to the procedure outlined in the section of Screening (page 85).

Results

Based on the intensity of the colour reaction from the experiments to optimize primary antibody detection a dilution of 1/500 of primary antibody was selected as optimal for immunoscreening. Following immunoscreening the clones from the cDNA library which reacted positively to the antibody were selected for further characterization. Results of the restriction digestion experiments with EcoRI, four of nine clones were found to have inserts varying in size from 500 to 700 bp (one with 500 bp insert, three with inserts of 700 bp).

Of these clones four were processed further by immunoblotting and immunodetection, using SDS-PAGE. A clone containing no insert DNA was also checked on the SDS-PAGE as a negative control. One clone (700 bp), designated as CRP-Imm-2, at 29.6 KDa in size strongly reacted with rabbit serum against rainbow trout CRP (Fig. 12, lane B). Clones, CRP-Imm-3 and CRP-Imm-4, also had reaction bands similar size to CRP-Imm-2, but the colour of reaction bands was less intense. There were also some reaction bands on the membrane which appeared in all sample lanes including the negative control lane (Fig 12).

Figure 12. Immunodetection of fusion proteins. A, Imm-CRP-1. B, Imm-CRP-2. C,Imm-CRP-3. D, Imm-CRP-4. E, negative control. F, rainbow trout CRP antigen.


A B C D E F
-31
-21.5

Discussion

The immunoscreening method depends on the specificity of the antibody. The production of specific antibody mainly depends on the purity of the antigen. The antibody used in the present experiment was produced by injecting highly purified rainbow trout CRP (see Chapter 2, page 23), which should minimize false positives. The results of immunoblotting and immunodetection used to confirm the positive clones found that the 29.6 KDa protein bands were unique to the positive clones (CRP-Imm 2, 3, 4). This strongly suggests these protein bands were produced from the inserted DNA and reacted with rabbit antibody to rainbow trout CRP. These positive clones likely contain trout CRP gene based on the reactivity of the rabbit antibody to rainbow trout CRP. However, there are many factors which could cause false positive reactions. (1) the expressed fusion protein may not be one of interest but has a cross-reaction with an antibody. (2) the antibodies to trout CRP were generated by mature CRP molecules, which was reported as a glycosylated protein (Murai *et al.*, 1990). Consequently, the antibody to the glycosylated protein could cause a false positive in the immunodetection system (Kimmel, 1987). (3) since a polyclonal antibody was used in the screening and this antibody was induced by rainbow trout CRP which contained small amounts of some other proteins. Perhaps rabbit antibody reacted with those proteins (Fig. 12).

The fusion protein consists of products of insert DNA and a

small portion of vector DNA. Since CRP mRNAs range from 1.0 Kb (*Xenopus*) to 2.4 Kb insert DNA (rabbit) the 700 bp insert is likely only part of the gene.

The variable intensity of the reaction of the 29.6 KDa band with rabbit antibodies with these positive clones may be due to, (1) the three clones contain different sections of the CRP gene, but are similar in size and each clone contains different numbers of the antigenic determinants. (2) the percentage of the fusion protein in the analyzed samples are variable, which could be due to a variation in the ability to express CRP by the individual clones since the insert protein could be toxic to the host and cause low production of the fusion protein (Mierendorf *et al.*, 1987).

The isolation of clones reacting with rabbit antibodies is strong indirect evidence that the CRP gene, or part of the gene has been isolated but nucleic acid sequence data is needed to confirm its isolation. Preliminary sequence data from the laboratory since I completed my thesis work has found the signature sequence for a pentraxin in CRP-Imm-2. Since the signature sequence is known for all the pentraxin family proteins, sequenced to date (Bairoch, 1991), this is compelling evidence that the CRP gene has been isolated.

CONCLUSION

1. Purification of rainbow trout CRP and a co-purifying molecule, SAP, has been achieved by using a PC-Sepharose column and a Sepharose CL-4B column.
2. Rainbow trout CRP was identified based on its binding to PC component of the PC-Sepharose column in a calcium dependent manner and its subunit size. CRP was further confirmed in the immunodiffusion reaction with SPS.
3. Rainbow trout SAP was identified based on calcium dependent binding to Sepharose and its subunit size, 30.2 KDa.
4. Rainbow trout CRP appears to have only one type of subunit with a molecular weight of 23.9 KDa, non-covalently associated.
5. Rabbit and mouse antibodies to rainbow trout CRP were raised and a capture ELISA detection system, sensitive to 3 ng of CRP, was developed for rainbow trout CRP.
6. Attempts to isolate the CRP gene using PCR with three synthetic CRP primers were unsuccessful.
7. A cDNA library with $8.6X10^4$ of recombinant and a genomic library with $1.8X10^6$ independent clones were built.
8. Three positive cDNA clones to rabbit antibody against rainbow trout CRP were identified and confirmed by immunodetection methods.

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