Isolation and Characterization of the Carotenoid-Binding Protein In Atlantic Salmon (Salmo salar)

by

Michelle Côté

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Table of Contents

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Amt. = amount
Ax = astaxanthin
BHT = butylated hydroxytoluene
Bmax = maximum number of binding sites determined by non-linear regression
BSA = bovine serum albumin
Cx = canthaxanthin
Da = daltons
d.d. water = distilled deionized water
DCC = dicyclohexyl carbodiimide
DMSO = dimethyl sulfoxide
DTT = dithiothreitol
EDC = 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
EtOH = ethanol
F-actin = filamentous actin
FPLC = fast protein liquid chromatography
G-actin = globular actin
GuHCl = guanidine hydrochloride
H-bond = hydrogen bond
HDL = high density lipoproteins
Kd = equilibrium dissociation constant determined by non-linear regression
kDa = kilodaltons
M = molar (mol/L)
MW = molecular weight
n = number of binding sites on the macromolecule in Scatchard analysis
PMSF = phenylmethylsulfonyl fluoride
ppm = parts per million
Ret. Acid = retinoic acid
RBP = retinol-binding protein
SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis
Tris-HCl = Tris hydrochloride
VHDL = very high density lipoproteins

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ABSTRACT

Atlantic salmon raised on fish meal without supplemented pigments lack the customary colour of their wild counterparts. Only 4-5% of the carotenoid pigments added to the fish meal diet (such as astaxanthin (Ax) and canthaxanthin (Cx)) are retained in salmon flesh. This low pigment retention value is explained in part by poor uptake of carotenoids from the intestinal tract and by poor retention in the muscle. The goal of the present study was to determine the binding site of Ax and Cx in salmon muscle tissue.

Several methods were used to attempt solubilization of the carotenoid-protein complex in Atlantic salmon. A series of low ionic strength washes that solubilized 60% of the salmon muscle proteins was analyzed by binding assay and SDS-PAGE. Analysis of the six low ionic strength fractions demonstrated binding with Cx to occur only in fractions that contained a singular common protein. This protein was tentatively identified as actin based on its abundance and molecular weight.

Actin was purified from both juvenile and mature salmon muscle tissues and binding studies provided strong evidence that actin is the major pigment-binding protein in salmon tissue. The maximum capacity of mature salmon actin was found to be 3 moles Ax/Cx per mole of F-actin. The affinity of mature actin for Ax was found to be equivalent to that for Cx. Work with juvenile actin showed that the affinity of juvenile actin for both carotenoids was lower than the affinity of mature actin for either Ax or Cx. The maximum capacity of juvenile actin was found to be 2 moles Ax and 1 mole Cx per mole of F-actin.

The present study provides strong evidence that changes in pigmentation during sexual maturation may be in part explained by a change in the ability of salmon actin to bind carotenoids, both in terms of affinity and capacity. Data collected in this study indicated that *in vitro* salmon muscle actin has a much greater capacity for carotenoids than *in vivo* salmon muscle actin. It is unlikely that the inability of actin to reach complete saturation with carotenoid is due entirely to poor absorption and transportation of the carotenoids through the digestive system or from the blood to the muscle. It is believed that pigmentation of muscle tissues is also related to changes in the relative amounts of different actin isoforms present in salmon muscle tissues and post-translational modifications of the actin structure during maturation.

1.0 Introduction

1.1 Atlantic Salmon Aquaculture

The number of Atlantic salmon in the ocean has reached an all-time low. If scientists' estimates are correct, and if the recorded downward trend continues, wild Atlantic salmon could be extinct in many rivers in Atlantic Canada in the next 10 years (Lee, 1996). In the late 1960's, scientists estimated that there were approximately 850,000 large salmon feeding in the North Atlantic (Lee 1996). However, by 1995 they estimated that there were only approximately 150,000 large salmon in the same area (Lee, 1996). The decline has continued even in the face of dramatic reductions in Canadian salmon catches.

Due to this downturn in world fish stocks, salmon aquaculture has expanded to meet the growing demand now facing the salmon market. The rearing of salmon to the adult phase first started in the early 1960's in Norway (Mills, 1989) and Atlantic salmon is farmed mainly in Norway, Chile, Scotland, Canada, and the United States. Nova Scotia currently produces approximately 1500 tonnes of aquaculture products annually with a value of $15.2 million (N.S. Department of Fisheries and Aquaculture, 1997). It is estimated that 160 full-time and 300 seasonal jobs are currently provided by the industry.

Aquaculture contributes 25% of the world's seafood (finfish and shellfish) landings, however Canada accounts for only 0.3% of the world's aquaculture production (N.S. Department of Fisheries and Aquaculture, 1997). This low production rate could be enhanced if the cost of running an aquaculture farm could be reduced. The production sales of finfish aquaculture products, including Atlantic salmon, in Canada over the last decade can be seen in Figure 1.1.1.

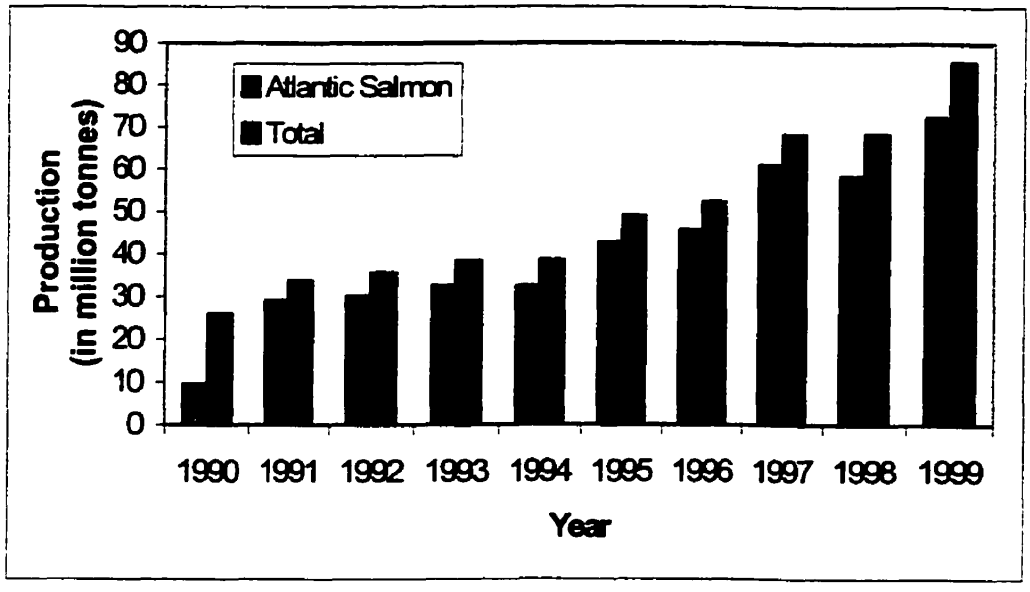
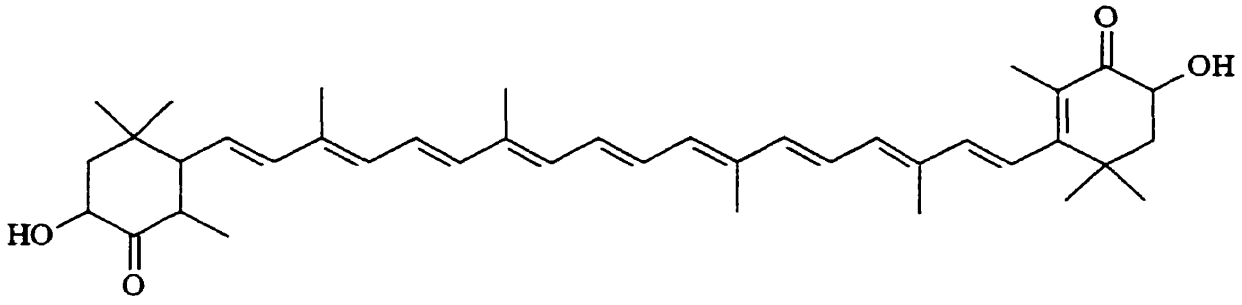


Figure 1.1.1 Finfish aquaculture production in Canada. (Data obtained from Fisheries and Oceans Canada, 2000)

1.2 Pigmentation of Salmon Flesh

Pigmentation of the flesh of salmonids results from the absorption and deposition of oxygenated carotenoids (Christiensen *et al*, 1995). The yellow to orange-red colours of carotenoids result from a chromatophore constituting a long chain of conjugated double bonds. In nature, carotenoids are synthesized by microalgae and then passed up the food chain. Salmon and other marine animals cannot synthesize carotenoids themselves and must obtain them through their diet. To obtain a flesh colour similar to that of wild salmon, aquaculture salmon are fed with fish meal that has been supplemented with colour additives. The two oxycarotenoids widely used as additives in fish meal to enhance the colour of aquacultured salmonids are canthaxanthin (Cx), β,β-carotene-4, 4'-dione, and astaxanthin (Ax), 3,3'-dihydroxy-β,β-carotene -4,4'-dione (see Figure 1.2.1) (Turujman *et al*, 1997).



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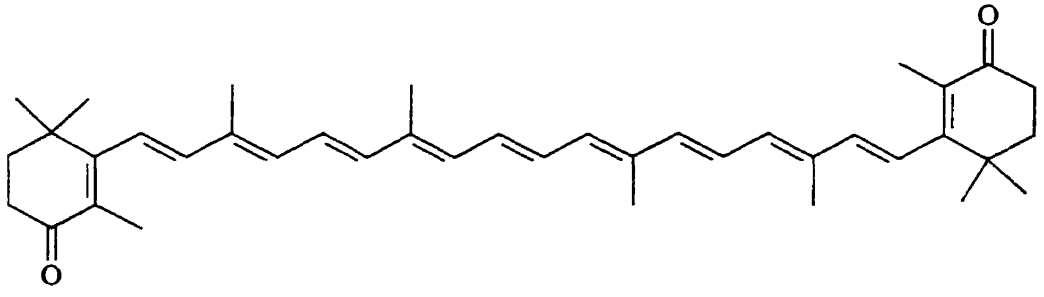

B

Figure 1.2.1 Structures of astaxanthin (A) and canthaxanthin (B).

Astaxanthin is normally found in the flesh of wild salmon although smaller amounts of Cx, β-carotene, lutein, tunaxanthin, and zeaxanthin are also found (Sheehan *et al*, 1998). Hoffman-LaRoche Ltd. produces all-*trans* astaxanthin, and it is used as the main source of pigment in aquaculture. Astaxanthin and Cx are usually fed at concentrations of 40-75 mg/kg of feed, and brightly pigmented salmon contain 5-20 mg of Ax or Cx/kg of flesh (Sigurgisladottir *et al*, 1994).

The grading or pricing of salmon is partially related to the intensity of the salmon's red hue. The pigmentation of cultured salmonids affects overall consumer product acceptance as well as variations in ultimate product price (Sheehan *et al*, 1998). The unacceptability of non-pigmented salmon flesh thus makes the red colour of the salmonid flesh of great economic importance to the fish-farming industry (Torrissen and Ingebrigtsen, 1992). In a 1994 study that examined carotenoid content and sensory

evaluation results, it was shown that salmon muscle with a higher Ax content was preferred ($p < 0.05$), presumably because of the natural red pigmentation. There was also some indication that Ax levels in the feed may affect fillet taste and texture (Sigurgisladottir *et al*, 1994). Therefore, fish farmers must provide the right pigments for their cultivated products in order to satisfy the consumer's expectation.

Scientific trials have shown that commercial Ax and Cx are adsorbed, transported and deposited in the same manner as the carotenoids consumed by wild fish (Sheehan *et al*, 1998). However, salmonids preferentially deposit more polar carotenoids like Ax, rather than Cx and zeaxanthin, and less polar carotenoids like β,β carotene (Schiedt *et al*, 1985). A study conducted by Gobantes *et al* (1997) showed that Ax and Cx have equivalent absorption rates in rainbow trout (*Oncorhynchus mykiss*), however the percent absorption of Cx is only 33.5% compared to that of Ax. The calculated half-life of removal of Cx from rainbow trout serum was less than half that of Ax which indicates that Cx is removed from the animal's system much more quickly than Ax. The digestibility of Ax in rainbow trout was shown to range from 40 to 60% compared to only 19 to 30% for Cx, depending on the formulation (Gobantes *et al*, 1997). These results were confirmed by a study by Bjerkeng *et al* (1992) that found Cx to have a higher metabolic turnover than Ax in rainbow trout. These authors attributed the difference in Ax and Cx pigmentation ability to the higher digestibility of Ax, the higher metabolic turnover of Cx, and a better ability of the muscle actomyosin to bind Ax than Cx. However, similar final concentrations of Ax and Cx in the flesh after a 140 week study indicated that the maximum storage capacity of the two carotenoids in the flesh is the same (Bjerkeng *et al*, 1992). Bjerkeng *et al* (1992) calculated a theoretical saturation level of nearly 100 mg Ax per kg of flesh. Due to the above findings, synthetic Ax is presently preferred as a pigmenter over synthetic Cx for salmonids (Johnson and An, 1991).

It is universally acknowledged that the pigmentation of salmonids changes throughout life (Bjerkeng *et al*, 1992). Pigmentation of young rainbow trout is far less efficient than with mature trout. Fry and fingerlings have limited carotenoid deposition in the flesh while significant amounts are deposited in the skin. However, post-juvenile fish deposit carotenoids mainly in their flesh. Studies on immature rainbow trout by

Nickell and Bromage (1998) showed that flesh carotenoid concentrations do not increase in proportion with increased Cx and Ax concentrations above 50 ppm. Studies have also shown that an innate variation of 20-25% exists in flesh pigmentation of salmon at a market weight of 400 g (Nickell and Bromage, 1998). Use of a low-pigment diet and increased feeding duration resulted in reduced inter-fish variation in pigmentation of Atlantic salmon, rainbow trout, and Chinook salmon. Studies also showed that fish fed Ax at an early stage of growth had significantly higher retention coefficients than fish fed Ax later (Nickell and Bromage, 1998).

It is estimated that the percentage of dietary pigment retained by farmed salmon is in the range of 4-5% (Hardy *et al*, 1990). This low pigment retention value was explained by Wathne *et al* (1998) in part by the poor uptake of carotenoids from the intestinal tract and by poor retention in the muscle. The savings that could have been achieved by increasing pigment retention to even 10% was estimated to be $13 million (US) in 1989 alone (Hardy *et al*, 1990). Once the salmon reaches 300-400 g, supplementation with carotenoid pigments increases feed costs by 7-10% (Sigurgisladottir *et al*, 1994). It is estimated that pigmentation of salmon with carotenoids increases the total cost of running a salmon farm by 10-15% (Hardy *et al*, 1990). This is due to the low efficiency of absorption and the requirement of a continual supply of carotenoids in the diet to achieve proper pigmentation (Hardy *et al*, 1990). In the face of expensive carotenoid-supplemented feed, low retention values indicate that most of the pigment is excreted as waste. Recent work has suggested that salmon absorb much higher levels of these pigments than are deposited and retained in the flesh (Hardy *et al*, 1990).

The mechanism of pigment binding to salmon flesh is not fully understood. No specific studies have been undertaken to examine the binding of carotenoids to components of the muscle in rainbow trout (Storebakken and No, 1992) or Atlantic salmon. It is therefore important for the salmon industry to be able to understand the mechanism through which carotenoids bind to the salmon flesh and factors that influence total pigment retention in salmonids. To achieve higher levels of pigment retention in farmed fish it is necessary to increase the bioavailability of the carotenoids present in the diet or to modify current pigmentation practices. Increased carotenoid retention would

allow for achieving the same flesh pigmentation at reduced total production costs. Also, salmon are exported throughout the world to markets with varying preferences for flesh pigmentation (Christiensen *et al*, 1995). An understanding of the method in which carotenoids bind to the flesh would aid the aquaculture industry in the development of satisfactory and uniform colour for every market. Studies undertaken to understand the binding mechanism between carotenoids and salmon tissue would also help food processors manipulate the stability of deposited carotenoids during shipping, storage and processing to ensure optimal acceptance of cultured products.

2.0 Literature Review

2.1 Carotenoids

Carotenoid research began in 1831 when Wackenroder isolated carotene from carrots (Pfander, 1992). Berzelius soon named the yellow pigments from autumn leaves *xanthophylls* in 1837 (Pfander, 1992). Since this time carotenoids have been the subject of many studies due to their various sources, physiological locations, functions and properties. Carotenoids are polyunsaturated hydrocarbons consisting of long, branched carbon chains containing several conjugated double bonds, closed at one (α-carotene) or both ends (β-carotene) to form cyclic ring systems, the ionone rings. The series of conjugated double bonds constitutes a chromatophore of variable length that gives rise to the characteristic yellow to red colours (Shahidi *et al*, 1998).

Carotenoids have essential roles in the protection against singlet oxygen-generated damage by photosensitized reactions (Packer, 1992), the absorption of potentially damaging radiation, and acting as antioxidants by which sensitive tissues and reactive compounds are protected from oxidation (Pfander, 1992). Carotenoids are known for their provitamin A activity, capability as free-radical scavengers, as stimulants for the immune response, and their action as anticarcinogenic agents (Scita, 1992). They are one of the most important natural marine pigments and are one of the main natural food colourants in use today.

In living organisms, carotenoids play other important roles, such as colouring the body, tissues, or biological fluids, and thereby allowing chromatic adaptation to the environment, and also by assuming various physiological functions as in respiration and vision. Carotenoids are responsible for the colouring of birds, fish, insects, and some invertebrates. Because only plants and microorganisms synthesize carotenoids, carotenoids found in animals are entirely composed of plant-source carotenoids that have been ingested at some point in the food chain. The presence of carotenoids in animals is the result of dietary habits, absorption, metabolic transformations, and symbiosis (Shahidi *et al*, 1998).

2.2 Carotenoproteins

Binding of carotenoids to hydrophobic sites of proteins allows them to be solubilized in aqueous systems (Shahidi *et al*, 1998). The complexes formed are termed *carotenoproteins* and many have been well characterized. Association of a carotenoid pigment with a protein may result in two important effects: it may cause the pigment-protein complex to become water soluble, and it may change the observed colour of the pigment. Many of the bright colours of marine animals are the result of such carotenoproteins. For example, the dark blue of lobster carapace is due to binding between astaxanthin and proteins (Zagalsky *et al*, 1990). The majority of known carotenoproteins have been isolated and characterized from marine invertebrates, especially crustaceans.

The carotenoproteins are stable products in which carotenoid molecules combine with the apoprotein unit in stoichiometric proportions as prosthetic groups (Lakshman and Okoh, 1993). Three types of these complexes have been characterized in review by Shahidi *et al* (1998) as carotenolipoproteins, chitinocarotenoids, and real carotenoproteins. Carotenolipoproteins are complex substances in which carbohydrates and lipids occur in addition to the main components. They are mainly found in the ovaries and eggs of crustacea, although they may also be present in the blood, cuticle, and epidermis. The second group of carotenoproteins is found in the exoskeleton of crustacea and is composed of chitin and carotenoid. Although chitin is not a protein, the structure and characteristics of the chitin-carotenoid relationship cause Ghidalia (1985) to classify chitinocarotenoids as carotenoproteins. The real carotenoproteins are formed when a carotenoid, usually Ax, is attached at specific sites to a glycoprotein. Real carotenoproteins are usually present on the external surfaces of crustacea and account for their colouration.

The increased stability of the carotenoid molecule bound to a protein has been well-documented (Cheesman *et al*, 1967). Astaxanthin, in its free state, is much more sensitive to the damaging effects of light, oxygen, heat, acid and alkali, than when it is bound to a protein, due to its long conjugated double bond system. Such destructive factors cause the destruction of carotenoids and the formation of artefacts (Johnson and

An, 1991). Astaxanthin and Cx are the most common carotenoids isolated from invertebrate carotenoproteins, however other carotenoid structures that are also found in the complexes are gradually being identified.

As was previously mentioned, free carotenoids are only slightly soluble in aqueous media. Buchwald and Jencks (1968) demonstrated that the addition of small quantities of concentrated Ax in water-miscible solvents (such as ethanol) to a large volume of water gave a stable suspension of pigment aggregates or crystals, which was usually almost or completely clear. Dispersion of Ax in ethanol into 8 M urea, 3 M guanidine hydrochloride, and bovine serum albumin (BSA) (7 mg/mL) gave spectra similar to that of an ethanolic Ax dispersion in water. Buchwald and Jencks (1968) also found that the presence of sodium chloride induced the formation of aggregates in which interaction of the carotenoid molecules caused a shift in the absorption maximum from 470 to 555 nm. High levels of sodium chloride resulted in the formation of a yellow product. The similarities between the absorption properties of the yellow aggregate and those of the yellow lobster pigment suggested that the mechanism of alteration of the optical properties of Ax is the same in the two systems. In lobster pigmentation, the protein served to replace the salt in the model system and to keep the pigment aggregate in solution, but did not appear to be directly responsible for the altered absorption spectra of the pigment.

The blue carotenoprotein α-crustacyanin from the carapace of the lobster *Homarus gammarus* is one of the best documented examples of the interaction between carotenoid and its associated protein. The carotenoprotein is an oligomer of 16 apoprotein subunits each with a molecular weight of about 20 kDa, with one Ax molecule being bound per apoprotein monomer (Zagalsky *et al*, 1995). It has been shown that the blue pigment undergoes a reversible or irreversible denaturation in the presence of acids, organic solvents, or heat to give purple, yellow, or red colours (Jencks and Buten, 1964). However, heating the pigment to higher than 60°C results in an irreversible denaturation reaction. Nelis *et al* (1989) found that the absorption spectrum of Ax differed greatly from the absorption spectrum of the Ax-crustacyanin complex. The carotenoprotein's well-documented spectral shift can be seen in Figure 2.2.1.

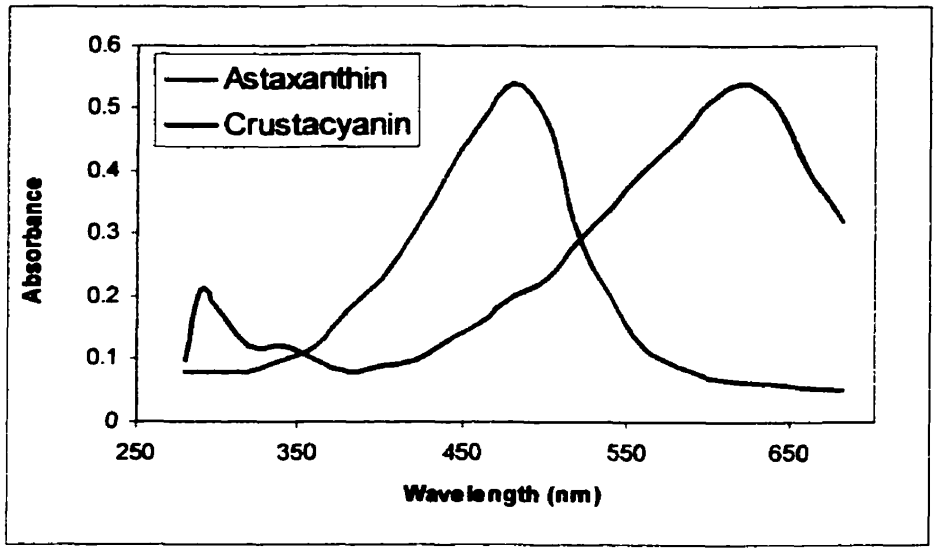

Figure 2.2.1 Absorption spectra of crustacyanin demonstrating spectral shift compared to astaxanthin alone (adapted from Shahidi *et al*, 1998).

Much work has been conducted on the crystal structure of crustacyanin. Zagalsky (1997) at the Royal Holloway University of London has determined that the subunits of this protein belong to the lipocalin superfamily of proteins. These proteins bind low molecular hydrophobic ligands within a protein cavity, or calyx. Zagalsky modeled the tertiary structures and Ax-binding pockets against the coordinates of retinol-binding protein using computer graphics. Other similarities between carotenoid-binding proteins and retinol-binding protein will be highlighted in Chapter 2.5. In crustacyanin the two apoproteins are arranged cavity-to-cavity with the protruding ends of each carotenoid interacting with the coils of the adjacent subunit. In each binding pocket, the carotenoid molecule associated with each apoprotein unit is closely packed between aromatic amino acids with a conserved tyrosine residue near the center of the polyenic chain (Zagalsky *et al*, 1995).

A carotenoprotein from the exoskeleton and muscular epithelium of the black tiger prawn *Penaeus monodon* was recently isolated and characterized by Nur-E-Borhan *et al* (1995) at the University of Tokyo. The tiger prawn is an important aquaculture species in Asia and is generally characterized by blue/black bands around the specimen

that determines the market price of the prawn. Previous work by these researchers had shown the main carotenoid in the exoskeleton of *P. monodon* to be Ax (Okada *et al*, 1994). The prawn turns red when heated or treated with organic solvent and this colour change suggested the presence of Ax in the form of carotenoprotein in the shell and body of the prawn. Nur-E-Borhan *et al* (1995) found the molecular weight of the carotenoprotein in the exoskeleton to be 120 kDa, containing six subunits with three sets of two types of subunits of different molecular weights. The stoichiometric molar ratio of Ax to apoprotein was determined to be 2:1. The molecular weight of the carotenoprotein from the muscular epithelium of *P. monodon* was about 20 kDa with no subunit structure and contained Ax as its prosthetic group (Nur-E-Borhan *et al*, 1995).

The structural characteristics of the carotenoids binding to the blue carotenoprotein originating in *Procambarus clarkii* were determined by Milicua *et al* (1985). They found that the blue carotenoprotein contained six Ax molecules per molecule of apoprotein. However, upon attempting reconstitution of the pigment-protein complex, they also found that minimal variation in the polyenic chain or the functional groups on the ring structures prevented the interaction between protein and carotenoid. This showed the high specificity of the *P. clarkii* pigment-binding site. The results of this experiment can be seen in Table 2.2.1. The percent reconstitution values shown in Table 2.2.1 are relative to the amount of astaxanthin originally bound to the apoprotein unit in *P. clarkii*.

Table 2.2.1 Carotenoprotein reconstitution obtained with the apoprotein from *Procambarus clarkii* using six different carotenoids (Milicua *et al*, 1985).

Carotenoid	% Reconstitution
Astaxanthin	42 ± 3
3-Hydroxyechinenone	None detectable
Canthaxanthin	None detectable
Actinioerythrol	42 ± 2
15,15'-Didehydroastaxanthin	None detectable
Crustaxanthin	None detectable

The first successful isolation of a vertebrate carotenoprotein was carried out by Lakshman and Okoh (1993) and involved the purification of a β-carotene-protein complex from fresh rat livers. Although the transportation of absorbed β-carotene by low-density lipoproteins had been previously reported in mammals, the mechanism of tissue uptake, storage and transport of intact β-carotene in a mammalian system was poorly understood. These researchers discovered a carotenoid-protein complex that existed in the membrane fraction of the rat liver cell. A research group in Norway has studied transport proteins in ^{14}C-labelled Ax in Atlantic salmon, but the results have not yet been published (Aas *et al*, 1999).

2.3 Fish Muscle Proteins

Fish muscle tissue constitutes the largest proportion of the fish body (Harder, 1975). The contractile elements of muscle cells are arranged into long muscle fibres which can be further subdivided into the individual contractile elements called the think and thin filaments. The cell membrane of the muscle cell is the sarcolemma. Myofibrillar proteins include contractile proteins such as myosin and actin, and several regulatory proteins such as tropomyosin, troponin, C-protein and actinin.

The contractile elements are arranged in repeating units called sarcomeres. These can be subdivided into thick and thin filaments. The major protein of the thick filament is myosin, which comprises 54% of the total protein of the myofibrils (Tonomura, 1973) and has a molecular weight of 470,000 Da (Hultin, 1984). The major protein of the thin filaments is actin, which comprises 20-25% of the myofibrillar proteins (Tonomura, 1973) and has a molecular weight of 43,000 to 48,000 Da (Hultin, 1984).

Actin exists in two different forms: globular actin (G-actin) and filamentous actin (F-actin). G-actin is the monomer of F-actin and can be assembled into F-actin by the addition of salts (Torigai and Konno, 1997). F-actin can be reconverted to G-actin by dissolving F-actin in ATP solution (Tonomura, 1973). F-actin is the natural state in which actin is found in the muscle tissue. The distance of a complete turn of a strand of G-actin is 13 molecules (Tonomura, 1973). Therefore 26 G-actin molecules come together to form a working F-actin filament.

In terms of actin purification, Ueda and colleagues obtained 1 to 2 mg actin per gram of freeze-dried tissue extracted with cold acetone (Ueda *et al*, 1967). This result was consistent for several different fish species. Other researchers have obtained up to 35 mg actin per 100 gram of the initial starting material (Watabe *et al*, 1983).

Six actin isoforms have been identified in vertebrates: cardiac α-actin, skeletal α-actin (found in striated muscle), vascular α-actin, enteric γ-actin (found in smooth muscle), and cytoplasmic β- and γ-actin. In muscle cells there exists a mixture of muscle (α and γ) and cytoplasmic (β and γ) actin isoforms (North *et al*, 1994). The contractile apparatus is predominantly composed of α and γ actin isoforms (Szymanski *et al*, 1998). Disturbance of the natural ratio affects the organization of bundle actin filaments (Höfer *et al*, 1997). Different actin isoforms have been shown to bind various actin-binding proteins differently. This ability of different isoforms to bind different proteins has been shown by Shuster and Herman (1995) who studied the affinity of ezrin for β-actin. Their studies showed that *in vitro* studies of actin systems should use the actin most closely related to the *in vivo* isoform. It is possible that the presence of different actin isoforms gives rise to different binding patterns depending on the molecule being studied.

Filaments of F-actin interact with myosin to give a complex called actomyosin. Actomyosin is the primary protein in myofibrils, with actin and myosin filaments arranged in parallel (Huxley, 1965). Each molecule of F-actin binds with one or two molecules of myosin in the actomyosin complex (Huxley, 1963; Encyclopedia Britannica, 2000). It is known that F- and G-actin interact with a multitude of proteins in the cell. At present there are 50 actin-binding proteins which account for approximately 20% of cellular proteins (About Actin, 1999). Actin affinity columns are often used to purify actin-binding proteins to determine if they have F- or G-actin binding capabilities.

2.4 Theories of Carotenoid-Binding Proteins in Salmon

Carotenoproteins are not the only forms in which carotenoids are present in the tissues of organisms. Henmi *et al* (1989a) have proposed that Ax and Cx combine with fish muscle actomyosin by means of a weak hydrophobic bond. This research group investigated how carotenoids combine with salmon actomyosin and the nature of the Ax and/or Cx-actomyosin complex. Actomyosin was observed to combine with many kinds of carotenoids and lipids and in this respect resembled a lipoprotein. Proof of hydrophobic involvement lay in the weak bathochromatic shift in the absorption spectrum of Ax, and in the easy release of Ax and Cx from actomyosin with acetone, Triton X-100, and sodium dodecyl sulfate. It was proposed that one β-ionone ring anchors to the bottom of a hydrophobic pocket in the actomyosin-binding site. The proposed model is illustrated in Figure 2.4.1. The authors propose the involvement of hydrogen bonds in the formation of the carotenoid-protein complex. Therefore, Ax, which may form two hydrogen bonds per β-ionone ring, combines more strongly to actomyosin than do other carotenoids.

A later study by Henmi *et al* (1989b) determined whether only actomyosin from salmonids is capable of combining with Ax and Cx. Of the fish actomyosins studied, it was found that those prepared from pigmented species showed higher binding ratios with the exception of Japanese common mackerel. These results can be seen in Table 2.4.1. One explanation for the carotenoid binding potential of actomyosin from non-pigmented fish could be the selective internalization of pigment across the plasma membranes of salmonid muscle fibers. It was also observed that the actomyosins having high surface hydrophobicity combined well with these carotenoids suggesting that carotenoids bind non-specifically to the hydrophobic binding sites on actomyosin. Henmi's group proposed that a carotenoid carrier lipoprotein be first bound to a receptor on the cell surface. The lipoprotein is then internalized by endocytosis and delivered to an unknown carrier system where Ax and/or Cx are selectively transported to myofibrillar proteins with carotenoid-binding sites.

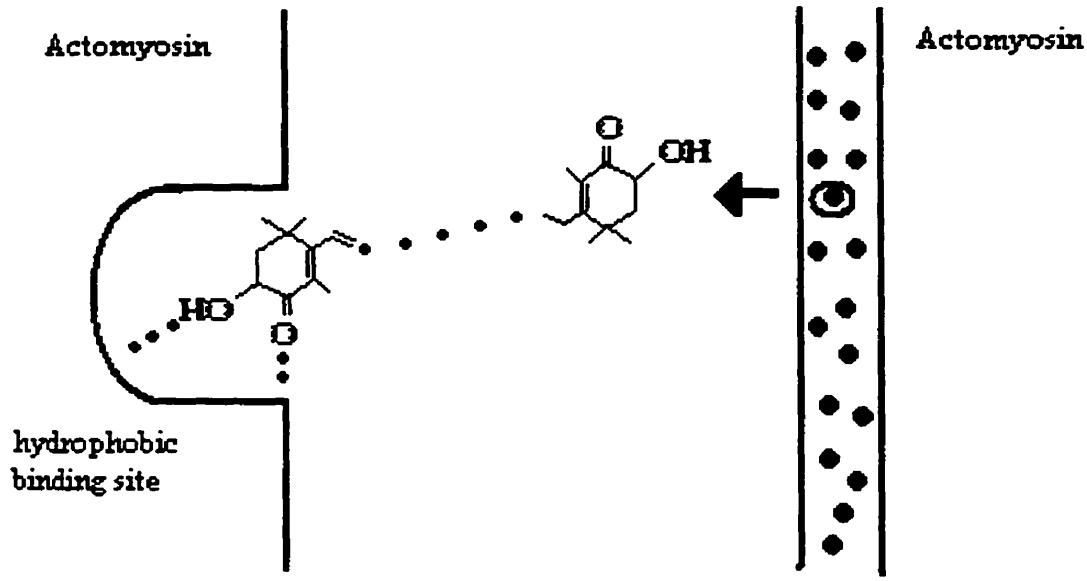

Figure 2.4.1 Proposed astaxanthin-actomyosin complex model (adapted from Henmi *et al*, 1989a)

Table 2.4.1 In vitro binding ratios of astaxanthin and canthaxanthin for various fish actomyosins (Henmi *et al*, 1989b)

Actomyosin Source	Amount astaxanthin bound to actomyosin (μg Ax/μg protein)	Amount canthaxanthin bound to actomyosin (μg Cx/μg protein)
Common mackerel	1.01	0.70
Coho salmon	0.86	0.53
Jack mackerel	0.80	0.57
Rainbow trout	0.73	0.43
Skipjack	0.72	0.52
Stone flounder	0.53	0.41
Carp	0.32	0.24

The suggestion that hydroxyl and keto groups at the β-end of the carotenoid increase the binding strength may help explain the higher deposition of Ax in salmon flesh compared to other carotenoids. Cx, lacking a hydroxyl group, would thus form a weaker complex and exhibit reduced retention in the muscle. Ax is widely accepted to be deposited in the myotome and further support is derived from the visual appearance of muscle from salmonids fed a diet enriched with Ax, where the myotome looks pink (Torrissen *et al*, 1992).

The carotenoid-carrying lipoprotein in the serum of chum salmon (*Oncorhynchus keta*) has been characterized by Ando *et al* (1986) at Hokkaido University, Japan. The isolated lipoprotein had a molecular weight of 70 kDa, with two subunits whose molecular weights were 24 and 12 kDa, present in a molar ratio of 2:2 as determined by SDS-PAGE in the presence of 2-mercaptoethanol. The carotenoid-carrying lipoprotein had an isoelectric point of 5.1. It was believed that the 70 kDa lipoprotein has two basic units and the carotenoid-carrying lipoprotein probably exists as a cluster of lipoproteins of various sizes, ranging from a total molecular weight of 30 to 500 kDa. Although the lipoprotein in this study is present in the serum of salmon and not necessarily the tissues, it is possible that a similar protein would be present in the tissues.

Clevidence and Bieri (1993) have studied the association of carotenoids with human plasma lipoproteins, a study that may also enhance the understanding of salmonid carotenoid associations. Their work showed that a wide variety of carotenoids found in plasma are also present to a large extent in human tissues, indicating that there is an effective transfer of carotenoids from plasma lipoproteins to tissues. The final destination and metabolic fate of a carotenoid thus depends on the relative affinity of that carotenoid for specific lipoprotein classes. Plasma carotenoids have a strong affinity for low-density lipoproteins, as was observed by the yellow colouring of the isolated low-density lipoprotein fraction in Clevidence and Bieri's work (1993). However, the mechanisms governing the selective association of the various carotenoids with the various lipoprotein classes have not been identified, nor have the maximum capacities of lipoproteins for binding the various carotenoids. Clevidence and Bieri (1993) suggested affinity chromatography to be an effective and high-recovery technique for assessing the carotenoid content of lipoproteins. They used a heparin-Sepharose affinity column, with

agarose-bound heparin as the ligand for retaining apolipoprotein B- and E-containing lipoproteins. This resulted in the retention of very low and low-density lipoproteins; to be later eluted with a higher ionic strength NaCl solution, while high-density lipoproteins passed through the column unretained.

2.5 Similarities Between Retinol-Binding Protein and Carotenoproteins

Retinol-binding protein has been isolated and characterized for a vast number of species. It is comprised of a single polypeptide chain with a molecular weight of roughly 21 kDa and a single binding site for one molecule of all-*trans*-retinol (Blaner *et al*, 1990). The retinol molecule is surrounded by the binding cavity, with the ring end of the retinol innermost. In the retinol-protein complex, only the hydroxyl group of the retinol molecule is solvent accessible. The metabolic function of retinol-binding protein is to facilitate the mobilization of retinol from retinoid stores in the liver, to prevent the abnormal interaction of retinol with tissues and to protect retinol from oxidation and other chemical changes (Vallet, 1996). The structure of retinol can be seen in Figure 2.5.1.

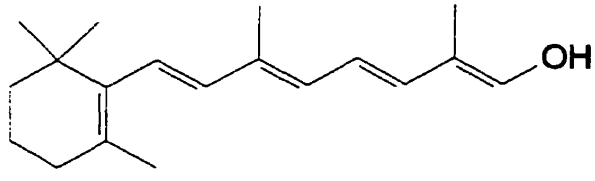

Figure 2.5.1 Structure of all-*trans* retinol.

In circulation, retinol-binding protein forms a 1:1 molar complex with another plasma protein, transthyretin. Transthyretin has been found to bind to retinol-binding protein through a 3-dimensional docking model in which two complementary 3-dimensional surfaces allow for specific recognition (Naylor and Newcomer, 1999). To

isolate retinol-binding protein, many forms of conventional chromatography have been used. Of particular interest to this study is the development of affinity columns which have isolated retinol-binding protein either by coupling transthyretin to Sepharose (Vahlquist *et al*, 1971) or by coupling retinoic acid, a derivative of retinol, to Sepharose (Fex and Hansson, 1978).

The cellular retinol-binding protein is an intracellular retinol carrier binding protein belonging to a family of hydrophobic ligand-binding proteins (Aalten *et al*, 1995). When the retinol-binding complex binds to the receptor, retinol dissociates from the complex and passes through the receptor and the cell membrane into the cell (Aalten *et al*, 1995). This mechanism is similar to the one proposed by Henmi's group (1989b) for the carotenoid-actomyosin complex in salmon (Section 2.4).

In comparing Figures 1.2.1 and 2.5.1, it can be seen that retinol is structurally related to carotenoids. Milicua *et al* (1990) commented on this similarity:

> *"Carotenoproteins have a certain degree of similarity to other complexes that play important roles in human physiology, such as low density plasma lipoproteins, the retinol-binding protein, and the visual pigment rhodopsin."*

It is perhaps likely that the interactions between retinol and retinol-binding protein would be similar to those of a carotenoid-binding protein. A likeness also exists concerning the hydrophobic nature that governs both the interactions between the two molecules and their associated proteins. Finally, Henmi's theory of how carotenoids are preferentially transported across the plasma membrane of actomyosin cells is similar to Aalten's proposal for retinol-binding proteins passing retinol through the cell membrane. Therefore, procedures for studying carotenoid-binding proteins in the present study will be adapted from earlier studies involving retinol-binding proteins.

2.6 Techniques

Many different techniques were used to analyze the proteins separated and isolated during the search for the carotenoid-binding protein in Atlantic salmon. Some general background information is useful for a better understanding of the detailed explanations found later in this thesis.

2.6.1 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Electrophoresis is based on the migration of charged proteins in an electric field. The technique is not often used to purify proteins in large amounts because simpler more practical alternative methods are usually available and chemicals used during electrophoretic methods often inactivate enzymes and denature proteins. Electrophoresis is however, especially useful as an analytical method because the isolated proteins can be visualized as well as separated. This permits a researcher to estimate quickly the number of proteins in a mixture or the degree of purity of a particular protein preparation. Also, electrophoresis allows the determination of crucial properties of a protein such as isoelectric point and approximate molecular weight.

Sodium dodecyl sulfate (SDS) is an anionic detergent, which binds strongly to proteins in a ratio of approximately one molecule of SDS for every two amino acids, or 1.4 g SDS per gram of protein (Reynolds and Tanford, 1970). The bound SDS contributes a large net negative charge, thereby nullifying the intrinsic charge of the protein. In addition, the native conformation of a protein is altered when SDS is bound and most proteins assume a similar rod-like shape, and thus a similar mass to charge ratio. Electrophoresis in the presence of SDS separates proteins almost exclusively on the basis of mass (molecular weight), with smaller polypeptides migrating more rapidly than larger ones. Weber and Osborn (1969) showed that SDS polyacrylamide gel electrophoresis (SDS-PAGE) could be confidently used for a variety of proteins in the determination of molecular weights to within 10%. These researchers plotted the electrophoretic mobilities against the logarithm of known polypeptide chain molecular weights to obtain a straight line. By knowing the electrophoretic mobility of an unknown protein, the molecular weight could be easily determined.

This technique was deemed to be useful in the identification of the carotenoid-binding protein because many proteins can be identified on the basis of their molecular weight, especially if the protein has been previously identified and characterized.

2.6.2 Solubilization of myofibrillar proteins

Muscle proteins have traditionally been separated into three distinct categories: soluble, myofibrillar/contractile or collagen/insoluble (Hultin, 1984). Soluble proteins are those that can be easily extracted from the muscle in water or dilute salt solutions. Insoluble proteins are not soluble in solutions of either high or low ionic strength. Instead, detergents must be used for solubilizing these proteins.

The classic procedure for the solubilization of myofibrillar proteins has been to dissolve them in a salt solution varying from 0.4 to 0.6 M at neutral, slightly acid or alkaline pH (Hultin, 1984). Procedures dating back to 1872 have involved the use of successive extraction of tissues with water, dilute neutral salts, and dilute alkali to solubilize the proteins present (Keller and Block, 1960). However, it has been discovered recently that these traditional categories do not always apply to myofibrillar proteins (Stefansson and Hultin, 1994). The significance of this recent finding was that it enabled almost complete solubilization of fish muscle proteins at low ionic strengths without the need for harsh detergents or salts.

Stefansson and Hultin (1994) have proposed that myofibrillar proteins have net negative charges at neutral pH. Therefore, in sufficient amounts of water, or solutions of very low ionic strength, the repulsive forces from the negatively charged side chains are great enough to force the individual protein units apart. Increasing the salt concentration or lowering the pH close to the isoelectric point thus reduces these repulsive forces, and precipitation occurs. Their method involved a series of successive washes of cod tissue with cold water at different pH values. This low ionic strength treatment was shown to give almost 100% solubility of cod muscle proteins. Feng and Hultin (1997) observed that the muscle proteins of mackerel were less soluble than those of cod at comparable ionic strengths. These researchers modified the procedure by Stefansson and Hultin

(1994) to wash the proteins at neutral pH with a moderate amount of NaCl. This was shown to solubilize over 90% of the mackerel light muscle proteins.

Feng and Hultin (1997) hypothesized that at least some of the proteins removed in the neutral salt wash were preventing the solubilization of the other muscle proteins in water. In following this hypothesis, it seemed reasonable to expect that the proteins involved as solubility inhibitors, or "possible solubility inhibiting (PSI) polypeptides", for proteins in water should be associated with structural elements of the muscle cell. This is indeed what they found. Six proteins were preferentially solubilized by sodium chloride solutions of moderate ionic strength at neutral pH under conditions that allowed later dissolution of myofibrillar and cytoskeletal proteins in water. Three of these six proteins were identified as M-protein, α-actinin, and desmin. It was observed that in almost all cases, the protein extractability of these polypeptides increased as the salt concentration increased, and at a given salt concentration, more polypeptide was extracted if the pH of the extracting solution was adjusted to neutrality.

A third method conducted in Hultin's laboratory by Krishnamurthy *et al* (1996) involved extracting chicken muscle proteins with neutral salt washes and the incorporation of a wash with histidine buffer at pH 7.4. Chicken myofibrillar proteins were shown to solubilize even less readily in the washes than cod and mackerel muscle proteins. These researchers were able to obtain greater than 90% solubilization of the total protein content in chicken breast muscle using this method. The incorporation of a buffered wash resulted in the solubilization and removal of several inhibitory proteins in chicken. These proteins include X-protein and C-protein of the thick filaments and α-actinin and amorphin of the Z-disk. The initial water washes were found to remove many sarcoplasmic proteins.

These extraction methods were adapted and adopted in the present study of the carotenoid-binding protein in salmon because they did not involve harsh detergents or high salt levels. Such substances could have resulted in denaturation and potential changes in the pigment-binding behaviour of the carotenoid-binding protein that could have presented further complications during recombination studies.

2.6.3 Affinity Chromatography

Affinity chromatography is a type of adsorption chromatography in which the molecule to be purified is specifically and reversibly adsorbed by a complementary binding substance (ligand) immobilized on an insoluble support (matrix). The principle of affinity chromatography using detergent is illustrated in Figure 2.6.3.1. Purification is often several thousand-fold and recoveries of active material are generally very high (Scopes, 1987). Affinity chromatography enables the purification of almost any biomolecule on the basis of its biological function or individual chemical structure. This technique presents one of the most rapid, efficient, and convenient means of generating pure protein. Indeed, one-step purifications of 1000-fold with nearly 100% recovery have been reported (Scopes, 1987).

The first application of affinity chromatography was the selective adsorption of amylase onto insoluble starch in 1910. The complex organic chemistry required for affinity separations prevented the technique from becoming generally established in biological laboratories until the 1960's (Scopes, 1987).

The high selectivity of affinity chromatography is derived from the natural specificities of the interacting molecules. For this reason, affinity chromatography can be used to purify substances from complex biological mixtures, separating native from denatured forms of the same substance, and removing small amounts of biological material from large amounts of contaminating substances. Any ligand with an affinity constant for the protein of 10^{-7} M or larger can be used for successful purification (Findlay, 1990).

Fex and Hansson (1978) isolated a retinol-binding protein from human urine and blood serum using affinity chromatography. Their column allowed retinol-binding protein to be purified directly from human urine and blood without any prior fractionation of the plasma proteins. The retinol-binding protein in plasma was able to bind to retinoic acid coupled to a Sepharose backbone. The ligand used by Fex and Hansson (1978) was all-trans retinoic acid coupled to Sepharose 4B, which bound 60-90% of the applied retinol-binding protein.

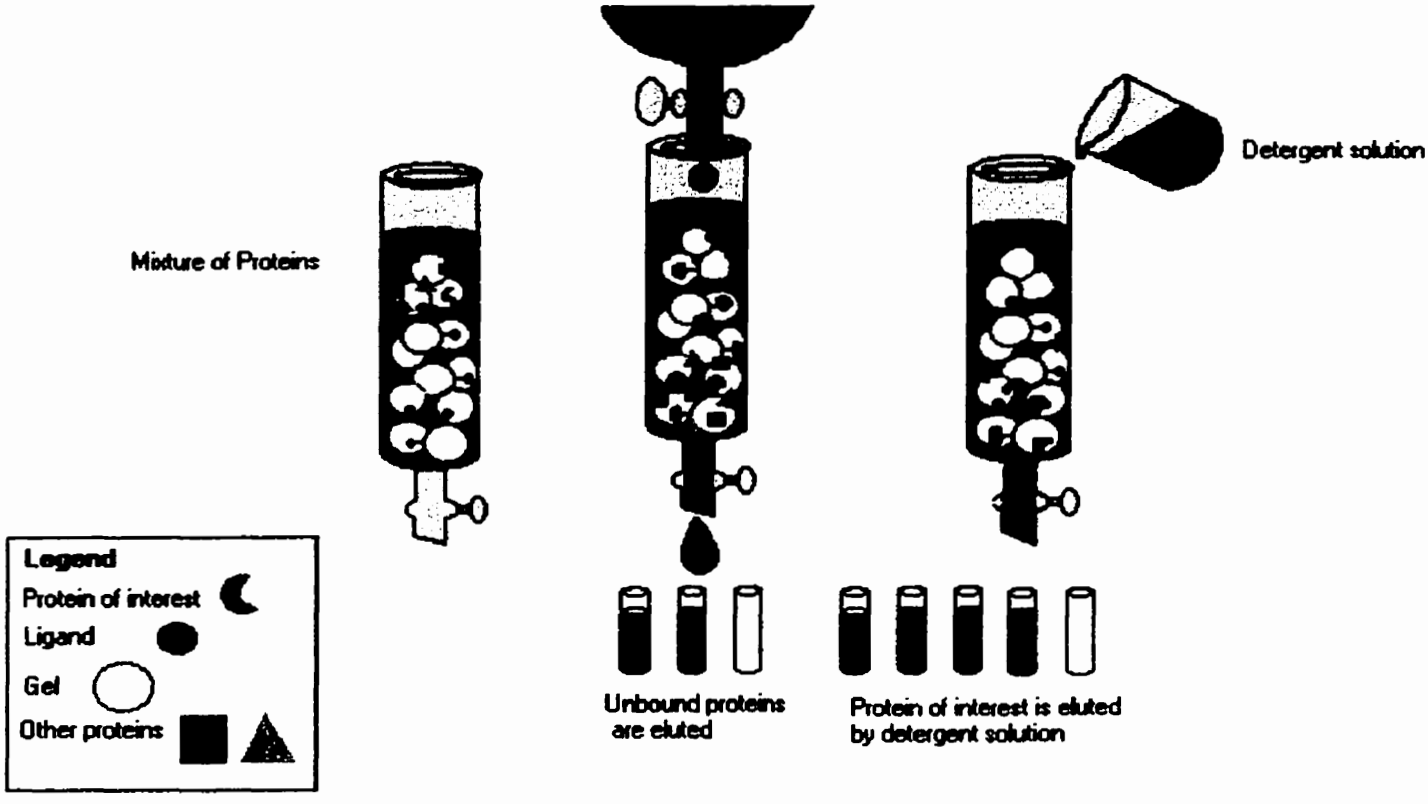

Figure 2.6.3.1 Principle of affinity chromatography demonstrating all proteins being washed away except those with a specific affinity for the ligand. The protein of interest is then eluted with a detergent solution (adapted from Lehninger, Nelson, and Cox, 1993).

2.6.4 Recombination/Binding Studies

Carotenoid-binding assays have been developed by many researchers attempting to reconstitute carotenoproteins by combining carotenoids and apoproteins. Similar assays have also been developed by researchers attempting to reconstitute the retinol-binding protein complex in many species.

Zagalsky *et al* (1995) reconstituted crustacyanin by combining the apoprotein with astaxanthin at 0°C. Astaxanthin was dissolved in acetone and added dropwise to a solution of aproprotein in phosphate buffer. The mixture was then dialyzed against the same phosphate buffer to remove the acetone. Excess Ax was removed by centrifuging the solutions at 16,000 x g for 1 h followed by subsequent passage through a 0.2 μm filter.

Many researchers studying retinol-binding proteins have developed their own methods for reconstituting the complex. Fex and Hansson (1979) prepared holo retinol-binding protein in their attempt to characterize two possible types of retinol-binding protein in human urine. Fex and Hansson dissolved retinol in ethanol and added the ethanolic retinol solution to crude retinol-binding protein or fresh urine. In this mixture, the ethanolic retinol to protein ratio was less than 1:20. The mixture was equilibrated for 18 h, in the dark, at 5°C. Shidoji and Muto (1977) also used an ethanolic retinol solution to prepare holo retinol-binding protein. These researchers incubated ethanolic retinol with piscine retinol-binding protein for 14 hr at 4-5°C.

Goodman and Raz (1972) devised a complicated recombination method to study the interaction of retinol with human plasma retinol-binding protein. In their study, an aqueous solution of retinol-binding protein was gently shaken with Celite that had been covered with a thin film of retinol. After 5 h, the Celite was removed by centrifugation and the aqueous phase was subjected to gel filtration on a small Sephadex column. The column fractions containing retinol-binding protein were then pooled and subjected to further study.

Henmi *et al* (1989a) combined actomyosin and carotenoids to study carotenoid-actomyosin complexes in salmon muscle. These researchers dissolved carotenoids in chloroform and then added octa-ethyleneglycol mono *n*-dodecyl ether. The chloroform

was removed under nitrogen and distilled water was added. The aqueous solubilized carotenoid solution was then added to actomyosin suspended in phosphate buffer. The solution was shaken for 12 hours at 5°C. At the end of this incubation period, the actomyosin was washed with water to remove any unbound carotenoid.

These recombination studies were used to formulate a binding assay for the present study that would help characterize the binding between the isolated carotenoid-binding protein(s) in Atlantic salmon flesh and dietary carotenoids. Saturation experiments were analyzed using non-linear regression, following the model:

$$Y = \frac{Bmax * X}{Kd + X}$$

This equation describes the equilibrium binding of a ligand to a receptor as a function of increasing ligand concentration. This equation was originally used to describe what is now known as the Michaelis-Menten model. Although Leonor Michealis was interpreting enzyme kinetics in 1913 (Stryer, 1988), the same model holds true for saturation of a substrate with ligand molecules. In this model, Y is the specific binding and X is the concentration of the ligand. Bmax is the maximum number of binding sites on the protein and is usually expressed in the same units as the Y-axis. Kd is the equilibrium dissociation constant and is expressed in the same units as the X-axis. When the ligand concentation equals Kd, half the binding sites on the protein are occupied. These parameters fit the binding isotherm, which is in the shape of a rectangular hyperbola.

3.0 Materials and Methods

3.1 Experimental Hypothesis and Design

An original hypothesis of the study was that the carotenoid-binding protein in Atlantic salmon might fall under the category of *carotenoprotein* detailed in the introduction of this thesis. If this had been the case, methods developed by researchers such as Nur-E-Borhan *et al* (1995) to liberate carotenoproteins in *Penaeus monodon* by collagenase action, and Lakshman and Okoh (1993) to liberate a β-carotenoid complex from rat livers, may have been useful in isolating the salmon carotenoprotein. Because of similarities already mentioned between carotenoproteins and retinol-binding proteins, it was also anticipated that the affinity chromatographic technique developed by Fex and Hansson (1978) would be useful in the purification of the salmon carotenoprotein.

After attempting isolation of the carotenoprotein by methods designed by Nur-E-Borhan *et al* (1995), a different approach was undertaken to isolate the carotenoid-binding protein in Atlantic salmon. If the carotenoid-binding protein was previously unknown, the affinity column approach developed by Fex and Hansson (1978) may have been useful in separating the unknown protein from the entire protein matrix. However, in the event that the carotenoid-binding protein was one of the well-documented myofibrillar proteins, myofibrils would be extracted by a standard method and the individual proteins fractionated using previously published procedures. A carotenoid-binding assay would then be developed to compare the binding abilities of the newly isolated protein with those of salmon myofibrils, thus giving a more complete picture of the binding properties of salmon tissue.

The individual proteins were to be fractionated using a series of three low ionic strength treatments developed by Stefansson and Hultin (1994), Krishnamurthy *et al* (1996), and Feng and Hultin (1997). Each of the three low ionic strength treatments varied slightly, each one designed to solubilize essentially all the muscle proteins of three different substrates: cod, mackerel, and chicken. It was expected that at least one of these three methods would successfully solubilize the carotenoid-binding proteins in Atlantic salmon. A diagram depicting the experimental design of this research can be seen in Figure 3.1.1.

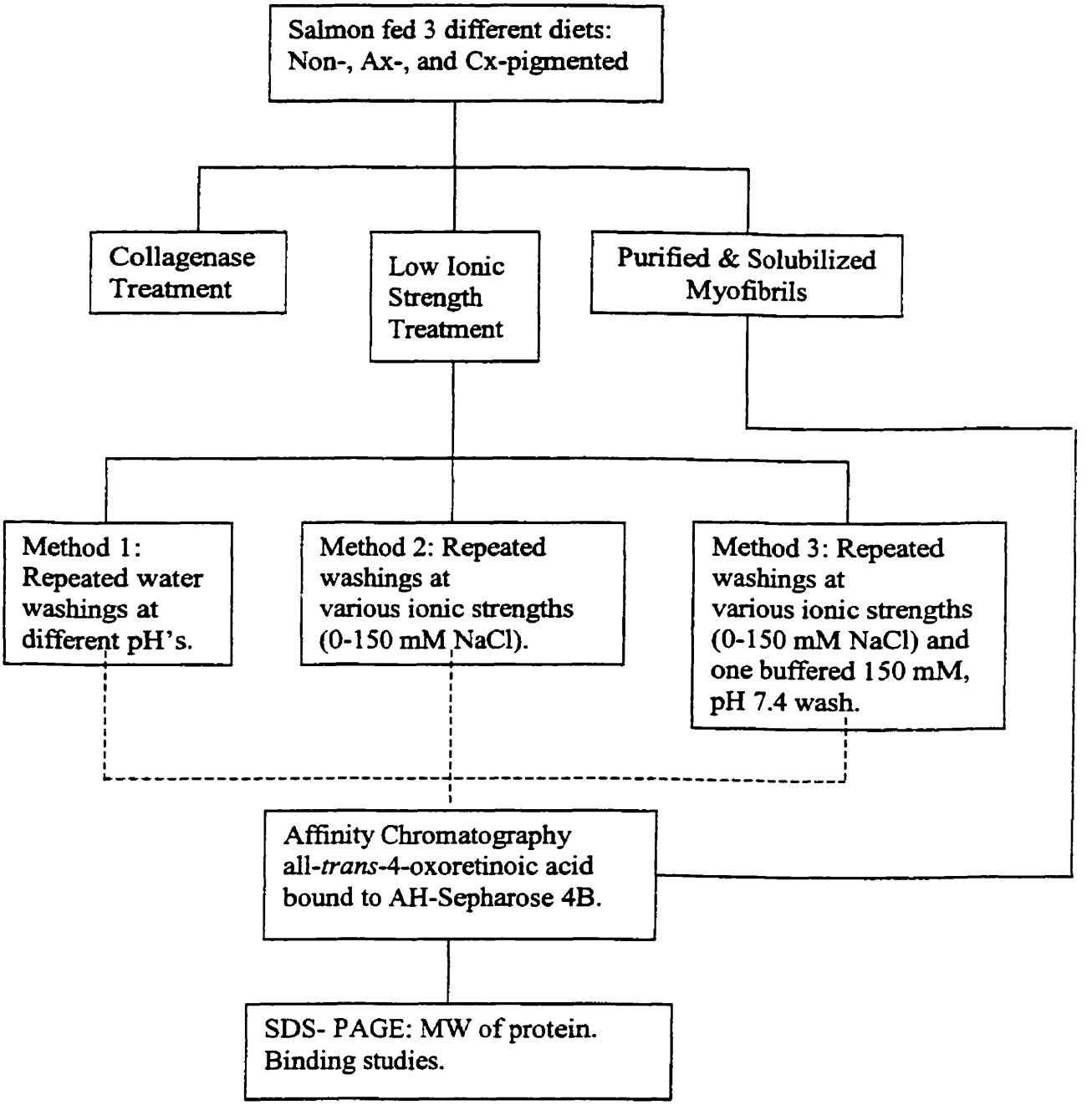

Figure 3.1.1 Flow diagram of extraction procedures for salmon muscle carotenoid-binding proteins.

3.2 Salmon

Atlantic salmon (*Salmo salar*) were raised in 2 m^3 tanks at the Aquatron facilities, Dalhousie University. The lighting was artificial and the photoperiod was 12 hours of light and 12 hours of darkness. These tanks can be seen in Figure 3.2.1. Recirculating aerated seawater (32 ‰) between 10-15°C and a flow rate of 10 L/min were used. At the time of the experiments, the fish were approximately three years old and had been fed a pigmented diet for over one year. Salmon smolt were obtained from Dr. Santosh Lall, Sandy Cove Research Station, National Research Council of Canada.

Figure 3.2.1 Tanks at Dalhousie's Aquatron facility used for rearing the Atlantic salmon used in these experiments.

Three feeds (Ax supplemented, Cx supplemented, and non-pigmented) were obtained from Corey Feed Mills Ltd. (Fredericton, NB) and stored in a freezer at the Aquatron facilities. A comparison of the composition of each of the three diets can be seen in Table 3.2.1. Each feeding was conducted once daily until apparent satiation, as can be seen in Figure 3.2.2. Following feeding, each tank was partially drained and

refilled to remove feces and excess feed. Every six weeks, salmon were removed from their tanks and the tanks were drained, scrubbed with iodine, and rinsed using high-pressure hoses. Nets were bleached to remove algal growth. The tanks were then refilled and the salmon were placed back into the clean tanks.

Table 3.2.1 Composition of the experimental diets produced by a commercial feed mill[1].

Carotenoid Supplement[2]		Diet Composition (%)		
Carotenoid	**Dietary Level (mg kg^{-1})**	**Protein**	**Fat**	**Fiber**
Ax	5.0	43	20	3
Cx	6.0	44	25	2
None	0	41	23	3

[1] Corey Feed Mills Ltd., Fredericton, NB

[2] Ax and Cx diets are based on trout and salmon feed formulations respectively.

Figure 3.2.2 Demonstration of the method in which salmon were fed on a daily basis.

The salmon were randomly selected on an individual basis prior to analysis. The selected individual was placed in a 0.15 m^3 tank half filled with water, and anaesthetized with the addition of 200 mL of a 95% ethanol, clove oil, and water mixture (12: 8: 80). After approximately 5 minutes, when the fish had become sufficiently subdued, the salmon brain was pithed with a sharp knife and the gills slit to allow the fish to bleed out into the tank (Fig. 3.2.4). Fish were gutted and filleted immediately and samples of muscle tissue were taken from the middle dorsal area. Random sampling was deemed inappropriate as studies have determined that the carotenoid concentration in Atlantic salmon is highly variable among the dorsal, ventral, and tail sections of the fish. Work undertaken by Refsgaard *et al* (1998) with 145 salmon showed that the highest muscle astaxanthin levels were found in the tail and dorsal regions while the lowest levels were found near the head. It was presumed that tissues with the highest carotenoid content would also have a high carotenoid-binding protein content, therefore these tissues were chosen for extraction purposes.

Figure 3.2.3 Salmon subdued with the clove oil/ ethanol mixture about to be pithed.

3.3 Equipment and Chemicals

All reagents and solvents were reagent-grade and were obtained from Fisher Scientific (Nepean, ON). All other chemicals were obtained from Sigma-Aldrich (Oakville, ON).

Astaxanthin, canthaxanthin, and all-*trans*-4-oxo-vitamin-A acid were donated by F. Hoffmann-LaRoche Ltd. (Basel, CHE).

The pH of the wash solutions and chromatography buffers was determined using an Accumet model 15 pH meter (Fisher Scientific, Nepean, ON).

Samples were centrifuged, washed, and concentrated using IEC Centra MP4R and Centra M2 centrifuges (International Equipment Company, Needham Heights, MA).

Affinity chromatography was accomplished using the Pharmacia (Piscataway, NJ) FPLC system. This system is comprised of a model P-500 pump, liquid chromatography controller model LCC-500, and a FRAC-100 fraction collector. A Pharmacia XK-16 column was used to pack the affinity matrix. The column and fraction collector were maintained at 5°C throughout the experiments. This system is illustrated in Figure 3.3.1.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis was carried out using the BioRad (Mississauga, ON) Mini PROTEAN II Cell.

A Hewlett Packard 8453 UV-Visible spectrophotometer equipped with Windows '95 and UV-Visible ChemStation software was used to determine all absorbances.

Non-linear analysis was conducted using Prism Graphpad Software, Version 3.02.

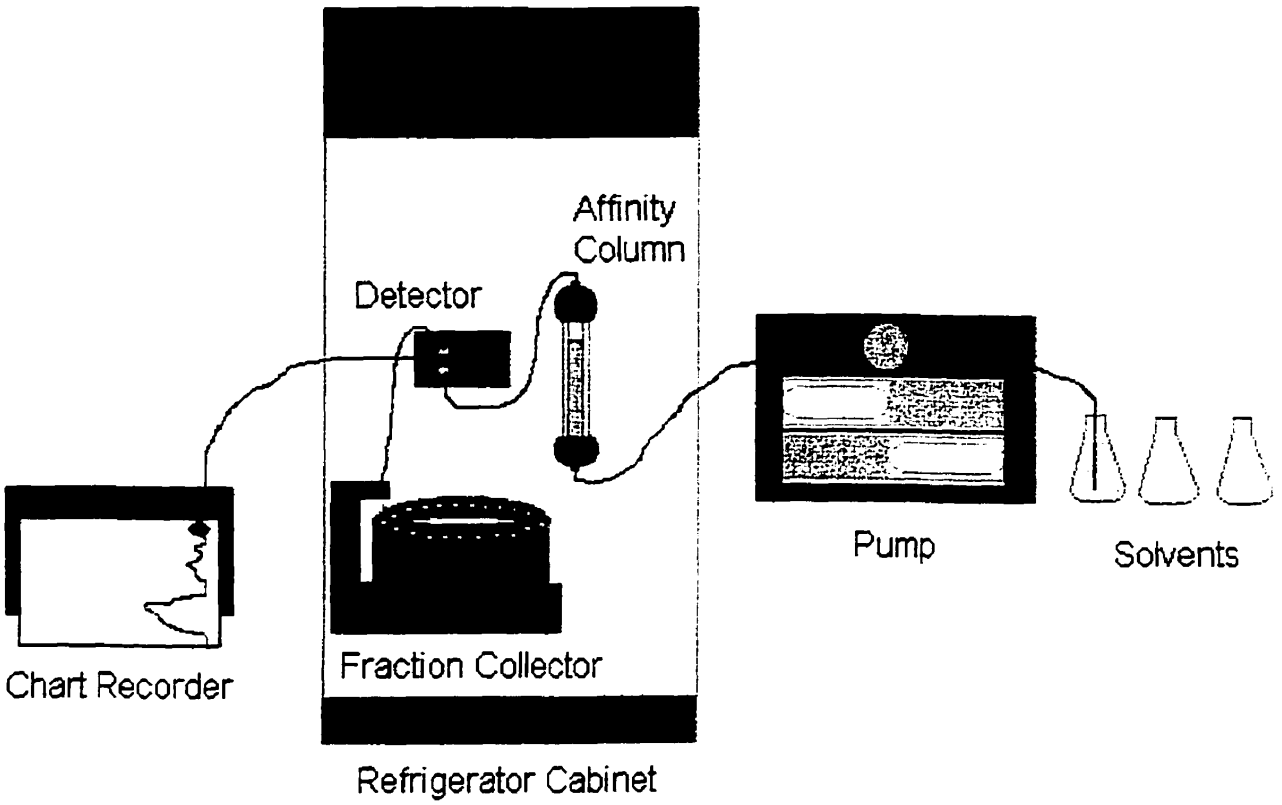

Figure 3.3.1 Affinity chromatography assembly using the Pharmacia FPLC system.

3.4 General Protein Analyses

Precautions were taken at every step to avoid denaturation of the proteins and excessive loss of the carotenoids present. Samples were placed on ice when not in use and protected from light to avoid pigment photo-oxidation as was recommended by Blaner *et al* (1990). Protein concentrations were measured according to the Bradford (1976) dye binding procedure using a commercially available Coomassie Brilliant Blue G-250 reagent solution with bovine serum albumin (Sigma-Aldrich, Oakville, ON) as the standard or according to the DC Protein Assay (BioRad Laboratories, Mississauga, ON), which is a modification of the Lowry *et al* (1951) assay.

3.5 SDS-PAGE

Protein samples were mixed 50/50 with sample buffer containing 50 mM dithiothreital (DTT) and heated at 100°C for two minutes. This sample buffer contains:

4.0 mL Distilled water
1.0 mL 0.5M Tris-HCl, pH 6.9
0.80 mL Glycerol
1.6 mL 10% SDS
0.4 mL DTT
0.2 mL 0.2% Bromophenol blue
---
8 mL Total

SDS-PAGE was performed on 15% polyacrylamide slab gels according to the Laemmli (1970) buffer system and the Mini-PROTEAN II system (BioRad Laboratories, Mississauga, ON). Apparent molecular masses were estimated with authentic molecular weight standards (# 161-0315) obtained from BioRad (Mississauga, ON).

Coomassie Blue, the standard dye for gel staining, was not found sensitive enough for all the experiments conducted in the study. Therefore a silver stain developed by Swain and Ross (1995) was used for the SDS-PAGE work. This stain incorporates high sensitivity with low background staining.

3.6 Binding Assay

In order to identify the carotenoid-binding protein in salmon muscle tissue, it was necessary to be able to detect the presence of the protein throughout the isolation procedure. This meant developing a carotenoid-binding assay in which binding between carotenoids and the carotenoid-binding protein could be observed and quantified.

A standard curve was obtained to determine the extinction coefficient of Cx at 450 nm in DMSO and water. Two mg of Cx was dissolved in 100 μL of DMSO and serial dilutions were made also in DMSO. Five μL of each of these dilutions was added to 100 μL distilled deionized water. The solutions were vortexed and their absorbances

read at 450 nm. An extinction coefficient of 57, 400 $M^{-1}cm^{-1}$ was determined for Cx in water.

There were problems when determining the extinction coefficient for Ax, therefore the value was adopted from work carried out by committee members at NRC. A standard curve was obtained to determine the extinction coefficient for Ax at 485 nm using tetrahydrofuran (THF) in water. Astaxanthin was similarly dissolved in THF and made into serial dilutions in THF. Two μL of the serial dilutions of Ax/Cx was added to 100 μL of water and a standard curve was determined. The extinction coefficients for Cx in water were determined to be 43,000 $M^{-1}cm^{-1}$. (See Appendix for calculation used to determine extinction coefficients).

To perform the binding assay with protein samples, two mg of Ax or Cx with solubilized muscle protein fractions were dissolved in 100 μL DMSO. Dimethyl sulfoxide was chosen as an appropriate solvent for the binding assay because Ax and Cx were highly soluble in this solvent. In addition, at low concentrations DMSO was not observed to precipitate the proteins to which it was exposed. Dimethyl sulfoxide was also found to give low background absorbance at the wavelengths to be used in the study (440 and 470 nm).

Two-fold serial dilutions were made in DMSO. Five μL of each of these dilutions were added to 100 μL of protein sample in a plastic microcentrifuge tube (protein concentration 1 mg/mL) to give final carotenoid concentrations of 4 to 20 μg carotenoid/100 μL sample. These solutions were vortexed and left sitting at room temperature for 0.5 h before reading their absorbances at 470 or 440 nm (λmax for Ax and Cx, respectively). This reading gave a measure of the total carotenoid (bound + free) present in the system. The spectrum obtained for these solutions showed a large peak at the λmax due to both bound and free carotenoids present in the sample. These solutions were then submitted to rapid gel permeation chromatography using 10 kDa MW exclusion Bio-Spin columns (BioRad Laboratories, Mississauga, ON). After gel filtration, the eluates were analyzed for carotenoid content at either 440 or 470 nm. The eluate absorbance value reflected the bound carotenoid parameter. Absorbances were corrected for protein absorbance by subtracting the absorbance value of the protein spectrum alone to give absorbance values attributable solely to the added carotenoids.

Using pre-determined extinction coefficients for Ax and Cx, absorbance values were transformed into molar quantities of carotenoid bound to the protein present in the sample.

A more detailed explanation of the calculations involved in the carotenoid-binding assay can be seen in Appendix A.

3.7 Preparation of Myofibrils

Myofibrils were prepared as outlined by Olson *et al* (1976) with some minor changes. Fifteen grams of salmon muscle tissue was homogenized in ice cold potassium phosphate buffer using a Stomacher lab blender Model 400 (Seward, London, GBR). The pH 7.0 potassium phosphate buffer was composed of: 100 mM KCl, 20 mM KH_2PO_4, 1 mM EDTA, disodium, dihydrate, and 0.2% sodium azide. The suspension was centrifuged at 3600 x g for 15 minutes and the soft upper layer of the resulting pellet was resuspended in buffer. The centrifugation/resuspension step was repeated five times to remove all the water-soluble proteins.

The myofibrils were then washed 3 times with cold 100 mM NaCl buffer to remove potassium ions. During the final wash, the myofibrils were resuspended in a minimal amount of extraction buffer mixed with glycerol (1:1 (v/v)), and stored at –30°C until needed. When needed, the vials were thawed, centrifuged for 15 minutes at 1300 x g and 6°C and the supernatants removed for analysis.

Myofibrillar suspensions were solubilized in a minimal amount of 30% NaOH and the concentration was adjusted to 1 mg/mL (using the method of Bradford (1976)) with cold distilled deionized water prior to the binding assays. The approximate dilution of the myofibrillar sample was 1/1000. The pH of the 1 mg/mL samples was not tested however three 100 mM NaCl washes sufficiently removes any potassium ions left in the sample.

3.8 Collagenase Treatment

Collagenase treatment of salmon muscle was attempted to solubilize the carotenoid-binding protein in Atlantic salmon. Nur-E-Borhan *et al* (1995) isolated a carotenoprotein from the muscular epithelium of the black tiger prawn by extraction with collagenase. The ease with which the carotenoprotein from the tiger prawn was released by collagenase action was persuasive evidence for the usefulness of this method.

Collagenase Type 1A from *Clostridium histolyticum* (380 units/mg solid (collagen digestion), Sigma-Aldrich, Oakville, ON) was dissolved at a concentration of 0.18% (w/v) in 20 mM potassium phosphate buffer containing 0.1 mM PMSF. Additional protease inhibitors were added to the collagenase solution to prevent possible non-collagenase protease activity. The PMSF concentration was increased to 2 mM, and aprotinin and leupeptin were each added at concentrations of 1 μg/mL based on recommendations by Lakshman and Okoh (1993). Fifty μg/mL butylated hydroxytoluene was also added to the solution to prevent oxidative breakdown of the carotenoid-protein complex, as was recommended by Lakshman and Okoh (1993). Since collagenase is a metalloprotease that requires the presence of zinc for inhibition (Polgár, 1989), it was not believed that these added inhibitors would adversely affect collagenase activity.

Seven hundred and twenty mL of the collagenase solution was added to 472 g of minced salmon tissue. Salmon was minced using a Moulinex (Cormelles-le-Royal, FRA), Type E12, Model No. 22085, grinder with a 2.5 mm pore size. The mixture was placed in a 28°C water bath for 2 hours with gentle shaking.

After incubation, the cloudy orange solution was collected by filtration through a Whatman No. 4 filter paper (Fisher Scientific, Nepean, ON) into a flask protected from light and held at 5°C. The proteins were then salted out with ammonium sulfate to 60% saturation. The pink precipitate was dissolved in a minimal amount of 20 mM potassium phosphate buffer (pH 6.8) containing 0.1 mM PMSF and then ultrafiltered through an Amicon (Nepean, ON) ultra-filtration device using a 300 kDa cut-off filter.

The pink >300 kDa fraction was prefiltered using a 0.45 μm filter and then applied to two different gel filtration columns. These columns were used to attempt size

fractionation of the proteins: an 8 mm x 30 cm column packed with Superose 12 prep grade (Pharmacia, Piscataway, NJ) and an XK 26-70 column packed with Sephacryl 400 HR (Pharmacia, Piscataway, NJ).

3.9 Low Ionic Strength Treatment

The method used for solubilization/extraction of salmon muscle proteins was similar to that reported for chicken by Krishnamurthy *et al* (1996). Two other methods (Stefansson and Hultin (1994); Feng and Hultin (1997)) were largely unsuccessful when applied to salmon tissue. A flow diagram of the Krishnamurthy method can be seen in Figure 3.9.1. After the final centrifugation of the washed salmon tissue, a final 1:50 extraction of the pellet in cold water was used to assess protein solubility. Success of solubilization was determined based on the colour of the pellet remaining after final centrifugation. If the remaining pellet after centrifuging the 1:50 extraction was pink, the extraction was deemed unsuccessful, since the pink colour was the result of carotenoids binding to the muscle proteins, meaning the proteins had not yet been taken up into solution.

The protein content of each of the solubilized fractions was determined by the method of Bradford (1976). Protein profiles of the fractions was determined by SDS-PAGE using the Laemmli (1970) method. Binding assays were conducted on the solubilized fractions to determine at what step the majority of the carotenoid-binding protein was removed. Binding between Cx and the samples was assumed to be representative of all carotenoid-binding, therefore Ax-binding assays were not repeated for the fractions. Comparisons of the ability and capacity of the fractions to bind Cx were made for extracts from Cx-pigmented and Ax-pigmented salmon flesh.

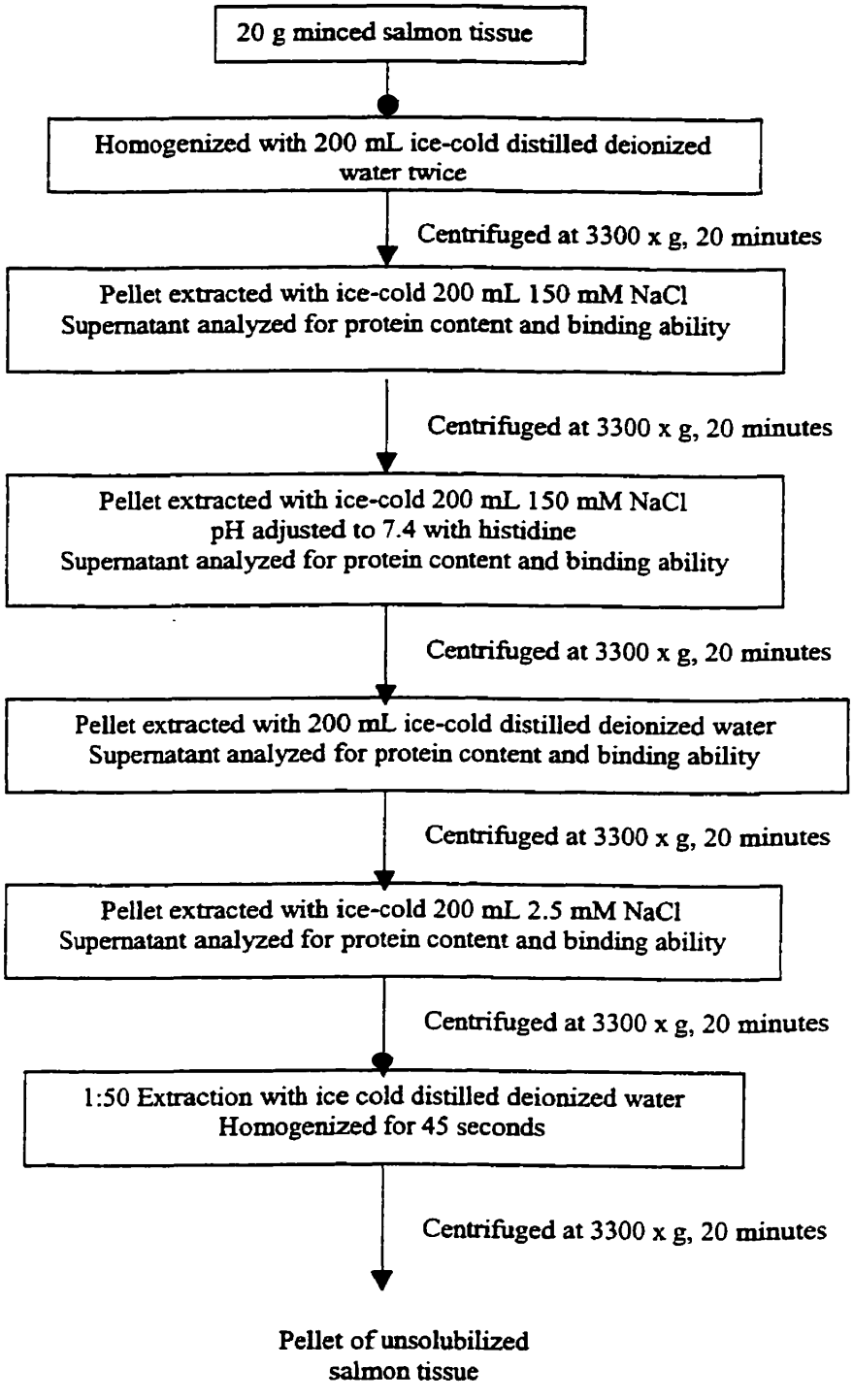

Figure 3.9.1 Flow diagram of the low ionic strength solubilization of Atlantic salmon tissue (adapted from Krishnamurthy *et al* (1996)).

3.10 Development of an Affinity Column

The ligand possibly capable of promoting the separation of the carotenoprotein from the other proteins in the salmon sample can be seen in Figure 3.10.1. It was believed that the Cx-protein complex would bind to all-*trans*-4-oxoretinoic acid due to the identical natures of the Cx and all-*trans*-4-oxoretinoic acid ring systems. The Ax-protein complex might also bind to this compound. However, the presence of a hydroxyl group on the aromatic ring might be necessary for the formation of an Ax-protein complex to occur. If this was the case, binding would not occur between the protein and the gel matrix embedded with all-*trans*-4-oxoretinoic acid ligands.

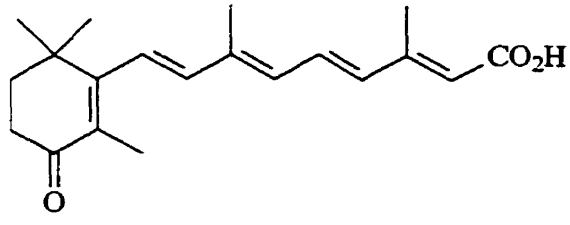

Figure 3.10.1 Structure of the retinoic acid derivative used in the development of an affinity column for the isolation of the carotenoid-binding protein in salmon: all-*trans*-4-oxoretinoic acid.

Two approaches were taken to build the affinity column for isolating the carotenoid-binding protein in salmon based on work carried out by Fex and Hansson (1978). In the first approach, coupling of all-*trans*-4-oxoretinoic acid (F. Hoffman–LaRoche Ltd, Basel, CHE) to dicyclohexyl carbodiimide (Sigma-Aldrich, Oakville, ON) took place in 1,4 dioxane (Fisher Scientific, Nepean, ON). Forty mg of the all-*trans*-4-oxoretinoic acid was dissolved in a minimal amount of 1,4-dioxane, as was 200 mg of dicyclohexyl carbodiimide. These were added to AH-Sepharose 4B (Pharmacia, Piscataway, NJ)-dioxane suspension (3 g AH-Sepharose 4B in 25 mL dioxane) and left for three days with gentle stirring at room temperature, protected from light. The Sepharose gel was then washed on a scintered glass filter with 300 mL 1,4-dioxane and

1000 mL distilled water to remove any unbound ligand and coupling agent. The clean gel was taken up in a minimal amount of 0.04 M Tris-HCl, pH 7.4, containing 0.5 M NaCl and 0.001 M EDTA.

Molar absorptivity coefficients of all-*trans*-4-oxoretinoic acid in 1,4 dioxane and in water were obtained by measuring the absorbance of a mixture of 10 μL of a 4 mg/mL ethanolic solution of all-*trans*-4-oxoretinoic acid in either 10 mL of 1,4-dioxane or distilled water at 360 nm.

Another coupling condition was tested in which AH-Sepharose 4B coupling of all-*trans*-4-oxoretinoic acid using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) took place in 50% dioxane/water. Six g AH-Sepharose 4B were allowed to swell by washing the gel on a scintered glass filter with 300 mL 0.5 M NaCl, followed by 300 mL distilled deionized water. Twenty-five mg of all-*trans*-4-oxoretinoic acid was dissolved in a minimal amount of 1,4-dioxane and added to the gel followed by 400 mg EDC dissolved in a minimal amount of d.d. water. The total volume was roughly 40 mL, 50% of which was 1,4-dioxane. The gel suspension was gently mixed at room temperature for 24 hours, protected from light. It was then washed on a scintered glass filter with 200 mL 1,4-dioxane and 500 mL distilled water. Finally, the cleaned gel was suspended in the Tris-HCl/ NaCl/ EDTA buffer.

The affinity column matrix was packed into an XK-16 column (Pharmacia, Piscataway, NJ) and the mobile phase pumped in an upward direction to permit higher flow rates and less compaction of the column packing material. The column was attached to the Pharmacia FPLC system illustrated in Figure 3.3.1, held in a refrigerated cabinet and protected from light.

To purify the carotenoid-binding protein from the salmon samples, 20 mL of the 1/50 extract was applied to the affinity column and flushed through the column with the Tris-HCl/ NaCl /EDTA buffer at a rate of 0.1 mL/min. After no more protein appeared to be coming off the column based on the baseline absorbance at 280 nm, the eluant was switched to the GuHCl buffer, at a flow rate of 0.1 mL/min. The elution position of the carotenoid-binding protein was followed by measuring the absorbance of each fraction at 280 nm and at 470 nm, the absorbance maximum for the carotenoid. Absorbance at 470

nm was monitored in the hopes that the carotenoid-binding complex might share the blue shift properties of the carotenoproteins.

After each run the column packing material was removed from the column and washed in order to remove remaining traces of sample and buffers. The washings were conducted on a scintered glass filter and consisted of one gel volume of each of the following: 0.1 N NaOH, Tris-HCl/ NaCl/ EDTA buffer, distilled water, and more Tris-HCl/ NaCl/ EDTA buffer. The column material was then poured back into the column and stored in the refrigerator for the next use. After several runs, no affinity peak was observed and the pressure began to increase due to protein build up on the column. The gel was treated with 1% SDS in an effort to solubilize the adsorbed proteins, but this was ineffective. It was observed that the guanidine hydrochloride left behind in the column after the elution process caused the SDS to precipitate, collecting in the column fittings, and causing major flow problems. The gel was treated with a 1 mg/mL protease solution (source: *Streptomyces griseus*, Sigma-Aldrich, Oakville, ON) which appeared to be successful. However, repeated use of protease might degrade the ligand-matrix bond. The column was then re-equilibrated and prepared for the next use.

After fractionating the protein that bound to the affinity column, the fractions were concentrated using a Centriprep-10 ultrafiltration device (Amicon, Nepean, ON). The fractions were then washed three times with distilled deionized water and concentrated even further using Minicon-3 ultrafiltration devices (MW cut-off 3000 Da) (Amicon, Nepean, ON).

3.11 Purification of Actin

Since SDS-PAGE (Chapter 3.5) suggested that the apparent molecular weight of the solubilized protein binding fraction (Chapters 3.6, 3.9) was roughly 43,700 Da, purification of actin was performed in order to test the hypothesis that actin was the carotenoid-binding protein in Atlantic salmon. Both mature and juvenile salmon muscle actins were prepared in order to compare the binding properties of carotenoid pigments with these proteins.

An acetone powder was prepared as outlined by Pardee and Spudich (1982). Purification of F-actin from this acetone powder followed the procedure described by Watabe *et al* (1983). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (Laemmli, 1970) was conducted on the final F-actin pellet to determine if the F-actin had been successfully purified without a high amount of contaminants.

4.0 Results

4.1 Collagenase Treatment

The salmon muscle tissue was exposed to collagenase action and the extract was concentrated before being applied to a gel filtration column to separate the carotenoid-binding protein from the protein matrix (Section 3.8). The Superose 12 prep grade (Pharmacia, Piscataway, NJ) column was unsuccessful in fractionating the proteins present in the >300 kDa coloured fraction. The column was easily overloaded and gave poor resolution, even with a greatly diluted sample. The > 300 kDa fraction appeared to be completely unretained on the column.

Because of this, another column was packed with Sephacryl 400 HR (Pharmacia, Piscataway, NJ). This column size/gel combination should have worked well for separating globular proteins in the molecular weight range of 10^4-10^7 Da. Although the proteins were better retained on this column, none of the fractions eluting from this column showed an absorbance at 470 nm, as they should have if the carotenoprotein was present in any of these fractions, due to absorbance by the bound carotenoids. These results led to the conclusion that the collagenase method presented in this thesis was unable to isolate the carotenoid-binding protein in Atlantic salmon. Therefore a series of low ionic strength washes was used to attempt solubilization of the carotenoid-binding protein in Atlantic salmon muscle tissue.

4.2 Low Ionic Strength Treatment

The first low ionic strength treatment developed by Stefansson and Hultin (1994) used with salmon tissue yielded a pink final pellet indicating that the carotenoid-binding protein was still present in the undissolved tissue. The average percent total protein recovered after the 3 washes and the 1:50 extraction was only 6 ± 1 % (n=3, ± 1 standard deviation). A subsequent treatment (Feng and Hultin, 1997) also resulted in a pink pellet after centrifugation of the 1:50 extraction in water, indicating that the complex had not yet been solubilized. However, this method was much more successful in solubilizing

the total protein content of salmon: 29 ± 3 % (n=2, ± 1 standard deviation). A third method (Krishnamurthy *et al*, 1996) resulted in a white final pellet after the 1:50 extraction, leading to the conclusion that either the pigment-protein complex was solubilized or the pigments were released from the protein. The percent total protein solubilized using this method was 57 ± 15% of the total proteins present (n=8, ± 1 standard deviation). The method was also useful in that it resulted in seven distinct fractions that could be analyzed separately.

As can be seen in Figure 3.9.1, the successful low ionic strength method involved seven successive steps consisting of two initial extractions in ice cold distilled deionized water, one extraction in 150 mM NaCl, one extraction in 150 mM NaCl buffered with histidine to give a final pH of 7.4, one extraction in ice-cold distilled deionized water, and a final wash with 2.5 mM NaCl. The final pellet was taken up in a 1/50 ratio with cold d.d. water. Each of these supernatant fractions was analyzed for protein content and pigment binding ability with Cx. Also, SDS-PAGE analysis was conducted on each of the wash fractions to determine which proteins were removed at each step in the washing process. The amount of protein solubilized in each step can be seen in Figure 4.2.1.

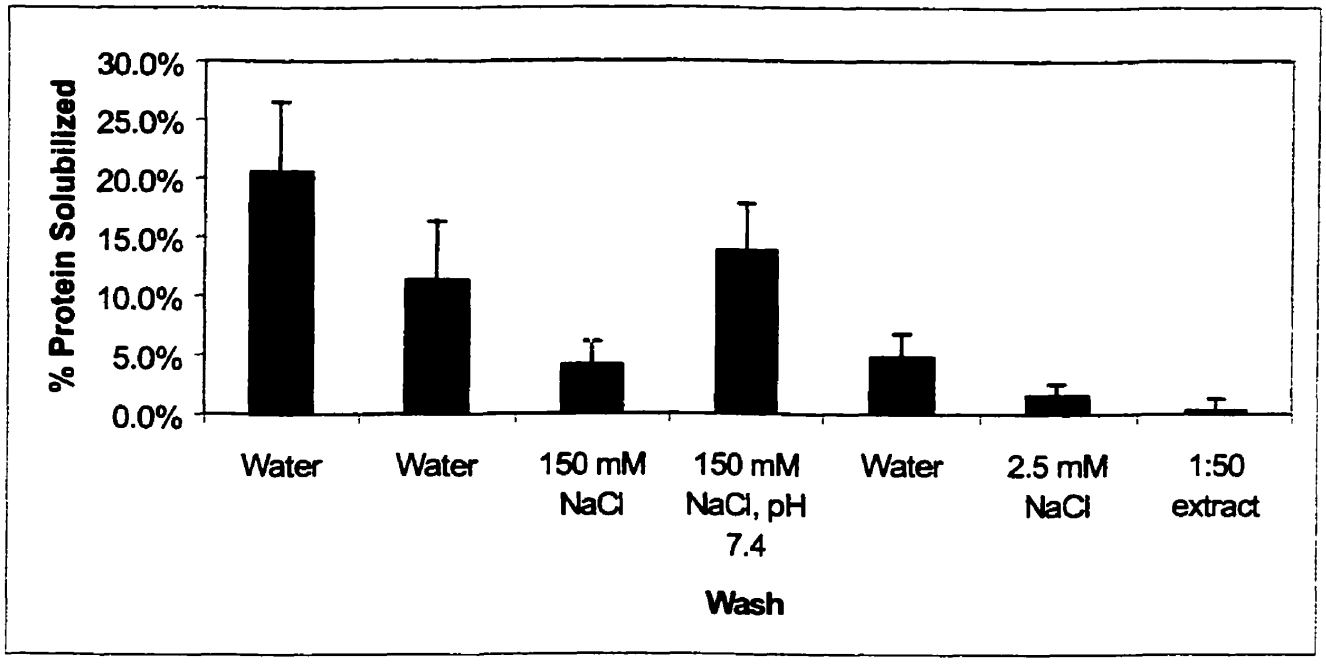

Figure 4.2.1 Protein content of the solubilized fractions from the low ionic strength treatment of Cx-pigmented salmon tissue. Protein content is expressed as a percentage of the total proteins present in Cx-pigmented salmon tissue (n=8, error bars indicate 1 standard deviation).

Binding assays were conducted on each of the solubilized fractions to determine at what step the majority of the Cx-binding proteins were extracted. Cx-binding was assumed to be representative of all carotenoid binding, therefore Ax-binding assays were not repeated for the washes. Comparisons of binding of each fraction with Cx were made for Cx-pigmented and Ax-pigmented salmon extracts. The results for the Cx-binding assays for each of the seven wash fraction can be seen in Tables 4.2.1 and 4.2.2. Data is presented as the total amount of carotenoid bound by each fraction.

Table 4.2.1 Results of the carotenoid binding assay for the solubilized fractions obtained from tissue prepared from Cx-pigmented Atlantic salmon. Binding assays were conducted with 100 μL of each fraction and 5 μL of 20 mg/mL Cx in DMSO. The amount of carotenoid bound to each sample is reported in μg carotenoid bound to 1 g of extracted protein. Protein concentrations of each fraction are also shown.

Sample (200 mL wash volume)	Binding of low ionic strength extract with Cx (μg Cx/g protein)	Protein Concentration (mg/mL)
Water	4.01	12
Water	4.65	6.6
150 mM NaCl	0.00	2.4
150 mM NaCl, pH 7.4	0.00	8.1
Water	1.81	2.8
2.5 mM NaCl	7.54	0.94
1:50 extract	4.89	0.16

Table 4.2.2 Results of the carotenoid binding assay for the solubilized fractions obtained from tissue prepared from Ax-pigmented Atlantic salmon. Binding assays were conducted with 100 μL of each fraction and 5 μL of 20 mg/mL Cx in DMSO. The amount of carotenoid bound to each sample is reported in μg carotenoid bound to 1 g of extracted protein. Protein concentrations of each fraction are also shown.

Sample (200 mL wash volume)	Binding of low ionic strength extract with Cx (μg Cx/g protein)	Protein Concentration (mg/mL)
Water	2.41	19
Water	1.63	6.6
150 mM NaCl	0.00	8.9
150 mM NaCl, pH 7.4	0.00	1.6
Water	0.00	0.96
2.5 mM NaCl	0.00	2.2
1:50 extract	0.00	0.18

Tables 4.2.1 and 4.2.2 show that the solubilization profiles of Ax- and Cx-pigmented salmon tissues are quite different. The protein content of the individual washes varies considerably between the tissues derived from the two different dietary treatments. Also, the pigment-binding protein was not solubilized to the same extent in the two tissues. Binding was observed to occur only in the first 2 fractions of a 7 step solubilization series for the Ax-pigmented salmon extract. Binding was not observed with the 1/50 fraction. Pigment binding did not occur in the stronger salt washes of the Cx-pigmented salmon tissue, but only in the water and dilute salt washes.

The SDS-PAGE profiles of the seven solubilized fractions of Cx-pigmented tissue can be seen in Figure 4.2.2. A comparison of the electrophoretic profiles shows that different proteins were removed during each solubilization step. An illustration of the SDS-PAGE gel shown in Figure 4.2.2 converted the protein bands of the SDS-PAGE gel to black bars of thicknesses varying with the intensity of the protein bands. This illustration helps highlight the major differences among these seven washes (Figure 4.2.3). The most prominent differences among the solubilized fractions occur in the middle and lower MW range with the presence or absence of protein bands belonging to proteins such as G-actin (44 kDa), troponin T (37-40), the myosin light chains (16-25 kDa), and other non-identified proteins. X-protein (151 kDa), tropomyosin (65-70 kDa), and troponin were removed in the initial washes. Only one band was consistently present in each of the washes where Cx-binding occurred, but was absent where no binding was observed. This band is highlighted in red in Figure 4.2.3. The band corresponds to a protein with a molecular weight of 43.7 kDa. This protein was tentatively identified as G-actin.

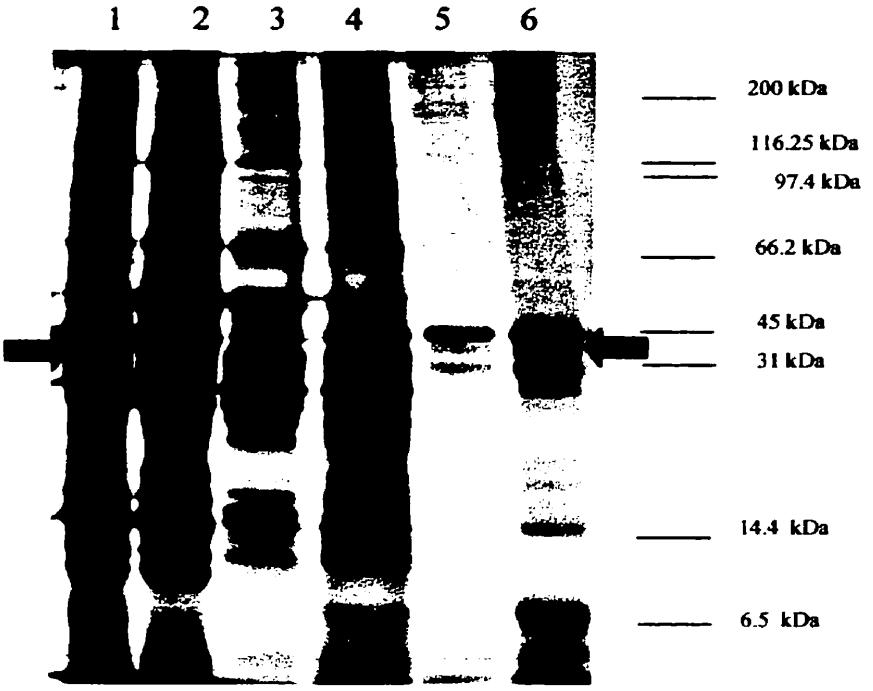

Figure 4.2.2 SDS-PAGE gel of the low ionic strength solubilized fractions from salmon muscle tissue. Salmon was fed a Cx-pigmented diet. Lane 1 = first water wash, lane 2 = second water wash, lane 3 = 150 mM NaCl wash, lane 4 = 150 mM NaCl pH 7.4 wash, lane 5 = third water wash, and lane 6 = 2.5 mM NaCl wash.

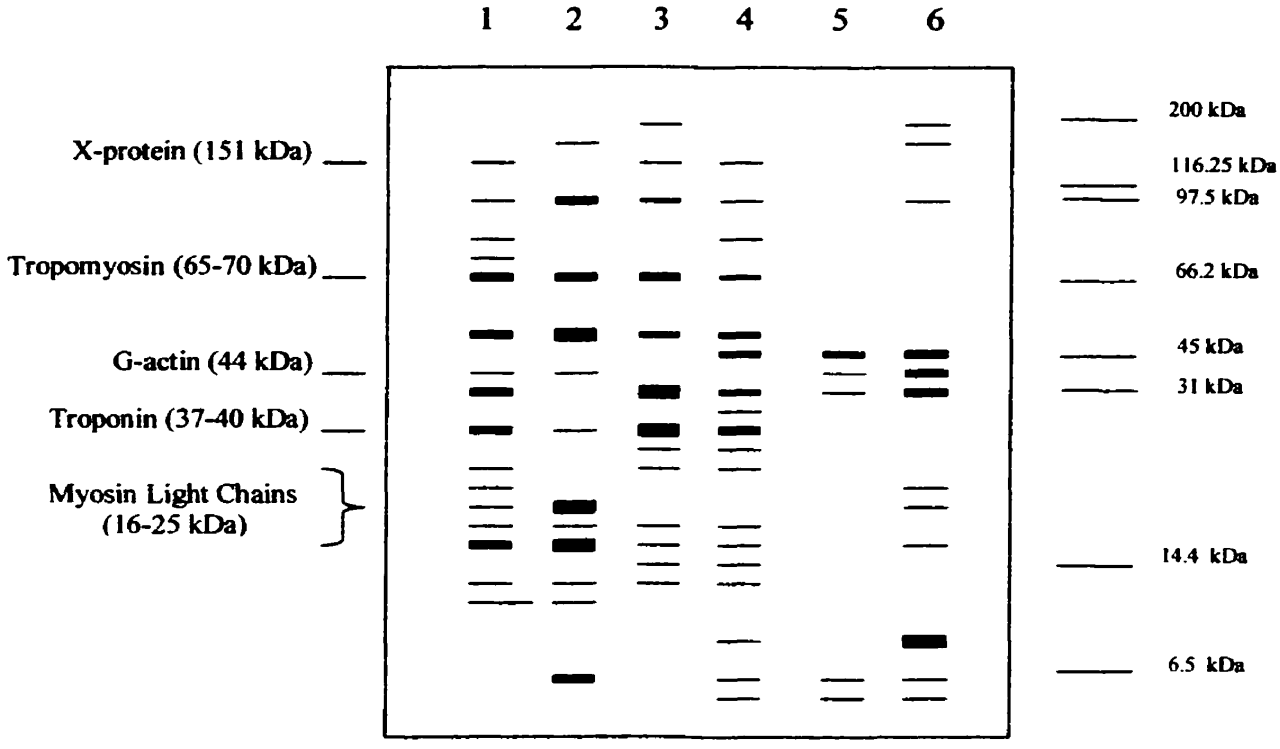

Figure 4.2.3 Illustrated SDS-PAGE gel of the seven low ionic strength solubilized muscle fractions of Cx-pigmented salmon. The only common protein for washes 1,2,5 and 6 (washes where binding with Cx was observed) is shown in red.

4.3 Affinity Column

An affinity chromatography column was prepared using all-*trans*-4-oxoretinoic acid as the ligand in an effort to isolate the carotenoid-binding protein in salmon. The structural similarities between retinoic acid and carotenoids can be seen in Figures 2.6.3.2 and 1.2.1. It was because of these similarities and the availability of all-*trans*-4-oxoretinoic acid that affinity fractionation was attempted. However, it was anticipated that ligand affinity would be stronger for Cx-binding proteins than for Ax-binding proteins since the substitution on the ring system of the Cx molecule is identical to that of all-*trans*-4-oxoretinoic acid.

Binding to the ligand was deemed to have occurred when a large peak corresponding to eluted proteins emerged after the run buffer was changed to the elution buffer (GuHCl). Changing the elution conditions from run buffer to GuHCl buffer led to an increased baseline absorbance at 280 nm. Elution profiles of low ionic strength muscle extracts from Cx-, non- and Ax-pigmented salmon can be seen in Figure 4.3.1.

These profiles show that no proteins in the low ionic strength extract from Ax-pigmented salmon bound to the affinity column because there was no peak at the GuHCl front, apart from the increased baseline absorbance already mentioned. The low ionic strength extracts from Cx- and non-pigmented salmon bound to the affinity column to the same extent, in as much as the peaks were approximately the same size for the same protein concentration. It is interesting to note that previously-frozen extracts (-15°C for 4 days) did not bind to the column nearly as well as their fresh counterparts, for the same amount of applied sample, and the same total protein concentration. The shape and height of the affinity peak of the previously frozen sample was significantly different from the fresh sample.

Low ionic strength muscle extracts prepared from Cx-pigmented and non-pigmented salmon extracts only bound to the column in which 36% of the retinoic acid derivative was cross-linked to the gel using DCC. Binding to the ligand was not observed to occur for the column in which 56% cross-linking occurred using EDC.

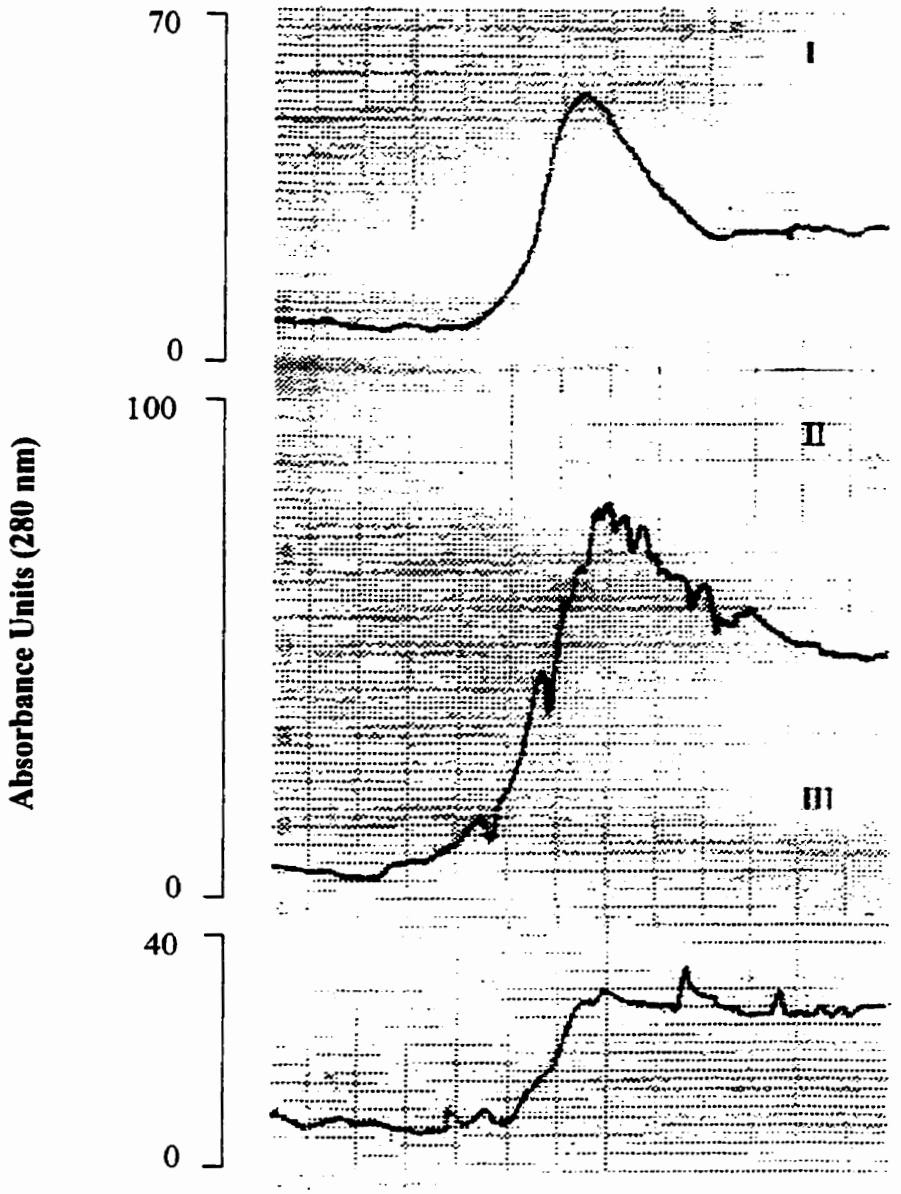

Figure 4.3.1 Comparison of affinity chromatography profiles for Cx-pigmented (I), non-pigmented (II), and Ax-pigmented (III) salmon muscle tissue extracts. Twenty mL of each 1/50 extract was applied to the affinity column. Peaks emerged in cases I and II after elution with 3M guanidine hydrochloride. No peak was observed in case III

indicating that binding did not occur between the Ax-pigmented extract and the affinity column.

The individual 2 mL fractions from each run on the affinity column were pooled into four larger fractions. The first pooled fraction (hereafter designated as Fraction 1) included fractions that initially eluted off the affinity column with the run buffer. This fraction included the large peak of unbound proteins that washed off the column initially. The second pooled fraction (known as Fraction 2) included fractions that eluted after the large unbound protein peak. The third pooled fraction (Fraction 3) included fractions that were collected when the run fraction was changed to the elution buffer. This fraction included the proteins that bound to the affinity column. The last pooled fraction (Fraction 4) included fractions that eluted after the bound proteins had been eluted off the affinity column. These fractions were pooled as shown in Figure 4.3.2.

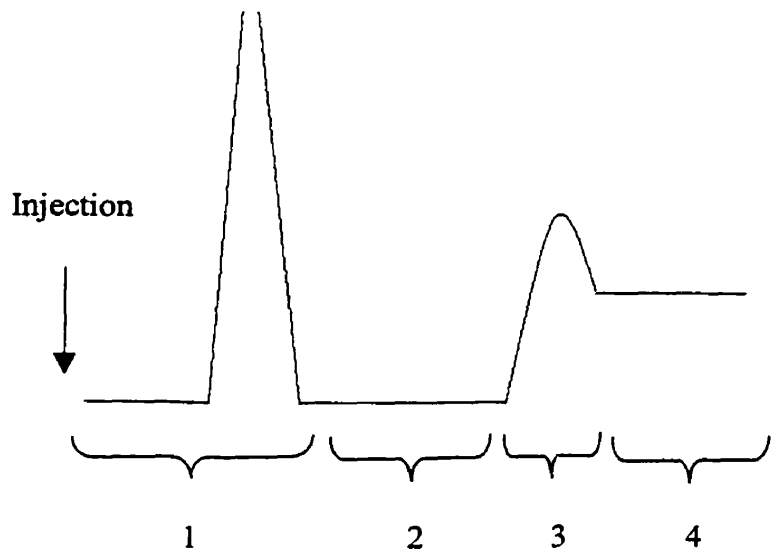

Figure 4.3.2 Representation of a chromatogram obtained from injection of a low ionic strength extract onto affinity column. Numbers indicate pooling of individual 2 mL fractions into four larger fractions.

These four fractions were run on SDS-PAGE to determine if the proteins contained within the fractions were significantly different from one another. This gel can be seen in Figure 4.3.3 which shows that fraction 3 (lane 4) contains different bands than are present in the three other fractions (lanes 2,3 and 5).

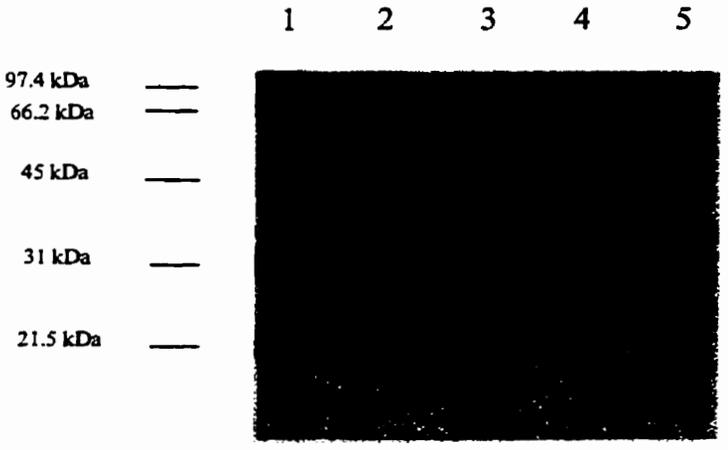

Figure 4.3.3 SDS-PAGE profile of the four different fractions pooled together from a Cx-pigmented affinity chromatography run. Where lane 1 = low range marker, lane 2 = fraction 1 (material that eluted unretained on the column), lane 3 = fraction 2 (material that eluted after the large unbound peak), lane 4 = fraction 3 (proteins that bound to the affinity column), lane 5 = fraction 4 (leftover material that eluted after the bound proteins).

A high MW doublet and several low MW bands are present in this fraction that are different than other proteins bands eluted in previous or later fractions. These notable proteins are indicated by brackets on the SDS-PAGE gel shown in Figure 4.3.3.

Fractions number three from six Cx-pigmented salmon were collected and the SDS-PAGE profiles (Figure 4.3.4) compared to determine the reproducibility of the

column. The gel shows that the column was isolating the same protein, or group of proteins, with every run.

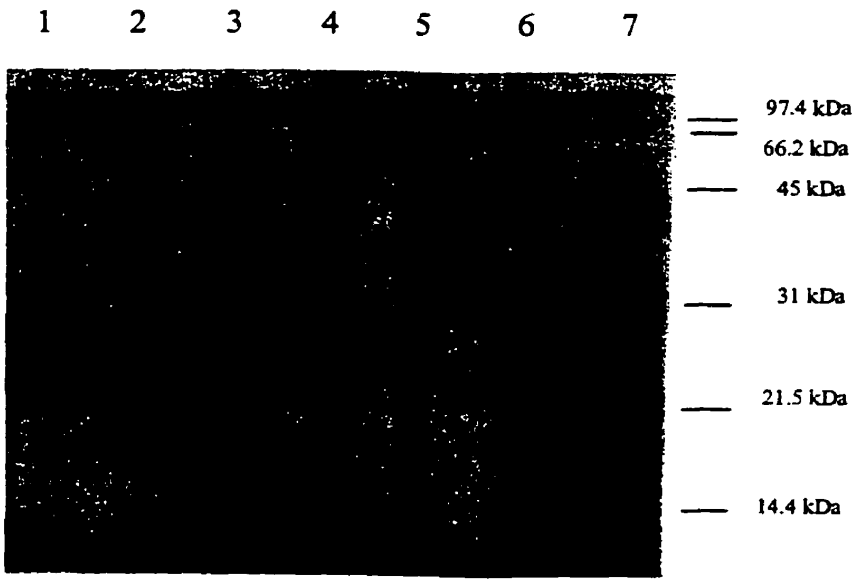

Figure 4.3.4 SDS-PAGE profile of fraction 3 (proteins that bound to the affinity column) from six different low ionic strength extracts of Cx-pigmented salmon muscle tissue over a 3-month period. Where lanes 1-6 = fractions number three from muscle extracts from six different Cx-pigmented salmon, lane 7 = high range marker.

Fractions number three from low ionic strength extracts from non- and Cx-pigmented salmon were compared and were seen to have the same protein bands observed in Figures 4.3.3 and 4.3.4. This gel can be seen in Figure 4.3.5.

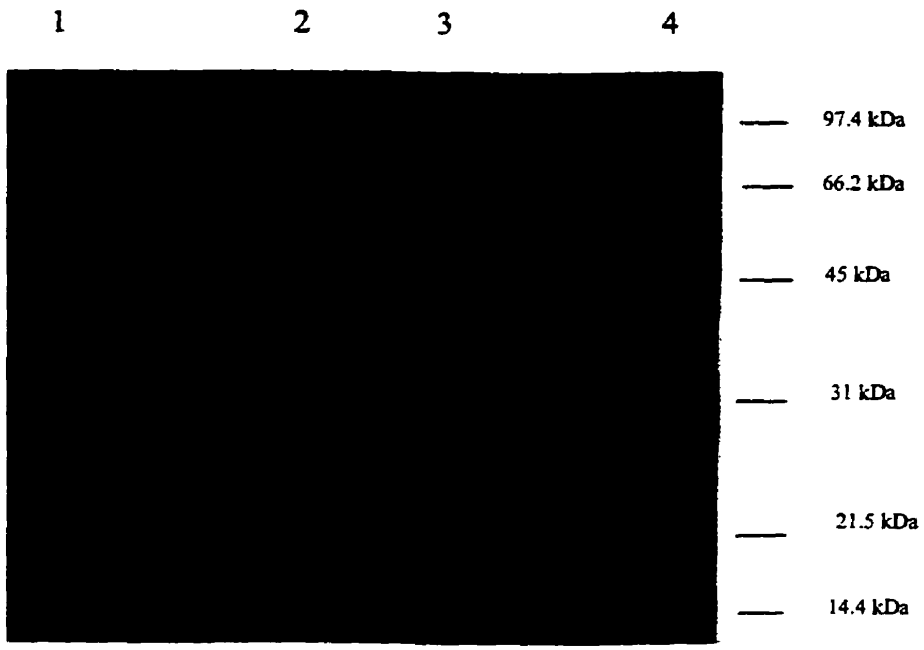

Figure 4.3.5 SDS-PAGE profile of the fraction 3 (proteins that bound to the affinity column) from low ionic strength extracts from non- and Cx-pigmented salmon muscle tissues. Where lane 1 and 4 = high range markers, lane 2 = fraction 3 from Cx-pigmented salmon tissue, lane 3 = fraction 3 from non-pigmented salmon tissue.

Binding studies were undertaken for the pooled fractions obtained from an extracted Cx-pigmented salmon. Binding was compared for Cx and retinoic acid using the 1:50 extract, fraction 1 (unbound proteins) and fraction 3 (bound proteins). The results can be seen in Table 4.3.1. Retinoic acid was used for comparison purposes since it was structurally similar to the compound bound to the affinity column.

Table 4.3.1 Ability of pooled affinity fractions to bind to Cx and retinoic acid. Affinity fractions were combined as described in Figure 4.4.2. One hundred μL of sample was combined with 5 μL of 20 μg/μL of Cx or retinoic acid.

Sample	Amt. Cx bound (μg Cx/ mg protein)	Amt. Ret. Acid bound (μg ret. Acid/ mg protein)
1:50 extract	3.4	8.6
Fraction 1	11.6	13.9
Fraction 3	0.0	85.1

Based on these results, it is clear that the affinity column was not separating a Cx-binding protein but was instead separating a retinoic acid-binding protein. The column successfully separated a protein and by doing so resulted in a ten-fold enrichment in retinoic acid-binding activity (as seen by the amount of retinoic acid bound per mg protein in fraction 3). The column appeared to remove impurities and proteins that may have inhibited the retinoic acid-binding ability of the retinoic acid binding protein. However, the total protein content of each of these fractions was not determined. If pigment binding enrichment occurred in a fraction with a low protein concentration, this would indicate a much greater significance for this column than if pigment binding increased simply because more of the protein was present in that fraction.

The results in Table 4.3.1 indicate that the affinity column built in this thesis was isolating retinoic-acid binding proteins. However, this protein does not fit the common profile of a retinol-binding protein. The proteins isolated in this thesis, as shown in Figure 4.3.4 showed a doublet at 55 kDa, a band at 32 KDa, and a series of bands at 25 kDa on the SDS-PAGE gel.

4.4 Salmon Myofibrils

Binding studies were undertaken with myofibrils prepared from mature salmon and *in vitro* binding was observed to occur between the myofibrils and synthetic Ax and Cx. Computer-aided non-linear regression was used to analyze the data produced during the binding study. The model used to analyze the data was described in Section 2.6.4 as a rectangular hyperbola. Figure 4.4.1 shows that the affinity between the myofibril and Ax is similar to the affinity observed between the myofibril and Cx. This can be inferred by comparing the two curves shown in Figure 4.4.1. Kd values were obtained by computer analysis. A two-tailed student's t-test found that these affinities, as indicated by the Kd values shown in Table 4.4.1, were equivalent (p=0.295).

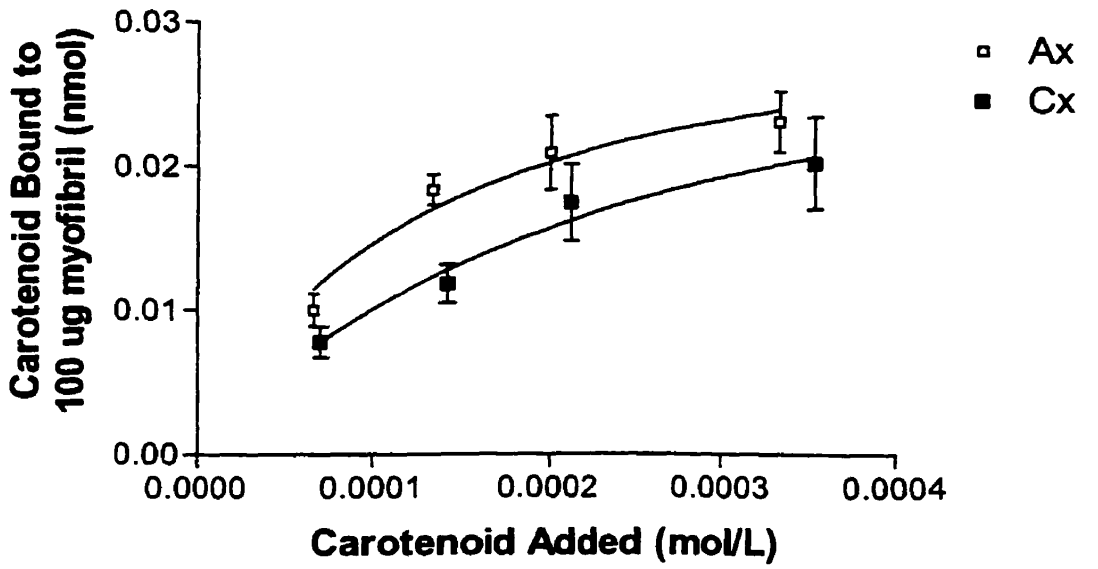

Figure 4.4.1 *In vitro* binding between myofibrils (1 mg/mL) and dietary carotenoids observed at A_{440} (λ_{max} for Cx) or A_{470} (λ_{max} for Ax). Carotenoid concentrations ranging from 4 to 20 μg per 100 μg solubilized salmon myofibrils were used.

Figure 4.4.1 also shows that there appears to be a saturation point on the myofibril where no more carotenoid is absorbed even if more carotenoid is added to the system. This trend is indicated by the assymptotal nature of the data. A two-tailed student's t-test

found that the myofibril saturation points, as indicated by Bmax values shown in Table 4.4.1, were equivalent for Ax and Cx ($p=0.425$). Bmax values were obtained by computer analysis. Values for Kd and Bmax can be seen in Table 4.4.1.

Table 4.4.1 Saturation (Bmax) and affinity (Kd) values obtained by computer-aided non-linear regression for the relationship between salmon myofibrils and Ax and Cx.

Carotenoid added to salmon myofibrils	Bmax (nmoles)	Kd (mol/L)
Ax	0.03 ± 0.01	0.00016 ± 0.00005
Cx	0.04 ± 0.01	0.0003 ± 0.0002

4.5 Studies with Actin

Electrophoretic evidence and pigment-binding studies (Section 4.2) suggested the possible involvement of actin in the retention of dietary Ax/Cx in salmon muscle. In order to confirm or disprove this assumption, F-actin was prepared from mature and juvenile salmon. Approximately 30 mg of F-actin was obtained per gram of acetone powder. Densitometric analysis of SDS-PAGE gels showed that the F-actin was mildly contaminated with some myosin and a very small amount of tropomyosin. Actin isolated from mature and juvenile salmon muscle tissue were found to be 80% and 76% pure, respectively, as determined by densitometric analysis of electrophoretic gels. However, it was believed that neither of these contaminants would be detrimental to future binding studies since myosin and tropomyosin were not present in the solubilized protein fractions where binding with carotenoids was observed in previous experiments. Therefore, the purification was deemed acceptable.

The gel obtained from the SDS-PAGE can be seen in Figure 4.5.1, where lane 1 is the mature F-actin, lane 2 is the juvenile F-actin, and lane 3 are the Bio-Rad molecular weight standards. Actin, myosin and tropomyosin bands are marked accordingly. The

SDS-PAGE results also show that the actins from the mature and juvenile tissue show no discernable difference in molecular weight.

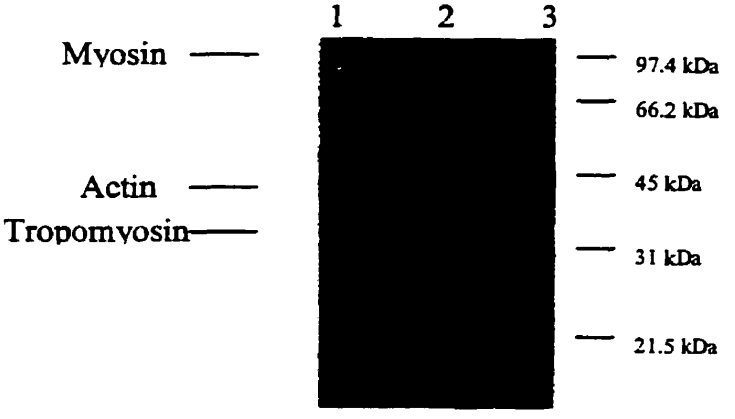

Figure 4.5.1 SDS-PAGE of actin isolated from mature and juvenile salmon muscle tissues. Where lane 1 = mature F-actin, lane 2 = juvenile F-actin, lane 3 = Bio-Rad molecular weight standards.

Binding studies were undertaken with Ax and Cx as detailed in Section 3.6. Computer-aided non-linear regression was used to analyze the binding data, following the model described in Section 2.6.4. The saturation profile for mature and juvenile salmon actin with Ax can be seen in Figure 4.5.2. The saturation profile for actin isolated from mature and juvenile salmon tissue with Cx can be seen in Figure 4.5.3. Affinity constants (Kd values) and saturation values (as designated by Bmax values) were generated for the profiles shown in Figures 4.5.2 and 4.5.3 using graphing software designed for biochemists (Prism Graphpad Software, Version 3.02).

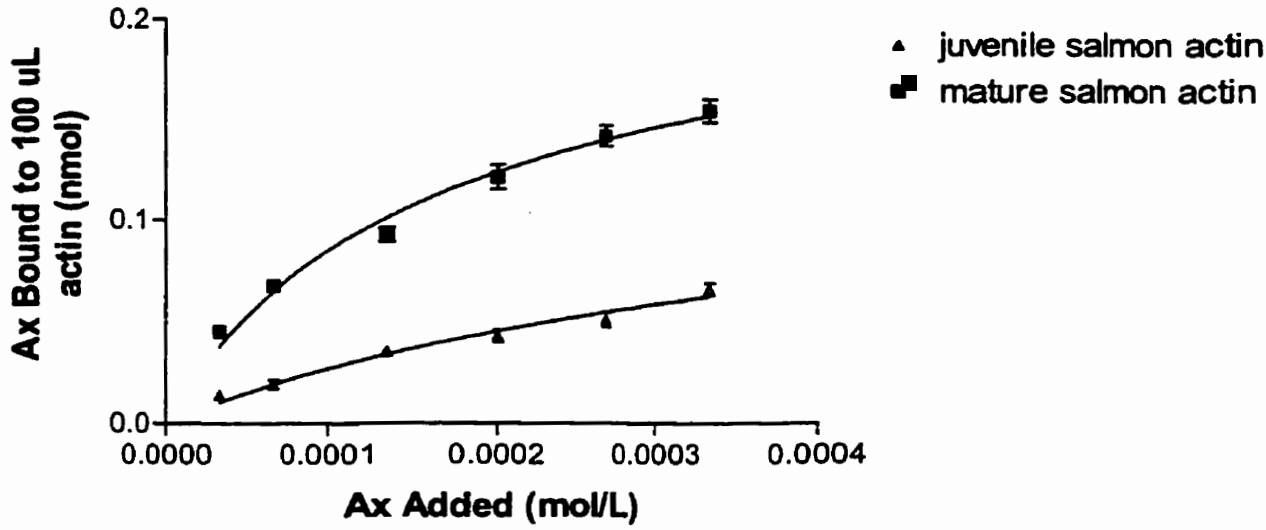

Figure 4.5.2 Saturation profiles illustrating the relationship between F-actin purified from mature (0.80 mg/mL) and juvenile (0.76 mg/mL) Atlantic salmon muscle tissues and Ax.

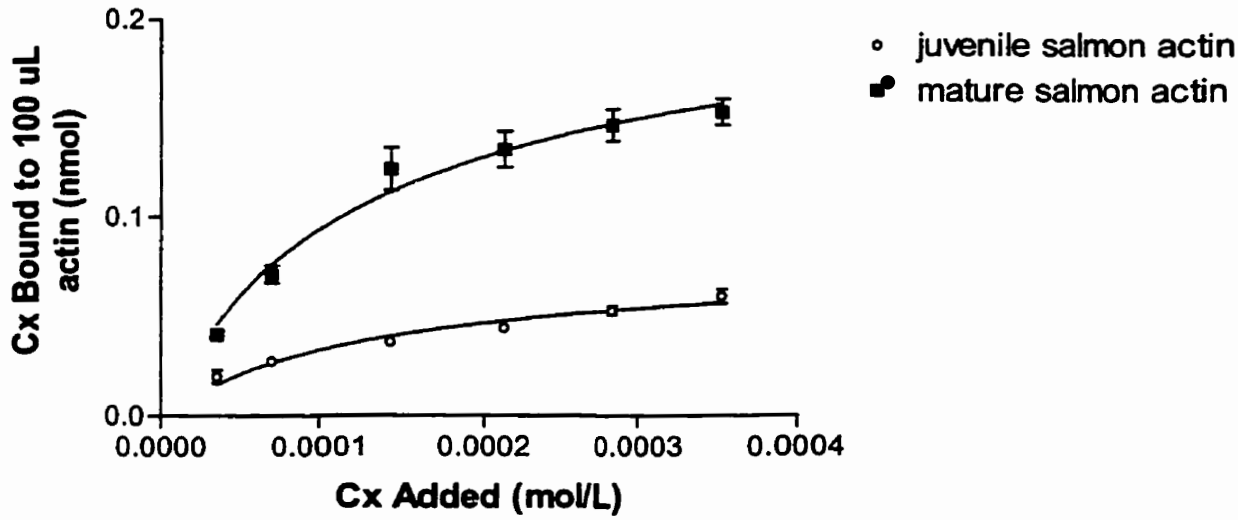

Figure 4.5.3 Saturation profiles illustrating the relationship between F-actin purified from mature (0.80 mg/mL) and juvenile (0.76 mg/mL) Atlantic salmon muscle tissues and Cx.

Table 4.5.1 Computer-generated affinity constants (Kd) for Ax and Cx with F-actin purified from mature and juvenile Atlantic salmon tissue. P values obtained from a 2-tailed student's t-test. Values greater than 0.05 mean data is equivalent at the 95% confidence interval.

Actin Type	Kd Value for Ax (mol/L)	Kd Value for Cx (mol/L)	p value
Mature	0.00017 ± 0.00003	0.00013 ± 0.00003	**0.145**
Juvenile	0.0004 ± 0.0001	0.00014 ± 0.00003	**0.020**
p value	**0.032**	**0.394**	-

Figures 4.5.2 and 4.5.3 indicate the difference in binding observed between F-actin purified from both mature and juvenile Atlantic salmon muscle tissues and Cx and Ax. These figures clearly demonstrate a difference between the ability of mature and juvenile salmon actin to bind carotenoids. The quantitative difference between the ability of mature and juvenile salmon actin to bind carotenoids can be seen in Table 4.5.1. Lower Kd values indicate a higher affinity between the carotenoid and the actin molecule. Thus in the relationship between carotenoids and F-actin purified from mature salmon muscle tissue, F-actin has an equivalent affinity for Ax and Cx. However, in the carotenoid-juvenile salmon F-actin relationship, juvenile salmon F-actin has a higher affinity for Cx than for Ax. Saturation values for Figures 4.5.2 and 4.5.3 can be seen in Table 4.5.2.

Table 4.5.2 Computer-generated saturation values (Bmax) for Ax and Cx with F-actin purified from mature and juvenile Atlantic salmon tissue. P values obtained from a 2-tailed student's t-test. Values greater than 0.05 mean data is equivalent at the 95% confidence interval.

Actin Type	Bmax Value for Ax (nmol)	Bmax Value for Cx (nmol)	p value
Mature	0.21 ± 0.02	0.23 ± 0.02	**0.284**
Juvenile	0.14 ± 0.03	0.079 ± 0.007	**0.018**
p value	**0.015**	**<0.001**	-

These saturation values were used to calculate the maximum amount of carotenoid that could possibly bind to F-actin. The maximum values of carotenoid binding can be seen in Tables 4.5.4 and 4.5.5. Sample calculations can be seen in Appendix B.

Table 4.5.3 Maximum capacity of mature salmon actin to bind dietary carotenoids.

Carotenoid	Mol/mol F-actin	mg/g F-actin
Ax	3.4	1.7
Cx	3.0	1.5

Table 4.5.4 Maximum capacity of juvenile salmon actin to bind dietary carotenoids.

Carotenoid	Mol/mol F-actin	mg/g F-actin
Ax	2.1	1.1
Cx	1.2	0.59

At the molecular level, binding of Ax and Cx with mature salmon F-actin showed that an average of 3.4 molecules of Ax can bind per molecule of F-actin and 3.0

molecules of Cx can bind per molecule of mature F-actin. These values were shown to be the same using a two-tailed student's t-test ($p=0.284$). Results obtained with juvenile salmon F-actin found 2.1 molecules of Ax are able to bind to each molecule of juvenile salmon F-actin while only 1.2 molecules of Cx can bind to juvenile salmon F-actin. These values were found to be different ($p=0.018$). The fact that juvenile salmon actin has the ability to bind carotenoids in any capacity suggests that the ability of actin to bind carotenoids is present from the beginning instead of being developed over time with exposure to dietary carotenoids.

Theoretical saturation levels for the amount of carotenoid bound to the flesh of salmon were calculated based on the assumption that salmonid flesh is 17% protein and 14% of this is actin (Huxley, 1963). These assumptions were used to compare the data in this thesis to saturation values calculated in other studies. Theoretical saturation levels for carotenoids in salmon flesh can be seen in Table 4.5.7. Sample calculations can be seen in Appendix B.

Table 4.5.6 Theoretical saturation levels of carotenoids in salmon muscle tissue.

Tissue	Saturation Value for Ax (mg Ax/ kg flesh)	Saturation Value for Cx (mg Cx/ kg flesh)
Mature	40	36
Juvenile	26	14

5.0 Discussion

Studies by Henmi *et al* (1989a) demonstrated that dietary carotenoids bind to actomyosin in salmon. These researchers showed that Ax in salmon muscle existed in the water-insoluble fraction, associated with actomyosin prepared from myofibrils, and released easily from the protein with treatment with Triton X-100, SDS and acetone. These facts suggested that Ax bound to actomyosin by means of a weak hydrophobic bond.

The specific protein within the actomyosin unit responsible for carotenoid binding was never determined. Therefore, Henmi's results were used to develop a procedure for isolating the carotenoid-binding protein in Atlantic salmon. Two main methods of attempting solubilization of the carotenoid-binding protein in Atlantic salmon muscle tissue were chosen. The first method employed collagenase to hopefully liberate the carotenoid-binding protein from salmon muscle tissue. The second method involved six successive washes that solubilized salmon tissue into various fractions of differing low ionic strengths and possibly solubilized the carotenoid-binding protein in the process.

5.1. Collagenase Method

The collagenase method used by Nur-E-Borhan *et al* (1995) to isolate the carotenoprotein in tiger prawn shrimp was unsuccessful when used to attempt solubilization of the carotenoid-protein complex in Atlantic salmon. Although the collagenase action appeared to release some pink pigment, two gel chromatography columns were unable to separate a carotenoid-protein complex from the protein matrix. The method became more complicated than desired and was abandoned in favour of a simpler approach.

However, now that it is known that the carotenoid in salmon tissue is not present as a *carotenoprotein*, but as an interaction between carotenoids and actin, it is clear that methods designed to specifically extract carotenoproteins would never have been able to

isolate the carotenoid-actin complex in salmon. Collagenase is an enzyme specific to collagen that would have released sarcoplasmic proteins, not myofibrillar proteins.

5.2 Low Ionic Strength Solubilization

A low ionic strength extraction method developed by Krishnamurthy *et al* (1996) was used to solubilize the carotenoid-binding protein in Atlantic salmon. Three low ionic strength methods were attempted, and although each of these methods reportedly solubilized over 90% of the substrate they were designed to solubilize, they were only moderately successful in solubilizing salmon tissue. Using a method that solubilized over 90% of chicken proteins (Krishnamurthy *et al*, 1996), work with salmon resulted in the solubilization of less than 60% of salmon proteins. Use of two other procedures that obtained greater than 90% solubilization of both cod (Stefansson and Hultin, 1994) and mackerel tissues (Feng and Hultin, 1997) gave results that were even more dismal. Salmon tissue thus reacts very differently under the same conditions than other muscle tissues. Personal communication with Hultin confirmed these disappointing results. Hultin believed that the high levels of collagen in salmon tissues compared to muscle tissues in other species were responsible for this phenomenon.

Solubilization trials with salmon tissue showed that Ax-pigmented salmon tissue could not be solubilized as easily as Cx-pigmented salmon tissue, using the same procedure. A greater percentage of the total proteins present in Cx-pigmented salmon tissue were solubilized compared to Ax-pigmented salmon tissue. This indicates that the presence of Ax may have an inhibitory effect on the solubility of the pigment-binding protein perhaps by increasing the hydrophobicity of the protein, and thereby preventing solubilization.

The low ionic strength solubilization method resulted in 6 different fractions of varying ionic strengths and pH's. A Cx binding assay was used to determine which fraction contained the highest carotenoid-binding ability; thereby suggesting which fraction contained the greatest amount of carotenoid-binding protein. In the low ionic strength solubilization of Cx-pigmented salmon muscle tissue, the greatest amount of Cx binding was observed in the very low salt fraction (2.5 mM NaCl) and the water washes

also demonstrated some Cx-binding ability. However, the pink colour of the Cx-pigmented salmon tissue was not solubilized until the final 1/50 fraction. This means that carotenoid-binding protein was solubilized in some washes without the total solubilization or release of the carotenoid present in the tissue.

In the case of the Ax-pigmented salmon tissue, Cx binding was only observed in the first two water extractions. The pink colour of the Ax-pigmented salmon tissue was never entirely solubilized, not even in the final 1/50 fraction. This led to two possible conclusions regarding Ax-pigmented salmon tissue. Astaxanthin may have such an inhibitory effect on the solubility of the Ax-protein complex that not much of the protein was solubilized in the 6 low ionic strength washes. It is also possible that the natural presence of Ax in the carotenoid-protein complex inhibits binding between the added Cx and the carotenoid-binding protein. If Ax was not released from the muscle tissues during the low ionic strength washes, Cx would not be able to bind to the already formed Ax-protein complexes. However, a diet was not used that contained both Ax and Cx, so this hypothesis cannot be tested.

5.3 Affinity Chromatography

Binding was observed to occur between the low ionic strength extracts from the Cx- and non-pigmented salmon tissue and the affinity column built with DCC. Binding was not observed to occur between any of the low ionic strength extracts and the affinity column built with EDC. Using DCC as the cross-linking agent resulted in 36% of the retinoic acid binding to the column while using EDC as the cross-linking agent resulted in 56% retinoic acid binding. One-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and DCC both give the same zero-length cross-linking between the ligand and the matrix. Thus it is not a matter of steric hindrance preventing binding from occurring when EDC is used as the crosslinking reagent as opposed to DCC. However it has been noted in some antibody affinity reactions that extensive coupling will lead to the loss of antibody binding sites (Amersham Pharmacia Biotech, 1999). Perhaps the same mechanism is occurring here.

Binding was only observed to occur between the affinity column and the low ionic strength extracts from the Cx- and non-pigmented salmon extracts. Binding studies on the 1/50 fraction revealed that the affinity column was actually isolating a retinol-binding protein and not a carotenoid-binding protein. Therefore the structure of all-*trans*-4-oxoretinoic acid was still too similar to the structure of retinoic acid to allow binding of a carotenoid-binding protein to occur. Zagalsky *et al* (1990) observed in their reconstitution work with apoproteins that carotenoids longer and shorter than Ax had lower affinities for apocrustacyanin. Therefore it was not sufficient for all-*trans*-4-oxoretinoic acid to have a similar ring structure as Ax, the ligand also needed to be approximately the same length in order for binding to occur.

The column was thus specific to retinoic acid-binding proteins, giving a 10-fold purification in the separation of a retinoic acid-binding protein. The column may be helpful in isolating and identifying vitamin A-binding proteins in salmon.

Retinol- binding proteins have been isolated and characterized from many species. The molecular weight of studied mammalian and piscine plasma retinol-binding proteins is constant, at approximately 20,000 Da (Shidoji and Muto, 1977). The retinoic-acid binding protein isolated from Atlantic salmon muscle tissue did not entirely match the profile of known retinol-binding proteins. The SDS-PAGE gel of the protein isolated in this thesis showed a doublet at 55 kDa, a band at 32 KDa, and a series of bands at 25 kDa. In the serum of the snapping turtle, retinol is bound by two proteins with molecular weights of 75,000 and 20,000 Da (Shidoji and Muto, 1977). Therefore it is not improbable that retinol-binding proteins in Atlantic salmon might have two molecular weights as determined by SDS-PAGE. However, even though the isolated Atlantic salmon protein is unpurified, none of the molecular weights of the protein bands on the SDS-PAGE correspond to a typical retinol-binding protein. This may indicate that a new muscle retinol-binding protein has been found in salmon. The 25 kDa band may correspond to a retinol-binding protein and the 55 and 32 kDa protein bands may belong to proteins bound to the 25 kDa protein.

5.4 Binding of Carotenoids with Myofibrils

Binding studies with purified salmon muscle myofibrils suggested that 100 μg of salmon myofibrils can be saturated with 0.038-0.04 nmoles of either Ax or Cx. However, it should be noted that with only four data points, the saturation values obtained for myofibrils with carotenoids are merely approximations. However, the trend agrees with results reported by Bjerkeng *et al* (1992) who found similar final concentrations of Ax and Cx in rainbow trout tissues after a 140 week feeding study, using identical carotenoid concentrations in the feed. Results with salmon myofibrils suggest that the maximum capacity of salmon tissues for the two dietary carotenoids is the same.

The affinity of salmon myofibrils for Ax was found to be over four times higher than that for Cx. This difference in affinity is reflected by work carried out by Gobantes *et al* (1997) who showed that Cx is removed from rainbow trout serum twice as quickly as Ax. This indicates that the affinity of rainbow trout muscle proteins for Ax is greater than that for Cx. It was hypothesized in the study by Gobantes *et al* (1997) that the higher affinity of rainbow trout muscle proteins for Ax than for Cx might partially account for the better digestibility of Ax (40 to 60%) compared to Cx (19 to 30%).

5.5 Binding of Carotenoids to Actin

Binding studies in the present study indicated that actin is one of the pigment-binding proteins in salmon muscle tissue. It is not surprising that this muscle protein is responsible for carotenoid binding in salmon tissue. Actin is known to be able to interact with numerous molecules in the cell. At present there are over 50 different proteins that bind to actin, accounting for approximately 25% of cellular protein (Johansson, 1998). These proteins take part in several cell processes such as muscle contraction, cell movement, cytokinesis, and cytoplasmic streaming, to name a few. The ability of actin to bind many types of molecules thus builds precedence for its ability to bind carotenoids. The maximum capacities of mature salmon actin for Ax and Cx were found to be equivalent ($p=0.284$), with 0.21-0.23 nmoles of Ax/Cx binding to 80 μg of actin. These

values were much higher than those reported by Henmi *et al* (1989a) because actin was used as opposed to actomyosin. The actomyosin complex results in carotenoid-binding sites becoming sterically hindered or blocked entirely by myosin molecules, thus reducing the amount of carotenoid that can bind to the complex. Also, carotenoid binding values found in this thesis represent total saturation of the actin molecule whereas Henmi and colleagues reported a binding value based on one carotenoid concentration.

Equivalent actin saturation values indicated that at maturity, Atlantic salmon tissue should pigment to the same extent with either carotenoid given a long enough feeding regime. A similar result was shown by Bjerkeng *et al* (1992) in their 140 week feeding study in which rainbow trout fed the same concentration of Ax or Cx reached similar final carotenoid levels in the flesh. The affinities of mature actin for Ax and Cx were also found to be equivalent in the present study ($p=0.145$). Bjerkeng *et al* (1992) found salmon fed Ax attained acceptable flesh pigmentation levels before salmon fed Cx, using similar carotenoid concentrations in the feed. This indicates that the difference in affinity observed between carotenoid *in vivo* is not due to actin binding Ax preferentially over Cx. Selection between carotenoids must take place prior to the final receptor protein.

The maximum capacities of juvenile salmon actin for Ax and Cx were found to be lower than the maximum capacities of the two carotenoids in mature salmon actin. Seventy-six μg of juvenile salmon actin was saturated with 0.14 nmol Ax and 0.079 nmol Cx. These values were not found to be statistically equivalent ($p=0.018$). This led to the conclusion that the ability of salmon actin to bind carotenoids changes as salmon mature. Bjerkeng *et al* (1992) showed that carotenoid deposition varies during different life stages of rainbow trout and they attributed this redistribution to changes in carotenoid absorption, transport capacity, affinity for carotenoids in various tissues, or to catabolism. Work carried out in the present study shows that changes in pigmentation during growth are in part explained by a change in the ability of salmon actin to bind carotenoids, both in terms of affinity and capacity.

The ability of juvenile salmon actin to bind Ax and Cx, shown in Section 4.5, was less than the ability of mature salmon actin to bind the same carotenoids. Based on Table 4.5.1, this is clear in the case of Ax but not so clear in the case of Cx. If the curves in

Figure 4.5.3 are compared, it is evident that juvenile salmon actin does not bind Cx as efficiently as mature actin. If the saturation values, Bmax, are held constant, the affinity of juvenile salmon actin for Cx is much lower than that of mature salmon actin. Once again, this clearly indicates that the ability of actin to bind carotenoids changes as salmon mature.

The calculated saturated binding levels of Ax and Cx in salmon tissues shown in Table 4.5.3 were slightly higher than are observed under both natural and aquaculture conditions. The levels of binding between carotenoids and rainbow trout muscle tissue reported by Bjerkeng *et al* (1992) were 19.6 mg Ax/kg flesh and 12.9 mg Cx/ kg flesh. However, these same researchers reported that one kg of muscle could theoretically bind 100 mg of Ax. Even though Bjerkeng *et al* (1992) were studying pigmentation of rainbow trout and not Atlantic salmon, pigmentation levels of the two fish are within the same order of magnitude, therefore a comparison is interesting. Using their same calculation, the results in the present study gave 40 mg Ax/kg mature salmon flesh, and 36 mg Cx/kg mature salmon flesh, which represent total saturation of the actin molecule with dietary carotenoids. These levels are double those reported by Bjerkeng *et al* (1992). Calculations by Bjerkeng *et al* (1992) did not use the actin molecule specifically, but the higher levels obtained in the present study indicate that other factors obviously come into play before a saturation point of the actin molecule is reached.

In Henmi *et al*'s studies (1989b), surface hydrophobicity of the protein was correlated with the binding characteristics of the protein and a model was put forward suggesting that carotenoids bind non-specifically to actomyosin binding sites by means of a hydrophobic bond. The results obtained in the present study show that actin binds carotenoids in a highly specific manner, with changing affinities and capacities for Ax and Cx depending on the maturity of the salmon. However, work carried out in the present study did not disprove that a hydrophobic interaction between actin and carotenoids occurs. Therefore, hydrophobicity is still the most likely reason for the formation of a carotenoid-actin complex.

It should also be noted that the 20-24% impurities present in the actin preparation used in this thesis may have given rise to altered values for Bmax and Kd. These

impurities may have hidden carotenoid binding sites, thus giving reduced binding values, or they have contributed to carotenoid binding, thus giving increased binding values.

5.6 Binding of Carotenoids to Actin Isoforms

Henmi *et al* (1989b) showed that the ability of actomyosin to bind carotenoids is not a function of species but that there are more carotenoid binding sites on salmon actomyosins than any other fish actomyosin studies, except mackerel. Now that the present study has strongly suggested that carotenoids form a complex with actin, it is possible that the ratio of actin to other muscle proteins is higher in salmon and mackerel than in other fish. It is also possible that salmon and mackerel have a larger proportion of a specific actin isoform responsible for the higher levels of carotenoid binding. The level of actin and the expression of actin isoforms have been reported to vary in different smooth muscle types (Fatigati and Murphy, 1984) and it is possible that different actin types and amounts might be responsible for pigmentation variations in salmon.

Lipoproteins studied at the feeding migration stage of chum salmon (when the salmon store energy reserves before migrating) had the same carotenoid transport ability as the counterpart at the upstream migration stage (when the salmon migrate upstream) (Ando and Hatano, 1988). Therefore, transport proteins appear to stay the same throughout the life cycle of chum salmon. This indicated to Ando and Hatano (1988) that changes in pigmentation throughout the life cycle may be due to receptor proteins and not transport proteins. As actin has been suggested to be the receptor protein in Atlantic salmon muscle tissues, and the ability of actin to bind Ax and Cx is present in both juvenile and mature actin, perhaps it is merely that actin conformation changes over the salmon life cycle.

It is possible that different actin isoforms dominate during different stages of life, thus changing the binding ability of carotenoids to salmonid muscle tissue. Disturbing the natural ratio of actin isoforms is known to affect the organization of bundle actin filaments (Höfer *et al*, 1997). It would seem plausible that changes in actin isoform ratios might also result in changes in carotenoid binding affinity. These ratios might be

expected to change throughout the life cycle of an animal. Different actin isoforms have been shown to bind different actin-binding proteins differently, such as the affinity of ezrin for β-actin (Shuster and Herman, 1995). Shuster and Herman (1995) demonstrated that *in vitro* studies of actin systems should use the actin most closely related to the *in vivo* isoform. It is possible that the presence of different actin isoforms gives rise to different binding patterns depending on the molecule being studied.

Nickell and Bromage (1998) showed that Ax deposition in small fish (< 50g) does not occur in a homogeneous manner within a population. Ax deposition progresses at a similar rate among individuals within a population of a minimum fish size. Differences in colour development within the fillet were attributed to differences in muscle morphology and physiology leading to variability in the number of Ax binding sites (Nickell and Bromage, 1998). Differences in muscle fibre number, size, and function within a fish have been reported (Totland *et al*, 1987). Results in the present study indicate that either actin generation does not occur homogeneously throughout the muscle tissues or different muscle fibres contain different amounts of actin. It is also possible that if different actin isoforms dominate throughout different stages of the life cycle, and not all fish develop at the same rate, different isoforms may dominate within a population until all fish reach the same stage.

Nickell and Bromage (1998) hypothesized that if there were a limit in the absorption and/or transport of Ax in the muscle, but no limit in its deposition, this would result in a homogeneous distribution of carotenoid. However, since this homogeneous distribution was not observed in Nickell and Bromage's studies, it was assumed that a limit does occur in deposition. Results found in the present study indicate that a saturation point of the actin molecule does exist, but will never be reached under natural conditions. Thus, it is not only a matter of the right actin isoform being present in the salmon tissue. Wathne *et al* (1998) hypothesized that poor utilization of Ax is caused by a limited capacity to bind and transport Ax to the lipoprotein and release it to the target tissues. It is possible that a unique carotenoid transport system develops over time with the growth of the fish, and young salmon have simply not yet developed the needed transport system, even though the receptor protein is there.

5.7 Differences between carotenoproteins, retinol-binding protein complexes and the carotenoid-actin complex

In the introduction of this thesis, it was proposed that the carotenoid-binding protein in salmon might be a *carotenoprotein* as described by researchers such as Zagalsky *et al* (1995) and reviewers such as Shahidi (1998). Although certain attributes of the actin-carotenoid complex are similar to carotenoproteins, many attributes are also different. As was discussed in Section 2.2, α-crustacyanin is composed of one carotenoid molecule bound per apoprotein monomer. The stoichiometric ratio of Ax/ Cx to mature F-actin was found to be approximately 3:1.

Binding of carotenoids within a hydrophobic cavity inside the protein structure fits the description of Ax-binding in α-crustacyanin, and is also the theory proposed for carotenoid binding in salmon by Henmi *et al* (1989a). However, a major difference between the two complexes is that actin appears to be able to bind at least two carotenoids whereas crustacyanin only forms a complex with Ax. Although only Ax and Cx were studied in the present study, it is reasonable to believe that other carotenoids would successfully form a carotenoid-actin complex. Milicua *et al* (1985) found several carotenoids able to reconstitute the carotenoprotein found in *P. clarkii*, provided the carotenoids were similar in chain length and functional groups to Ax. The ability of actin to bind with a multitude of molecules demonstrates actin to be very versatile. Further evidence for the probability of actin binding other carotenoids lies in the natural presence of β-carotene, lutein, tunaxanthin and zeaxanthin in wild salmon (Shehaan *et al*, 1998). These carotenoids must be binding to a protein in salmon muscle tissue and perhaps this protein is actin.

The actin-carotenoid complex also differs from carotenoproteins in that the absorption spectrum of the complex does not display a bathochromatic shift during the binding/release of Ax and/or Cx to actin. The carotenoid-actin complex is thus different from typical carotenoproteins. No bathochromatic shift was observed when Ax was associated with serum lipoproteins either (Ando and Hatano, 1988). The λmax exists as 480 nm both before and after binding.

The MW of the carotenoid-actin complex was shown to be nearly double that of the retinol-binding protein complex. Retinol-binding protein is 21 kDa and has one binding site that is able to bind one molecule of retinol. The carotenoid-binding protein in mature and juvenile salmon muscle tissue was shown to have a molecular weight of 43.7 kDa, and was able to bind 3 molecules of Ax or Cx. The binding characteristics of the two complexes were not alike in that a small bathochromatic shift is detected when retinol is released from RBP.

6.0 Conclusions

Several methods were used to help solubilize salmon muscle proteins in the attempt to identify a carotenoid-binding protein in Atlantic salmon. Of the methods attempted, a low ionic strength treatment developed by Krishnamurthy *et al* (1996) solubilized almost 60% of the proteins present in Atlantic salmon tissue. The collagenase method developed by Nur-E-Borhan *et al* (1995), while useful in isolating the carotenoprotein in tiger prawn shrimp, was unable to solubilize actin in salmon tissue. Also, the affinity column developed to isolate the carotenoid-binding protein in salmon by binding all-*trans*-4-oxoretinoic acid to a Sepharose backbone was unsuccessful in fractionating the desired protein. However, the column did isolate a retinoic acid-binding protein consistently with a tenfold purification factor.

The carotenoid-binding protein in Atlantic salmon muscle tissue was found to be a protein with a molecular weight of 43.7 kDa that was tentatively identified as actin. After purifying salmon F-actin, binding studies were undertaken to prove that actin was indeed binding dietary carotenoids. These studies showed that mature salmon F-actin binds Ax or Cx in a 1:3 ratio (protein:carotenoid). Juvenile salmon F-actin was found to bind less Ax and Cx than its mature counterpart. The affinities of mature salmon actin for Ax and Cx were found to be equivalent and much higher than the affinities of juvenile salmon actin for both carotenoids. The changes in affinity and capacity of the actin molecule to bind carotenoids as salmon mature, indicates that the absorption and transportation of carotenoids is a system that evolves over time. Young salmon that do not show pigmentation even when exposed to pigmented feed probably have not yet developed both of these abilities.

Bjerkeng and colleagues (1992) reported that the maximum amount of Ax that could bind to the flesh was 100 mg Ax per kg of flesh, even though in nature only 6 to 25 mg Ax were observed to bind per kg of salmon flesh. Calculations carried out in this thesis showed that 40 mg Ax and 36 mg Cx were able to bind per kg of salmon flesh *in vitro*. Obviously other factors come into play before a saturation point of the actin molecule is reached. It is likely that the inability of actin to reach complete saturation with carotenoid is due to poor absorption and transportation of the carotenoids through the digestive system and/or from the blood to the muscle.

These studies have made major headway in understanding the binding between carotenoids and salmonid flesh. Future studies need to be undertaken to determine the exact site of binding of carotenoids on the actin molecule. The site and exact type of binding (H-bond or hydrophobic interaction) must be identified before manipulation of the site can begin. This manipulation will guide researchers in preserving the carotenoid-actin complex during frozen storage and will also help them increase the amount of carotenoid bound to the actin molecule.

6.1 Unanswered Questions and New Questions

1) Is only salmon actin able to bind carotenoids or do other fish actins possess the same abilities? Is salmon actin structurally different than other fish actins (primary, secondary and tertiary structure) leading to more hydrophobic pockets in which carotenoids can bind, or is there proportionately more actin in salmon muscle? Studies by other researchers have shown that actin isoforms exist. Do one or two isoforms have a better ability to bind carotenoids? Are these isoforms only present in species that show the ability to bind carotenoids?

2) What is the exact site at which carotenoids bind on the actin molecule? What is the nature of the non-covalent carotenoid-actin interaction?

3) What is the stability of the actin-carotenoid complex? Are changes in this complex responsible for pigment fading during frozen storage? Could the stability be enhanced? How does frozen storage affect the structure of actin? Could this affect the stability of carotenoids during frozen storage?

4) More rigorous binding tests need to be carried out to determine actual binding kinetics as the data presented in this thesis is still preliminary in nature.

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Appendix A – Determining Extinction Coefficients for Ax and Cx

The extinction coefficient for Ax in water was determined as described in Section 3.6. A standard curve was prepared by dissolving 2 mg Ax in DMSO and making serial dilutions in DMSO. Five µL of each dilution was added to 100 mL of distilled deionized water. The solutions were vortexed and their absorbances read at 470 nm. An example of the dilutions and the standard curve obtained can be seen in Figure A.1.

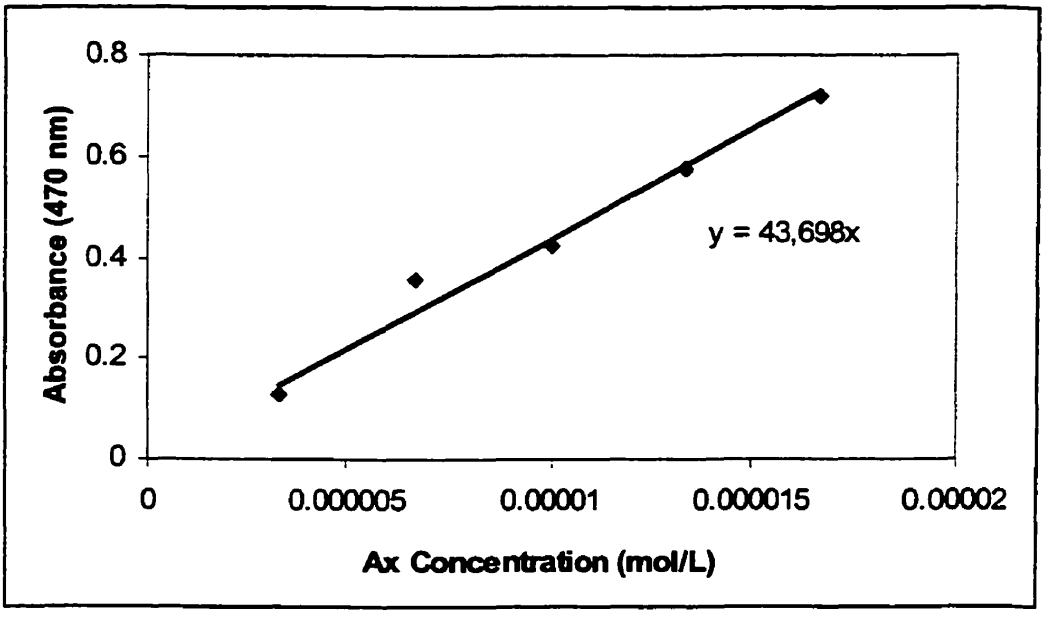

Figure A.1. Standard curve prepared to determine the extinction coefficient for Ax in water. Slope indicated on graph is the extinction coefficent for Ax at 470 nm in water.

For Cx, the extinction coefficient was determined at NRC. Details can be found in Section 3.6. The standard curve for Cx was obtained in the same manner, however THF was used to solubilize the carotenoid instead of DMSO.

Several important assumptions were made when using the carotenoid extinction coefficients. The first was that the use of DMSO versus THF would not drastically affect the value of the extinction coefficient. The second was that the value of the extinction coefficient would not change in sodium phosphate buffer compared to distilled deionized water. The third assumption was that the value of the extinction coefficient would not vary whether the carotenoid was free in solution or bound to protein.

Appendix B – Determining values from the binding assay

Absorbance values due solely to carotenoids in the binding assay were determined in the following manner. The sample and carotenoid solution were mixed together and the absorbance profile was taken. The mixture was applied to the Bio-Spin column and centrifuged, and then absorbance profile was taken again. A sample chromatogram before centrifugation can be seen in Figure B.1. As described in Section 3.6, absorbance values were corrected for protein absorbance by subtracting the absorbance value of the protein spectrum alone to give absorbance values attributable solely to the added carotenoids.

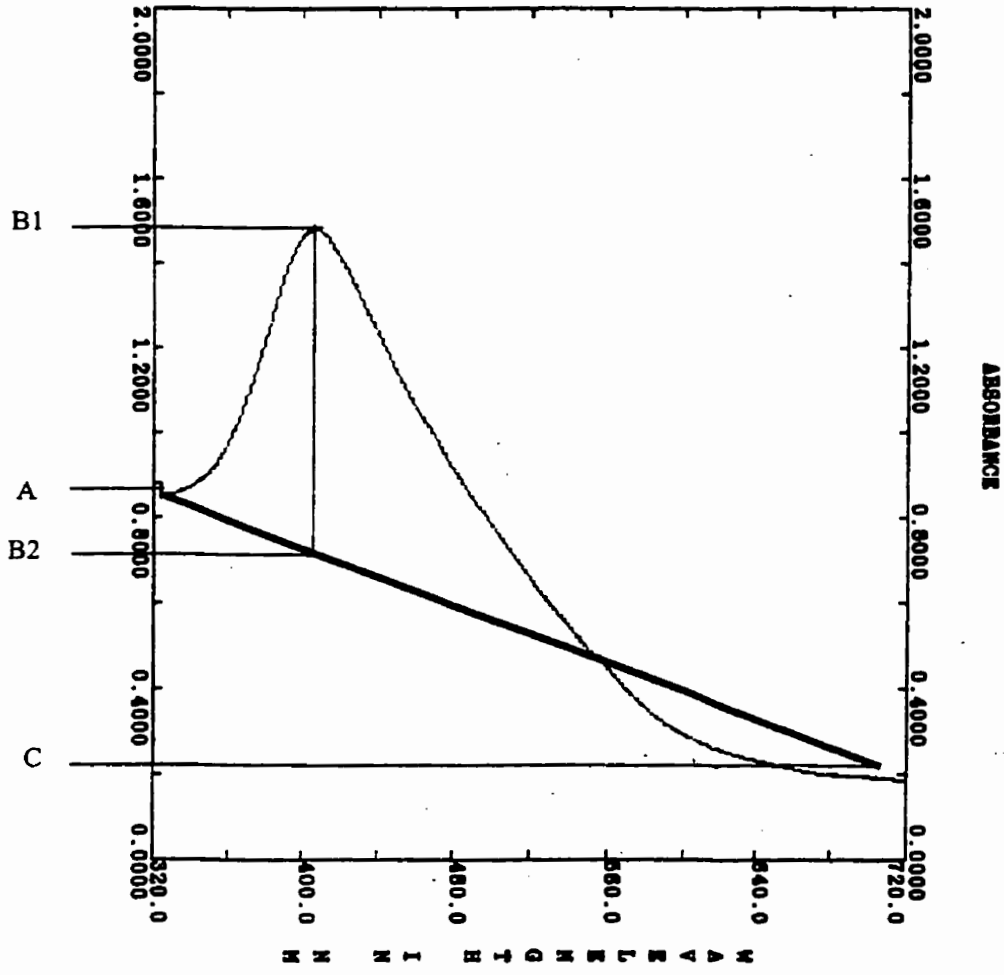

Figure B.1 Sample spectrophotometric wavelength scan of a mixture of myofibrils and Ax.

On Fig. B.1, the equation of the line drawn between points A and C was used to determine how much of the absorbance seen in the chromatogram was due to Ax. Extinction coefficients for Ax and Cx in water were determined as described in the text and in Appendix A. From these extinction coefficients, it was posssible to convert absorbance units to mole of Ax bound to the sample. An example of this calculation follows:

Sample Calculation for mature salmon actin and 2 μg Ax added:

Absorbance due to actin?

Absorbance (350 nm) = 0.10338 (Point A on Figure B.1)

Absorbance (680 nm) = 0.033127 (Point C on Figure B.1)

Equation of line between 350 and 680 nm: $y = -0.0002x + 0.1779$

Absorbance due to Ax?

Total Absorbance (470 nm) = 0.16131 (Point B1 on Figure B.1)

Actin Absorbance (470 nm) = 0.13890 (Point B2 on Figure B.1)

Absorbance due to Ax only = 0.16131 – 0.13890

Absorbance due to Ax only = 0.02241

How much Ax bound to actin?

$A = \varepsilon c l$ where ε was determined experimentally as described in Appendix A

$0.02241 = 43{,}700\ M^{-1}cm^{-1} * c * 1\ cm$

$c = 5.13\ E^{-7}\ M$

Therefore $5.13\ E^{-7}$ mol Ax bound per L of mature salmon actin.

Appendix C - Sample calculations for salmon actin

Parameters obtained by non-linear regression shown in Figures 4.5.2 and 4.5.3 give rise to the maximum number of binding sites on the F-actin molecule in nmoles carotenoid. This value was converted to mole carotenoid per mole F-actin through the following calculation:

$$\frac{\text{mole Ax/Cx}}{\text{mole F-actin}} = \text{nmole Ax/Cx} * \frac{1}{\mu\text{L F-actin}} * \frac{\mu\text{L}}{\mu\text{g F-actin}} * \text{MW Ax/Cx}$$

Moles of carotenoid per mole F-actin was converted to mg carotenoid per g F-actin through the following calculation:

$$\frac{\text{mg Ax/Cx}}{\text{g F-actin}} = \frac{\text{mole Ax/Cx}}{\text{mole F-actin}} * \frac{1}{\text{MW F-actin}} * \text{MW Ax/Cx} * \frac{1000\text{ mg}}{\text{g}}$$

Saturation values of carotenoids in salmon tissue were calculated in the following manner:

$$\frac{\text{mg Ax/Cx}}{\text{g flesh}} = \frac{\text{mg Ax/Cx}}{\text{g F-actin}} * \frac{14\%\text{ actin}}{\text{protein}} * \frac{17\%\text{ protein}}{\text{flesh}}$$

The following data was used for the above calculations:

E Ax = 43,700 $M^{-1}cm^{-1}$

E Cx = 57,400 $M^{-1}cm^{-1}$

MW Ax = 598.86 g/mol

MW Cx = 564.86 g/mol

MW F-actin = 1,136,000 g/mol