The Role of Muscle Proteins in the Retention of Carotenoid in Atlantic Salmon Flesh

by

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Dedicated to my daughter *Prama Roy*

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ABSTRACT

Dietary carotenoid (astaxanthin and canthaxanthin) is required for flesh pigmentation of Atlantic salmon and is an expensive component in commercial salmonid feeds. A better understanding of the interaction between astaxanthin and salmon muscle proteins is important to achieve better retention of carotenoid in salmon flesh. This study was undertaken to determine *in vitro* astaxanthin binding to the muscle proteins of both adult and juvenile Atlantic salmon (*Salmo salar*), haddock (*Melanogrammus aeglefinus*) and halibut (*Hippoglossus hippoglossus*) for comparison, as well as to investigate membrane bound astaxanthin transport protein(s) in adult salmon using proteomics approach. Several methods such as gel filtration chromatography, fluorometry, immobilization of actin for the binding assay and displacement of hydrophobic probe were examined to assess *in vitro* astaxanthin binding to salmon muscle actin. None of these methods were found suitable for the binding assays. Astaxanthin binding studies using the gel filtration chromatography or ultrafiltration method were limited by the aggregation of astaxanthin in experimental conditions. An ultrafiltration method was developed for astaxanthin-protein binding assay using 200 mM sodium cholate for dispersion of astaxanthin aggregates based on the 100% passage of dispersed astaxanthin (5.03 μM) through a 30,000 nominal molecular weight limit filter in the presence of 200 mM sodium cholate.

Astaxanthin binding to the salmon muscle actin or solubilized fractions of salmon muscle proteins was assessed using the ultrafiltration method. The results suggest that proteins other that actin or actomyosin can bind astaxanthin. As well, actin or solubilized fractions of muscle proteins did not show saturation in astaxanthin binding. The characteristics of astaxanthin binding to muscle proteins of salmon, haddock and halibut suggest that carotenoid binding to the muscle proteins do not limit the deposition of carotenoid in salmon flesh.

Investigation of membrane bound astaxanthin transport proteins in adult salmon using proteomics approach revealed that proteins were found to be up-regulated in salmon in response to dietary astaxanthin. Mass spectrometry analysis revealed that the up-regulated protein (24-25 kDa) from astaxanthin fed salmon showed extensive homology with the myelin protein zero. Further investigation is necessary to elucidate the transport mechanism of astaxanthin from the blood into the muscle.

LIST OF ABBREVIATIONS

ANS	= 8-anilino-1-naphthalenesulfonate
ATP	= adenosine triphosphate
CMC	= critical micelle concentration
DC	= detergent compatible
DMSO	= dimethyl sulfoxide
DTT	= dithiothreitol
EDTA	= ethylenediaminetetraacetic acid
EGTA	= ethyleneglycol-*bis*(β-aminoethyl)-N,N,N′, N′-tetraacetic acid
ELISA	= enzyme linked immunosorbent assay
GRASP	= Genome Research on Atlantic Salmon Project
HDL	= high density lipoproteins
HEPES	= N-(2-Hydroxyethyl)piperazine- N′-(2-ethanesulfonic acid)
HPLC	= high performance liquid chromatography
IPG	= immobilized pH gradient
kDa	= kilo Daltons
LDL	= low density lipoproteins
MW	= molecular weight
NaCh	= sodium cholate
NaTCh	= sodium taurocholate
NaTDCh	= sodium taurodeoxycholate
NMWL	= nominal molecular weight limit
S.D.	= standard deviation
SDS-PAGE	= sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TCA	= trichloroacetic acid
TMS	= tricaine methanesulfonate
VHDL	= very high density lipoproteins
Vhr	= volt hour
VLDL	= very low density lipoproteins
v/v	= volume/volume
w/v	= weight/volume
w/w	= weight/weight

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Chapter 1: Introduction

Flesh pigmentation of salmonids is generally regarded as the most important quality criterion next to product freshness because of consumers' preference for pigmented flesh. Pigmentation of salmonid flesh results from the absorption and deposition of carotenoids. Salmonids, like other vertebrates, are unable to synthesize carotenoid *de novo* and depend entirely on carotenoids in their feed. Astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione) is the predominant carotenoid in wild salmonids (Khare et al., 1973; Schiedt et al., 1981, 1986; Scalia et al., 1989) and is derived mainly from ingested zooplankton. Flesh pigmentation of farmed salmonids is achieved by dietary supplementation of either astaxanthin or canthaxanthin (β,β-carotene-4,4'-dione) usually in the range of 35-75 mg kg^{-1} diet. Dietary supplementation of astaxanthin accounts for approximately 15-20% of feed cost and 6-8% of total production cost for the salmon industry (Torrissen, 1995).

Retention of dietary carotenoids is poor in Atlantic salmon, less than 10% of supplemented carotenoids are deposited in the flesh. Retention of dietary carotenoids in the flesh of rainbow trout and Atlantic salmon reported by different investigators are summarized in Table 1.1. Retention of carotenoids in animals depends on four main interacting factors, 1) efficiency of absorption of carotenoids from the intestinal tract, 2) carotenoid transport capacity, 3) carotenoid deposition mechanisms of various tissues and 4) metabolism and excretion of carotenoids (Torrissen et al., 1989).

1.1 Carotenoid chemistry

Carotenoids, the isoprenoid polyenes, are formed by joining eight C_5-isoprene units in a regular head to tail manner except in the center of the molecule, where the order is tail to tail. Carotenoids are C_{40} hydrocarbons containing a long chain of conjugated double bonds and an ionone ring, mostly, at both ends. Structures of some selected carotenoids are shown in Fig. 1.1. The long conjugated double bonds, i.e. polyene system render carotenoids extremely unstable and susceptible to oxidative degradation

Table 1.1 Retention of carotenoids in salmonids

Species	Initial weight (g)	Final weight (g)	Carotenoid (mg kg^{-1} diet)	Pigment	Feeding period (days)	Retention (%)	References
Atlantic salmon	583-616	1073-1122	30	astaxanthin	113	6.0-7.4	Bjerkeng et al. (1999a)
Atlantic salmon	144	740	60	astaxanthin	112	3.9	Bjerkeng and Berge (2000)
Rainbow trout	135	235	12.5-200	astaxanthin canthaxanthin	42 42	2.8-12.5 2.3-7.0	Choubert and Storebakken (1989)
Atlantic salmon	67	104	25 56 78	astaxanthin astaxanthin astaxanthin	22 22 22	4.69 3.64 2.45	Wieruszewski (2000)

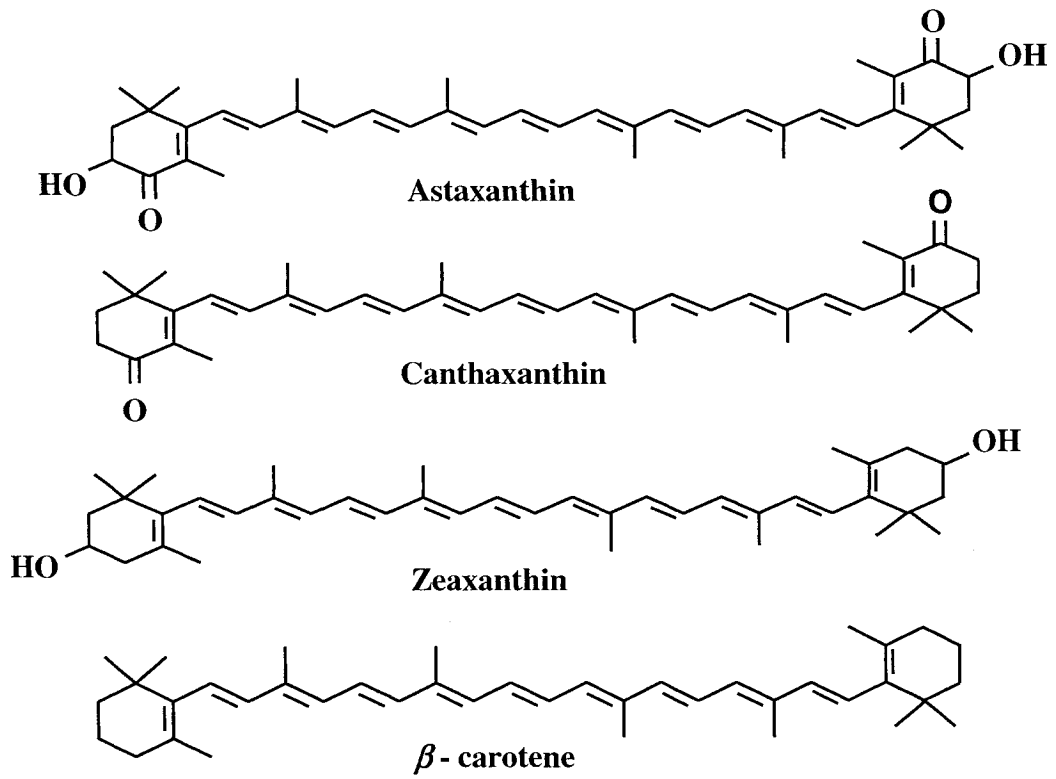

Fig. 1.1 Structures of some selected carotenoids.

and isomerization by light, heat, acids and bases (Briton, 1985; Weedon and Moss, 1995). Carotenoids are the most widespread pigments in nature, found throughout the plant and animal kingdoms. Despite their wide distribution, *de novo* synthesis of carotenoids is confined to certain microorganisms, fungi, algae and higher plants.

Geometrical (*E-/Z-* or *cis/trans*) and optical isomers (*R-* or *S-*) are commonly found among the carotenoids. In nature, ca. 80% of the astaxanthin is present as *trans* (*E*) and 20% as *cis* (*Z*) isomers (Schiedt et al., 1981). Structures of some geometrical isomers of astaxanthin are shown in Fig. 1.2. Synthetic astaxanthin Carophyll pink

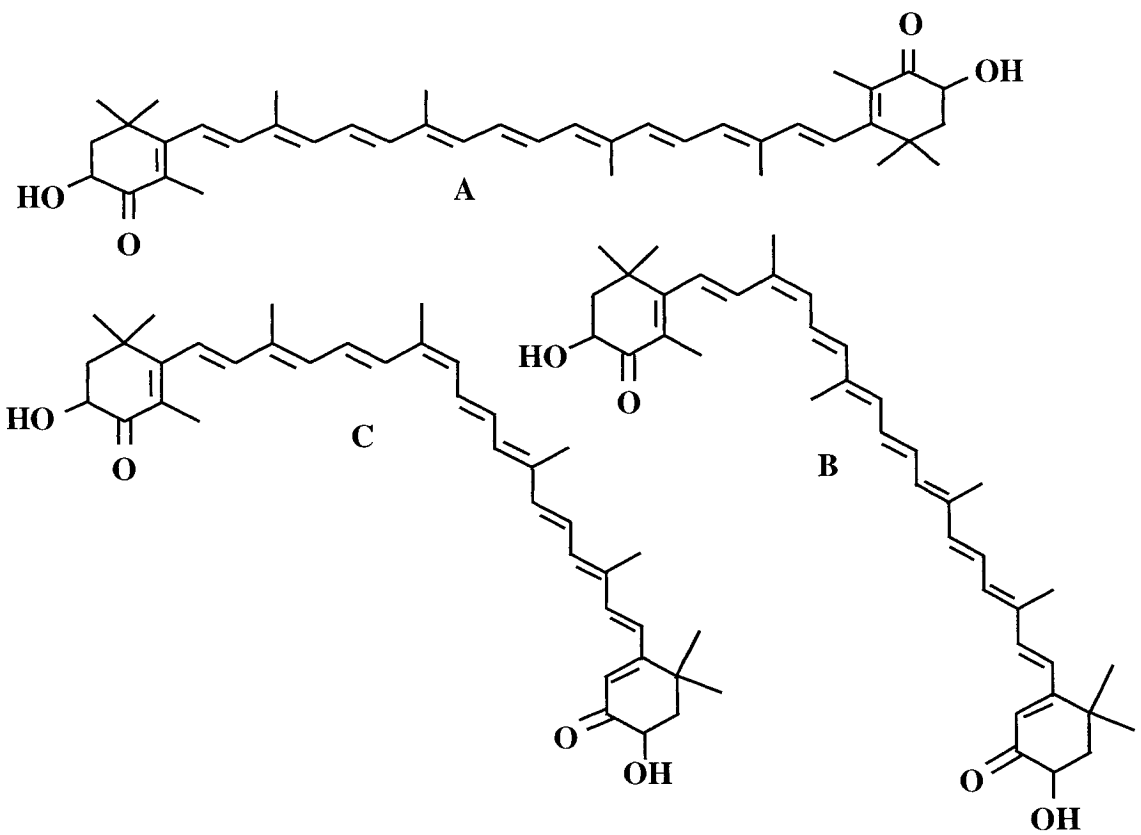

Fig. 1.2 Structures of all-*E*-astaxanthin (A), 9*Z*-astaxanthin (B) and 13*Z*-astaxanthin(C) [adapted from Bjerkeng et al., 1997].

(F. Hoffmann-La Roche Ltd., Basel, Switzerland) contains 75% *E* and 25% *Z* isomers of astaxanthin (Bjerkeng et al., 1997). Astaxanthin has two identical chiral carbon atoms and exists in three optical configurations: two enantiomeric forms (3*S*, 3′*S* and 3*R*, 3′*R*) and one meso form (3*R*, 3′*S*). The (3*S*, 3′*S*)-astaxanthin is predominately produced by algae (Renstrøm et al., 1981) which accounts for the highest accumulation of (3*S*, 3′*S*)-astaxanthin in the flesh of wild salmonids (Schiedt et al., 1981). Synthetic astaxanthin is a racemic mixture of (3*R*, 3′*R*), (3*R*, 3′*S*) and (3*S*, 3′*S*) with a ratio of 1:2:1.

1.2 Carotenoid absorption

Absorption of dietary carotenoid in salmonids is usually measured by plasma or serum carotenoid concentration or by the apparent digestibility of dietary carotenoid. Serum astaxanthin concentration was higher when rainbow trout were fed diets containing higher levels of lipid (Barbosa et al., 1999). The absence of dietary fat or very low levels of fat in the diet substantially reduces absorption of carotenoid in humans (Erdman, 1988; Erdman et al., 1993; Prince and Frisoli, 1993). Dietary fat stimulates the bile flow from the gall bladder that facilitates emulsification of fat and carotenoid into lipid micelles within the small intestine and hence increases intestinal carotenoid absorption (Erdman et al., 1993). Recent investigation by Olsen et al. (2005) showed that dietary supplementation of taurocholic acid did not significantly increase blood astaxanthin concentration compared to the control diet in Atlantic salmon.

The apparent digestibility coefficient of carotenoid in rainbow trout increased with increasing dietary lipid level (Torrissen et al., 1990; Choubert et al., 1991). Torrissen et al. (1990) fed rainbow trout with diets containing different levels of canthaxanthin and reported that the apparent digestibility was decreased with increased dietary levels of canthaxanthin. Decreased apparent digestibility of astaxanthin with increased dietary concentration of astaxanthin was also reported for Atlantic salmon (Wieruszewski, 2000). It is likely that micellar solubilization of carotenoids decrease with increasing concentration of carotenoids and hence decreases the apparent digestibility of carotenoid.

The digestion and rate of absorption depend on the raw materials of the pigment source (Johnson et al., 1980; Torrissen et al., 1981). Efficiency of carotenoid absorption also varies with the type and form (free or esterified) of dietary carotenoid, dietary level of carotenoid and fish species, which are summarized in Table 1.2. Influences of genetic factors on carotenoid deposition in the flesh of salmonids reported by Torrissen and Naevdal (1984, 1988) suggest that genetic variation may influence intestinal absorption of carotenoid.

Absorption of ingested carotenoids by passive diffusion across the intestinal membrane has been reported for rainbow trout (Choubert et al., 1994a), rat (Hollander

Table 1.2 Plasma or serum carotenoid levels in salmonids

Species	Carotenoid (mg kg^{-1} diet)	Carotenoid	Fish weight (g)	Serum carotenoid conc. (μg mL^{-1})	References
Rainbow trout	89	astaxanthin	400	8.40	Choubert et al. (1994a)
	116	canthaxanthin		3.58	
Atlantic salmon	50	astaxanthin	1376	0.45[a]	Kiessling et al. (1995)
	75	astaxanthin		0.68[a]	
Atlantic salmon	40-100	astaxanthin	749-814	2.84-3.65[b]	March and MacMillan (1996)
Chinook salmon	40-100	astaxanthin	404-483	3.27-5.86[b]	
Rainbow trout	40-100	astaxanthin	564-590	5.78-8.70[b]	
Atlantic salmon[c]	5-63	astaxanthin	2000-2500	1.0-2.9[b]	Storebakken and Goswami (1996)
Atlantic salmon[d]	30	astaxanthin	841	1.84 - 2.21[b]	Bjerkeng et al. (1999a)
Atlantic salmon	30	astaxanthin[e]	1362	2.9	Bjerkeng et al. (1999b)
	50	astaxanthin[e]	1248	2.8	
Rainbow trout	50	astaxanthin	217	2.0	White et al. (2002)
	50	astaxanthin[f]		1.3	
Rainbow trout	30	astaxanthin	141	1.61	White et al. (2003b)
		astaxanthin[g]		1.54	
		astaxanthin[h]		0.99	
Atlantic salmon	60	astaxanthin	800-1600	1.20[a]	Kiessling et al. (2003)
		canthaxanthin		1.85[a]	

[a] blood astaxanthin concentration (μg mL^{-1})
[b] plasma astaxanthin concentration
[c] both previously unpigmented and pigmented salmon
[d] fed on diets with different dietary fish oil (herring, capelin, sandeel and Peruvian high PUFA oils) along with supplemented astaxanthin (30 mg kg^{-1} diet)
[e] astaxanthin with 400 mg kg^{-1} α-tocopheryl acetate
[f] esterified astaxanthin (from *Haematococcus pluvialis*)
[g] astaxanthin monoester isolated from *Haematococcus pluvialis*
[h] astaxanthin diester isolated from *H. pluvialis*

and Ruble, 1978) and mammals (Parker, 1996). Choubert et al. (1994a) reported that the concentration of carotenoid in the serum of immature rainbow trout increased with the increasing concentration of dietary carotenoid (12.5-200 mg kg^{-1}). A high correlation ($r = 0.995$) was reported between dietary astaxanthin concentrations (0-75 mg kg^{-1}) and whole blood astaxanthin concentration in Atlantic salmon (Kiessling et al., 1995). Storebakken and Goswami (1996) also reported a correlation ($r = 0.90$) between dietary astaxanthin concentrations and plasma astaxanthin concentration. Despite this correlation, the plasma or blood carotenoid levels indicate poor absorption of dietary carotenoid. Poor absorption of dietary carotenoid is one of the hindrances to optimal flesh pigmentation in salmonids.

It appears that routes of administration influence the bioavailability of carotenoid. Maltby (née Wieruszewski) et al. (2003) reported that the plasma astaxanthin concentration of Atlantic salmon was higher when astaxanthin was administered via intraperitonial injection compared to oral administration. This finding clearly indicates that the gastrointestinal wall is an effective barrier for carotenoid absorption.

1.3 Transport of carotenoid

In blood, lipoprotein fractions are associated with the transport of carotenoids. Astaxanthin is found to be associated with different fractions of lipoproteins depending on the physiological state of the fish. In the serum of upstream migrating chum salmon, astaxanthin was found to be exclusively transported by the high density lipoprotein (HDL) fraction (Nakamura et al., 1985) or by HDL and very high density lipoprotein, VHDL (Ando et al., 1986a). In immature rainbow trout, canthaxanthin or astaxanthin was associated with all of the lipoprotein fractions e.g. very low density lipoprotein (VLDL), LDL, HDL and VHDL (Choubert et al., 1992, 1994b). The amounts of canthaxanthin associated with serum lipoprotein fractions of VLDL, LDL and HDL were found to be 33, 49 and 18%, respectively in canthaxanthin fed immature rainbow trout (Chavez et al., 1998). It has been reported that juvenile trout contain mostly VLDL and LDL (Frémont and Marion, 1982) whereas adult trout have high levels of HDL in their serum (Chapman et al., 1978). These observations may explain the reported differences

in association of carotenoid with different lipoprotein fractions between immature and spawning migrating fish.

Chavez et al. (1998) reported that *in vitro* saturation of rainbow trout lipoprotein with canthaxanthin was proportional to the amount of amphiphilic components of the surface and lipoprotein does not have an affinity for carotenoid, but has an ability to bind the pigment, as a function of its phospholipid content. Small bathochromic shifts in the absorption spectrum of lipoprotein-bound astaxanthin compared to that of astaxanthin extracted from lipoprotein were reported by Choubert et al. (1994b) and Nakamura et al. (1985). This observation suggests that carotenoids bind non-specifically to the lipoproteins. It has been reported that carotenoids are associated with plasma lipoproteins but apparently not associated with specific carotenoid-binding proteins of plasma (Olson, 1994; Parker, 1996). It is well known that some carotenoids can be pro-vitamin A as they are metabolized to yield retinol. Several studies conducted with mammals showed that retinol (vitamin A) is transported in blood by a specific protein, retinol-binding protein (Kanai et al., 1968; Peterson, 1971; Muto and Goodman, 1972; Muto et al., 1973). Retinol is bound to specific cellular retinol-binding proteins (CRBP and CRBP II) within the cells (Chytil and Ong, 1987; Wolf, 1991).

The findings of Ando et al. (1986b) suggested that vitellogenin, a female-specific serum protein, might participate in transporting carotenoids from the muscle to the ovaries in mature chum salmon. The mechanism of transfer of carotenoids from muscle to the skin and gonad via the serum is not known yet.

Two apolipoproteins, molecular weights of 24,000 and 12,000, have been isolated from the carotenoid-carrying lipoprotein of the serum HDL fraction of upstream migrating chum salmon on sodium dodecyl sulphate-polyacrylamide gel electrophoresis, SDS-PAGE (Ando et al., 1986a; Ando and Hatano, 1988a). The identity of bilirubin-binding protein in the HDL fraction of the serum of spent males with the carotenoid-carrying lipoprotein of HDL fraction has been reported by Ando and Hatano (1988b). It has been reported that a major proportion of astaxanthin in the plasma of Atlantic salmon is associated with a protein, which is tentatively identified as albumin by Native-PAGE (Aas et al., 1999). These researchers found that the protein associated with the highest level of radioactive astaxanthin in the HDL fraction obtained from the iodixanol-gradient

showed the similar migration length to bovine serum albumin (BSA) in Native-PAGE. Though Aas et al. (1999) performed the SDS-PAGE of the same HDL fraction from the iodixanol-gradient, however these researchers only reported about the information of the autoradiography, not about the molecular weight of astaxanthin associated protein in SDS-PAGE. As a result, comparison of the SDS-PAGE findings of Aas et al. (1999) with Ando et al. (1986a) or Ando and Hatano (1988a, 1988b) is not possible. In addition, so far no further information regarding the affinity of the above reported protein for carotenoid has been published yet. The *in vitro* binding study between carotenoid and the reported protein would make clear whether carotenoid is transported by a specific protein in the blood. Considering the findings of Ando et al. (1986a, 1986b), Ando and Hatano (1988a, 1988b), Chavez et al. (1998) and Aas et al. (1999) all together, it indicates that the interaction between carotenoid and protein in the blood is non-specific.

1.4 Deposition of carotenoid

Salmonids deposit a major proportion of ingested carotenoid in their flesh. Carotenoids are deposited in the flesh in the free form whereas in the skin, carotenoids are present predominantly as esters. Astaxanthin is found to be accumulated more efficiently than canthaxanthin in the flesh of rainbow trout (Foss et al., 1984, 1987; Torrissen, 1986; Choubert and Storebakken, 1989). Recently, *in vitro* studies using isolated perfused liver of rainbow trout, Page and Davies (2003) found that the liver uptake of astaxanthin showed saturation earlier than that of canthaxanthin. These findings may partly explain the better utilization of astaxanthin than canthaxanthin in rainbow trout. However, Buttle et al. (2001) and Baker et al. (2002) reported higher accumulation of canthaxanthin in the flesh of Atlantic salmon compared to astaxanthin. Blood canthaxanthin level was also reported to be higher than astaxanthin in Atlantic salmon at the same dietary inclusion level (Kiessling et al., 2003). In Atlantic salmon, astaxanthin may have metabolically transformed to idoxanthin, which was not detected in these studies and may cause lower absorption and accumulation of astaxanthin than canthaxanthin. The evidence of rapid reduction of astaxanthin to idoxanthin has been reported in Atlantic salmon (Aas et al., 1999).

Deposition of astaxanthin was low from astaxanthin dipalmitate by comparing synthetic astaxanthin dipalmitate and free astaxanthin as pigment sources for rainbow trout (Schiedt et al., 1985; Foss et al., 1987) and Atlantic salmon (Storebakken et al., 1987). A similar observation was reported for rainbow trout fed astaxanthin mono- and diesters and free astaxanthin isolated from copepod, *Calanus finmarchicus* (Torrissen and Braekkan, 1979). This differential deposition might be due to the limited rate of hydrolysis of astaxanthin esters to free astaxanthin in the intestine of salmonids (Torrissen et al., 1989).

1.4.1 Fish size

Small salmonids cannot efficiently accumulate carotenoids in their flesh; they accumulate carotenoids mainly in their skin. The ability of small salmonids to deposit carotenoid efficiently in their flesh differs among species. Rainbow trout weighing less than 120 g deposited small amounts of carotenoids (< 3 mg kg^{-1}) in the flesh, while depositing carotenoid preferably in the skin (Bjerkeng et al., 1992). No carotenoids were detected in the muscle of Atlantic salmon (~1.5 years old) weighing 80 g after feeding 8 weeks with diets containing different levels of carotenoids (Storebakken et al., 1987). However, a minor deposition of carotenoid in the flesh (0.6 mg kg^{-1}, mean values of three dietary concentrations) was observed at the next sampling following 16 weeks of feeding when salmon reached a body weight about 120 g. At the end of this experiment (after 56 weeks), salmon weighing about 406 g deposited 2.0 mg kg^{-1} carotenoid in their flesh (Storebakken et al., 1987). After feeding three year classes of Arctic charr (*Salvenius alpinus*) with 63 mg astaxanthin kg^{-1} diet for a period of 13 weeks, 1+ year class fish weighing about 80 g deposited 2.3 mg kg^{-1} carotenoid in their flesh (Hatlen et al., 1995). In comparison, 2+ and 3+ year classes Artic charr deposited 3.8 and 6.6 mg kg^{-1} carotenoid in their flesh, respectively. Artic charr of 1+ year class were less efficient in utilizing dietary astaxanthin for flesh pigmentation than the older fish (Hatlen et al., 1995).

Recently, Ytrestøyl et al. (2004) reported that 0+ Atlantic salmon smolts contained more carotenoids than 1+ smolts after correction for mass differences ($P<0.0001$) when salmon were transferred to seawater as 0+ and 1+ smolts fed a diet

supplemented with 30 mg kg^{-1} astaxanthin and 30 mg kg^{-1} canthaxanthin for 35 weeks. However, the biochemical and physiological basis of these differences is unknown and need to be elucidated.

Accumulation of carotenoids in the skin of small salmonids demonstrates their ability to absorb dietary carotenoids hence muscle deposition is limited by factors other than absorption. Small salmonids weighing between 21-40 g efficiently absorbed carotenoid from their diet; the plasma carotenoid concentration was reported to be 3.6-4.5 μg mL^{-1} (March et al., 1990). The reported plasma carotenoid concentration of small salmonids is higher than that of Atlantic salmon (2.9 μg mL^{-1}) weighing 2-2.5 kg reported by Storebakken and Goswami (1996).

1.4.2 Sexual maturation

During maturation, salmonids redistribute carotenoids from their flesh to the skin and ovaries (Crozier, 1970; Sivtseva and Dubrovin, 1981; Kitahara, 1983) and this redistribution results in depletion of flesh carotenoid concentration. Kitahara (1983) reported a decrease in flesh carotenoid concentration of chum salmon from 6.3 mg kg^{-1} in immature female fish to 0.36 mg kg^{-1} at spawning time. The flesh carotenoid concentration in mature rainbow trout (both male and female) fed with diet containing canthaxanthin was 3.9-6 mg kg^{-1} whereas it was 20.2 mg kg^{-1} in immature fish (Bjerkeng et al., 1992). During sexual maturation, female salmonids mobilize flesh carotenoids to skin and ovaries whereas male deposit carotenoids mainly in their skin. Skin carotenoid concentrations were higher both in male and female mature rainbow trout compared to immature trout. Gonad (ovaries) carotenoid concentration was higher in mature female fish than mature male fish (Bjerkeng et al., 1992).

1.4.3 Genetic factors

Genetic variation in flesh carotenoid concentrations has been reported for different species (Torrissen and Naevdal, 1984, 1988; McCallum et al., 1987), although, Choubert and Blanc (1985) observed that immature triploid and diploid rainbow trout exhibited no significant difference in flesh coloration after feeding the same diet supplemented with canthaxanthin. Based on flesh pigmentation, Chinook salmon

(*Oncorhynchus tshawytscha*) are classified to two groups: (1) red-fleshed and (2) white-fleshed types. These color types are under strong genetic control (Withler, 1986). The white-fleshed ones do not deposit carotenoids in their flesh. After administering a single oral dose of astaxanthin to different genotypes of Chinook salmon, however, March et al. (1990) found no significant differences in the plasma astaxanthin concentrations among the different groups and concluded that the failure of certain genotypes to pigment their flesh could not be attributed to their inability to absorb astaxanthin from the digestive tract.

1.5 Metabolism of carotenoid

In fish, carotenoid can be metabolized by enzymes which catalyze three types of reactions: (a) substitution of carotenoid end group (oxidative and reductive pathways), (b) cleavage of the polyene chain of carotenoid to yield retinol and even the apocarotenoids, (c) alteration of the ring structure of the end group (Davies, 1985). In oxidative pathways, β-carotene and other carotenoids are converted to astaxanthin. Oxidative metabolic pathways have been reported for goldfish (Hata and Hata, 1972), fancy red carp (Hata and Hata, 1976), prawn (Katayama et al., 1972), lobster and crab (Katayama et al., 1973a, 1973b). In salmonids, except for the assumption of Guillou et al. (1992), reductive metabolic pathways are well documented (Schiedt et al., 1985, 1988a, 1988b; Ando et al., 1989; Metusalach et al., 1996; Aas et al., 1997). Guillou et al. (1992) reported that salmonids probably possess oxidative pathways of carotenoid based on the detection of ^{3}H-astaxanthin in the liver of all rainbow trout after force feeding one meal containing ^{14}C-astaxanthin and ^{3}H-canthaxanthin or ^{3}H-zeaxanthin which indicated that ^{3}H-canthaxanthin and ^{3}H-zeaxanthin were precursors of astaxanthin.

In salmonids, the intestinal wall seems to be a major site for bioconversion of carotenoids into vitamin A (Schiedt et al., 1985; Al-Khalifa and Simpson, 1988; White et al., 2003a). Vitamin A-depleted rainbow trout converted astaxanthin and canthaxanthin into vitamin A and this conversion was not observed in rainbow trout fed vitamin A supplemented diet (Schiedt et al., 1985). However, *in vitro* studies using everted intestine of rainbow trout showed that astaxanthin was transformed to retinol (Al-Khalifa and Simpson, 1988; White et al., 2003a). The metabolic transformation of different

carotenoids into vitamin A has also been reported for freshwater fish (Gross and Budowski, 1966; Barua and Goswami, 1977). In mammals, β-carotene is mainly converted by the enzyme β-carotene-15,15'-dioxygenase into retinal (Lakshman et al., 1972; During and Harrison, 2004). It appears that the conversion mechanism of astaxanthin and canthaxanthin into vitamin A involves the reductive metabolic transformation of these carotenoids into the intermediate β-carotene and then enzymatic cleavage.

A low level of carotenoid has been detected in the liver of salmonids (Sivtseva, 1982; Schiedt et al., 1988b). There have been conflicting findings on the possible role of the liver in the metabolism of carotenoids in salmonids. Hardy et al. (1990) reported that large amounts of canthaxanthin metabolic derivatives were excreted in the bile of rainbow trout. In contrast, Schiedt et al. (1985) reported the highest concentration of carotenoid in the bile (20-40 $\mu g\ g^{-1}$), which was exclusively free astaxanthin. The discrepancy between these two findings could be due to individual variation or different strains of the species. Later, Schiedt et al. (1988b) detected astaxanthin as well as its metabolites in the liver of Atlantic salmon fed a diet containing astaxanthin. The reductive metabolites of canthaxanthin have also been found in the liver of canthaxanthin-fed Arctic charr (Metusalach et al., 1996).

High levels of radioactivity have been observed in the bile of rainbow trout and Atlantic salmon after ingestion of labeled astaxanthin and canthaxanthin (Schiedt et al., 1985; Hardy et al., 1990; Torrissen and Ingebrigtsen, 1992). These observations have led the investigators to conclude that bile may be the major excretory route of carotenoids in salmonids. The radioactivity in the posterior kidney of Atlantic salmon suggested urinary excretion of astaxanthin metabolites in Atlantic salmon (Torrissen and Ingebrigtsen, 1992). However, *in vitro* metabolism studies of Atlantic salmon showed that xenobiotic-metabolizing enzymes of liver homogenate, phase I enzymes (CYP-450 enzymes) and phase II enzymes (UDPGA-glucuronosyl transferase, sulfotransferase and acetyltransferase) could not metabolize astaxanthin (Ding et al., unpublished data). The hepatic cytochrome P4501A enzyme system in rainbow trout was not found to be induced after exposure to astaxanthin both *in vivo* and *in vitro* (Amcoff et al., 1998). Similarly, a recent study by Page and Davies (2002) showed no inductive effect of either

astaxanthin or canthaxanthin on liver or kidney xenobiotic-metabolizing enzyme, cytochrome P450 monooxygenase (phase I) and conjugating enzyme (phase II) activities in rainbow trout. However, dietary supplementation of astaxanthin or canthaxanthin increased liver xenobiotic-metabolizing enzyme activities in rats and mice (Astorg et al., 1994, 1997; Gradelet et al., 1996; Jewell and O'Brien, 1999). The lack of inductive effect of astaxanthin or canthaxanthin on liver xenobiotic-metabolizing enzyme activities in salmonids is likely to be a species-specific pattern.

Metabolites of carotenoids are detected in various tissues of salmonids. Canthaxanthin metabolite, 4′-hydroxyechinenone, has been isolated from the muscle of Atlantic salmon (Ytrestøyl et al., 2004). Idoxanthin (3,3′,4′-trihydroxy -β,β-carotene-4-one) is found to accumulate in various tissues of Atlantic salmon (Schiedt et al., 1988a, 1988b; Bjerkeng and Berge, 2000) and Arctic charr (Bjerkeng et al., 2000) fed astaxanthin supplemented diet. Ando et al. (1989) also detected a considerable amount of idoxanthin in the muscles and ovaries of genus *Salvelinus*. However, trace amounts of idoxanthin have been detected in the flesh of rainbow trout (Bjerkeng et al., 1997). Schiedt et al. (1985) proposed that astaxanthin is reduced to zeaxanthin (β,β-carotene-3,3′-diol) via β-adinoxanthin (3,3′-dihydroxy-β,β-carotene-4-one) in rainbow trout.

1.6 Carotenoid-protein binding

Carotenoid is associated with proteins in the various tissues of animals. Carotenoid is associated with protein in the carapace of some crustaceans and lipo(glyco) protein in the ovaries, eggs and blood of invertebrates (Cheesman et al., 1967; Zagalsky et al., 1990). The association between carotenoid and carapace protein showed a stoichiometric relationship while carotenoid was associated non-stoichiometrically with lipo(glyco)protein. The blue coloration of the carapace of the lobster, *Homarus gammarus* (L.) is provided by the astaxanthin binding protein, α-crustacyanin (absorption maximum 632 nm). A large bathochromic spectral shift (160 nm compared with astaxanthin absorption spectrum in hexane) of α-crustacyanin is an indication of the specific interaction between astaxanthin and apocrustacyanin (Zagalsky et al., 1995).

Rao et al. (1997) partially characterized a specific cellular carotenoid-binding protein of 67 kDa from ferret liver with a high specificity for β-carotene. The protein-

carotene complex showed a 32 nm bathochromic shift in its absorption maximum compared with that of β-carotene in light petroleum. These researchers reported that purified cellular carotenoid-binding protein bound β-carotene mole per mole at the high affinity site whereas 145 mole of this protein bound 1 mole of β-carotene at the low affinity sites.

In fish, carotenoids are associated with muscle protein in salmonid flesh. Henmi et al. (1987) suggested that carotenoids are bound to actomyosin of salmon muscle by a weak hydrophobic interaction. It has been proposed that one β-ionone ring of the carotenoid binds to the hydrophobic binding site on actomyosin of salmon muscle (Fig. 1.3; Henmi et al., 1989). It was proposed that the hydroxyl group at C3 position and the carbonyl group at C4 position in one β-ionone ring of astaxanthin form two hydrogen bonds with a side chain of protein whereas canthaxanthin forms one hydrogen bond using the carbonyl group at C4 (Henmi et al., 1989). Thus, these researchers concluded that astaxanthin binds more strongly to actomyosin than canthaxanthin or other carotenoids. The model of astaxanthin-actomyosin complex (Fig. 1.3) proposed by Henmi et al. (1989) indicates that the primary interaction between β-ionone ring and actomyosin is hydrophobic which takes place initially. Then, formation of hydrogen bonds, which is a hydrophilic interaction, strengthens the initial hydrophobic interaction.

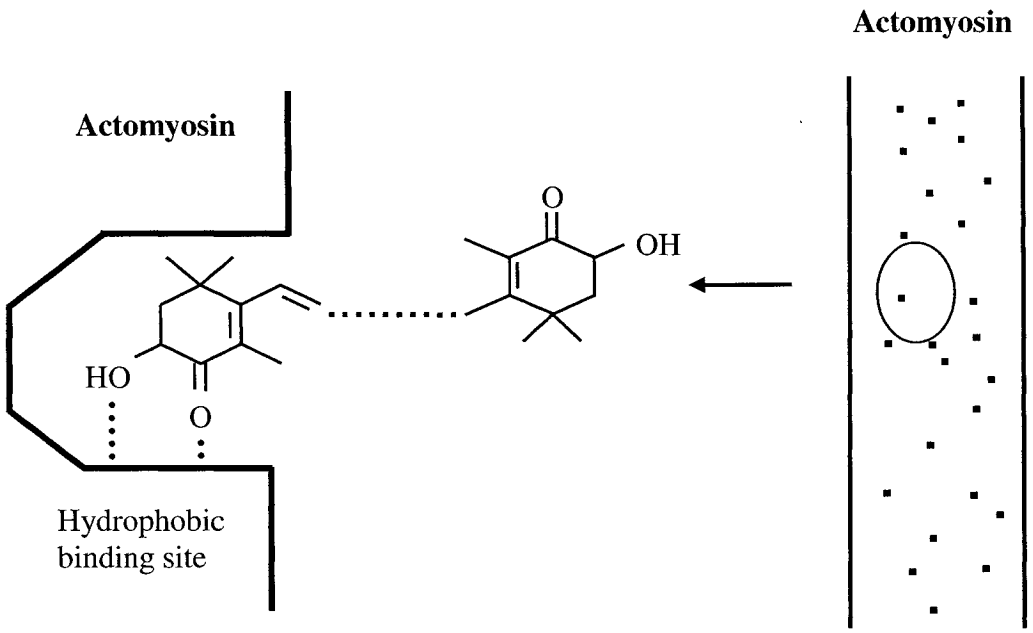

Fig. 1.3 Proposed astaxanthin-actomyosin complex model (adapted from Henmi et al., 1989).

Henmi et al. (1989) combined astaxanthin with salmon actomyosin and reported that bound astaxanthin was 0.70-0.86 μg mg^{-1} actomyosin. Henmi et al. (1990a) observed a correlation between the amounts of carotenoids bound to actomyosin of different fish and the surface hydrophobicity of actomyosin and presumed that carotenoids bind non-specifically to the hydrophobic binding sites on actomyosin. In Henmi's studies, only actomyosin was used for the binding studies. However, their

works do not account for astaxanthin binding to other muscle proteins, some of which may have a higher affinity for astaxanthin.

Recently, Côté (2000) characterized actin as a major carotenoid-binding protein in Atlantic salmon flesh and reported that purified actin of juvenile Atlantic salmon showed less affinity for carotenoid than actin purified from the muscle of mature salmon. The researcher used a gel filtration chromatography method to separate actin-bound carotenoid (astaxanthin or canthaxanthin) from free carotenoid.

The level of flesh pigmentation is recognized as a major factor affecting consumers' acceptance as well as the price of farmed salmonids. Flesh carotenoid concentration of salmonids reflects the absorption of dietary carotenoid, metabolism and binding of carotenoid (astaxanthin or canthaxanthin) to the muscle protein(s). It is apparent that slow and poor intestinal uptake of dietary carotenoids and metabolism of carotenoids in different tissues is responsible for poor deposition and retention of carotenoid in salmonid flesh. Carotenoid binding characteristics of the muscle proteins of salmonids may also be responsible for poor retention of dietary carotenoid in the flesh. Although significant research efforts have been focused on the flesh deposition of dietary carotenoids (astaxanthin and canthaxanthin) in various species of salmonids, very little information is available on the carotenoid binding ability of the muscle protein(s) of salmonids. To improve flesh pigmentation and to reduce the high cost of astaxanthin and canthaxanthin supplementation in commercial salmonid feeds, it is important to understand the characteristics of astaxanthin binding to salmon muscle protein(s) and their role in the deposition and retention of astaxanthin in salmonid flesh. It is also important to examine whether muscle membrane protein(s) play(s) a role in transporting astaxanthin from the blood into the muscle.

1.7 Objectives

This thesis research focused on the development of a reliable binding assay to quantify bound astaxanthin of salmon muscle protein(s), comparison of *in vitro* astaxanthin binding to the muscle protein(s) of salmon (juvenile and adult) and white-fleshed fish to examine the role of muscle protein(s) in flesh deposition of astaxanthin

and to identify muscle membrane protein(s) that may be involved in transporting astaxanthin from blood to muscle. The specific objectives of this project were:

1. To develop a method for an astaxanthin-protein binding assay to determine binding of astaxanthin to salmon muscle protein(s).
2. To compare the *in vitro* astaxanthin binding ability of juvenile and adult Atlantic salmon and white-fleshed fish species, haddock and halibut.
3. To identify astaxanthin transport/receptor protein(s) in muscle membranes using a proteomics approach.

CHAPTER 2

Screening of different binding assays to assess interaction between astaxanthin and salmon muscle actin

2.1 Abstract

Several methods were examined to identify a suitable method to characterize the binding between actin and astaxanthin. The methods included gel filtration chromatography, fluorometry, binding assay using immobilized actin and displacement of a hydrophobic probe. With gel filtration chromatography, aggregation of astaxanthin under the experimental conditions was a major problem for the separation of bound astaxanthin from free astaxanthin since the apparent molecular weight of aggregated astaxanthin or astaxanthin micelles was in the same range as protein-astaxanthin complexes. The fluorometry method was not successful for the characterization of binding between actin and astaxanthin because the changes in intrinsic fluorescence of actin were caused by the addition of solvent while adding astaxanthin solution (prepared in solvent) to actin. An inadequate amount of actin was found to be immobilized on the enzyme-linked immunosorbent assay (ELISA) plate after 72 hr incubation period. In the binding study using actin immobilized on Sepharose 4B beads, adsorption of astaxanthin by the beads due to hydrophobic interactions and inconsistent volume of beads during pipetting of slurry were the major problems for application of this approach. Displacement of the hydrophobic fluorescent probe 8-anilino-1-naphthalenesulfonate (ANS) was not effective because astaxanthin quenched the fluorophore preventing detection of displacement. The above methods are not suitable for use in astaxanthin-actin binding assays.

2.2 Introduction

Salmonids with brightly pigmented flesh always have consumer preference and high market price. Carotenoids are supplemented in the diet of farmed salmonids to achieve their flesh pigmentation. Pigmentation of the flesh of salmonids results from the

accumulation of dietary carotenoids in their muscle tissue. It has been reported that carotenoids bind to myofibrillar protein actomyosin in salmon muscle (Henmi et al., 1987, 1989).

Henmi et al. (1989) combined carotenoid with salmon actomyosin and measured the actomyosin-bound carotenoid. However, quantitative determination of protein-bound astaxanthin by the procedure of Henmi et al. (1989) is difficult since the actomyosin used in their study was not soluble and astaxanthin may have bound to exposed surfaces of insoluble aggregates. As well, astaxanthin may be expected to dissociate from protein during washing since the interaction between actomyosin and astaxanthin is reportedly weak (Henmi et al., 1987, 1989, 1990b). Using a gel filtration chromatography method for the separation of protein-bound carotenoid from free carotenoid (astaxanthin and canthaxanthin), Côté (2000) characterized actin as a major carotenoid-binding protein in Atlantic salmon flesh. This researcher also reported that purified actin of juvenile and adult salmon have different binding affinities for carotenoid. Thus the method of Côté (2000) was examined for the astaxanthin-actin binding assay in the present study. However, a major problem was encountered in molecular mass-based separation of actin-bound astaxanthin from free astaxanthin by gel filtration chromatography method as astaxanthin formed aggregates in aqueous solution due to its hydrophobic characteristics.

To avoid the problem in separating protein-bound astaxanthin from free astaxanthin, emphasis was given in the present study to several other approaches including those that do not involve the separation of bound and unbound ligand for characterization the binding between ligand and macromolecule. The second approach involved the examination of the intrinsic fluorescence of actin in response to astaxanthin. In this approach, the fluorescence of actin was expected to change upon binding to astaxanthin. The binding affinity of retinol to retinol-binding protein has been determined by an intrinsic fluorometric method in several studies (Cogan et al., 1976, Ong and Chytil, 1978). The third approach involved the immobilization of actin on solid supports for binding studies between actin and astaxanthin. The fourth approach involved the use of hydrophobic fluorescent probes to determine the binding affinity of actin for astaxanthin by displacing the bound probe from the binding site(s) of actin with the gradual addition of astaxanthin. The fluorescent probe, 1-anilino-8-

naphthalenesulfonic acid (ANS), has been used for determination of protein surface hydrophobicity (Li-Chan et al., 1985; Careche and Li-Chan, 1997) and the investigation of the drug-protein interaction (Essassi et al., 1990). All of these approaches were examined and the observations discussed critically.

2.3 Materials and methods

2.3.1 Fish samples

Atlantic salmon (*Salmo salar* L., Saint John River strain) were reared in seawater at Marine Research Station of the Institute for Marine Biosciences, National Research Council, Sandy Cove, Nova Scotia. The fish were fed a diet supplemented with astaxanthin (Carophyll pink; F. Hoffmann-La Roche Ltd., Basel, Switzerland) at a rate of 70 mg kg^{-1} diet. Fish (average weight of 2.4 kg) were selected randomly on an individual basis at the sampling time and anaesthetized using 50 mg L^{-1} seawater of tricaine methanesulphonate (TMS) and transported on ice to the laboratory for about 30 minutes. Fish were decapitated, bled and gutted immediately after arrival at the laboratory. After filleting the fish, muscle tissue was taken from the mid-dorsal section for preparation of acetone powder.

2.3.2 Isolation and purification of salmon muscle actin

Muscle tissue was coarsely ground using a chilled (4 °C) meat grinder and acetone powder was prepared from 200 g ground salmon muscle according to the method reported for rabbit muscle by Pardee and Spudich (1982). Details of the acetone powder preparation are represented in a flow diagram (Fig. 2.1).

Actin was extracted from the dried acetone powder using low ionic strength buffer (buffer A - 2 mM Tris-HCl, 0.2 mM $CaCl_2$, 0.2 mM Na_2ATP, 0.5 mM 2-mercaptoethanol and 0.005% NaN_3, pH 8.0) as described by Watabe et al. (1983) with minor modification of the actin polymerization step. Actin extracted in buffer A was polymerized in the presence of 0.1 M KCl, 1 mM $MgCl_2$ and 1 mM Na_2ATP (actin polymerization buffer) for 2 hr at 4 °C rather than for 30-40 minutes at room temperature. Finally, polymerized actin was stored at 4 °C with 0.02% NaN_3. Protein concentration

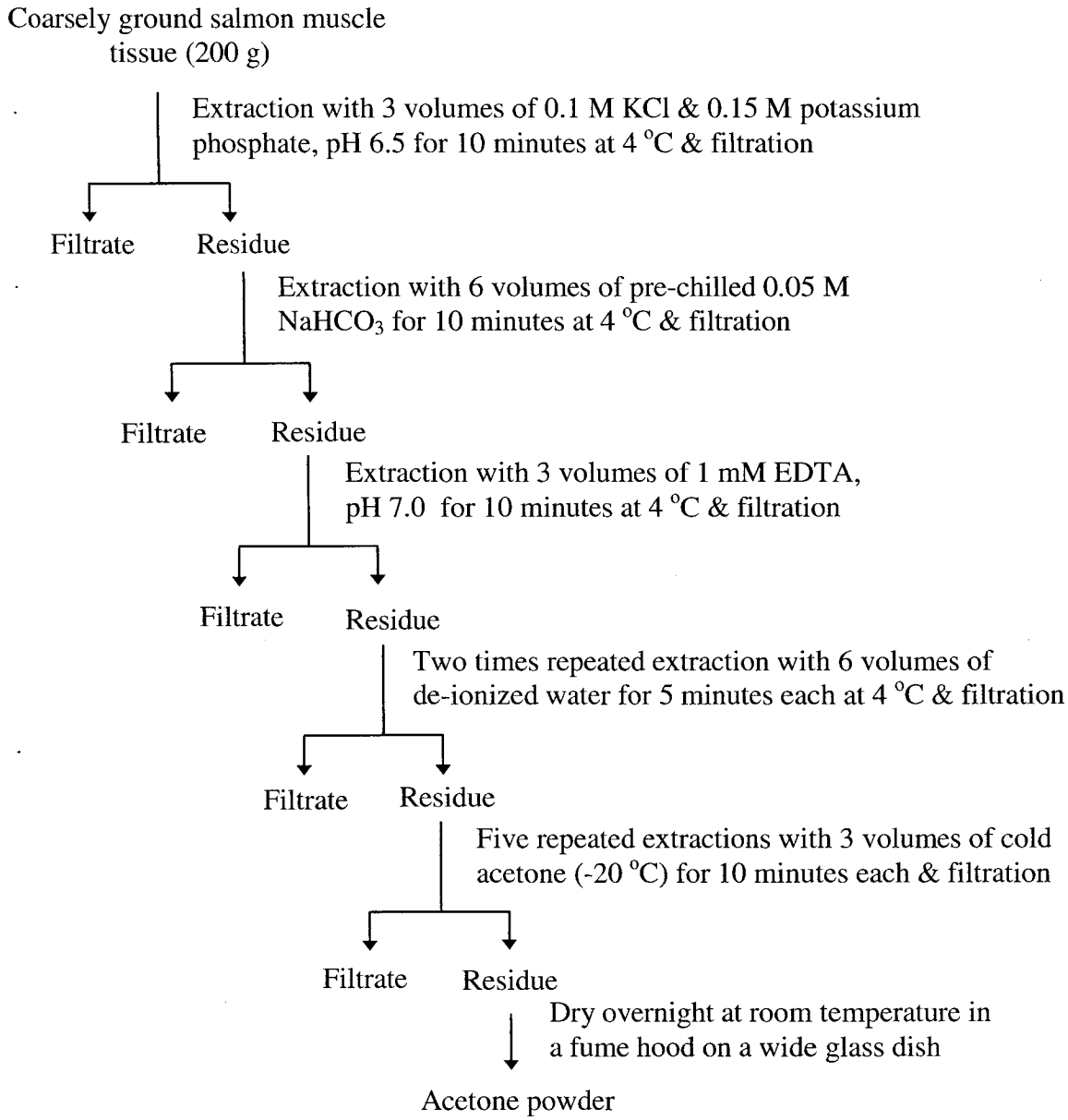

Fig. 2.1 Flow diagram for preparation of acetone powder from Atlantic salmon muscle tissue (adapted from Pardee and Spudich, 1982).

was determined by the method of Bradford (1976) using bovine γ-globulin (Bio-Rad Laboratories, Mississauga, ON) as a standard.

2.3.3 Electrophoresis

Actin was subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% polyacrylamide slab gel according to the method of Laemmli (1970) to assess purity. Protein bands were visualized by staining with 0.1% Coomassie Brilliant Blue G-250 (Bio-Rad Laboratories). The apparent molecular weight of actin was estimated by comparison with unstained Precision protein standards (Bio-Rad Laboratories).

2.3.4 Binding assays

Several methods were assessed for their applicability to characterize the binding between astaxanthin (F. Hoffmann-La Roche Ltd., Basel, Switzerland) and purified salmon muscle actin. Astaxanthin solutions were always prepared fresh just prior to binding assays and a Hamilton microsyringe was used to deliver the required volume of astaxanthin solution. All assays were performed under yellow light.

2.3.4.1 Gel filtration chromatography method

The binding assay between astaxanthin and actin was performed according to the method of Côté (2000) with some modifications. Côté (2000) dissolved astaxanthin in dimethyl sulfoxide (DMSO) and added 5 μL of astaxanthin solution to 100 μL of actin for the binding assay. However, astaxanthin was dissolved in tetrahydrofuran (HPLC grade) and 2 μL of astaxanthin solution was added to 100 μL of actin in the present study since the solubility of astaxanthin was significantly higher in tetrahydrofuran than in DMSO. Precipitation of protein was not observed after the addition of 2 μL of tetrahydrofuran to 100 μL of actin. A series of astaxanthin dilutions were prepared in tetrahydrofuran from an astaxanthin stock solution. A 2 μL aliquot of astaxanthin was added to 100 μL of polymerized actin (conc. 0.8 mg mL^{-1}) in amber vials to achieve final astaxanthin concentrations of 3.35 to 16.75 μM. The samples were mixed by brief vortexing and allowed to stand at room temperature for 30 minutes. One aliquot of the

sample was diluted 1:1 using the actin polymerization buffer and the absorbance of the sample was measured at 485 nm (λmax of astaxanthin in experimental condition). This absorbance data gave a measure of the total astaxanthin (bound + free). A second aliquot of the sample was passed through the micro Bio-Spin chromatography columns (6 kDa MW exclusion; Bio-Rad Laboratories). The eluate of the column was diluted 1:1 using actin polymerization buffer and the absorbance was measured at 485 nm to determine the actin-bound astaxanthin. The absorbances were corrected for actin absorbance at 485 nm. All absorbances were measured in a micro quartz cell using an Ultrospec 2000 uv/visible spectrophotometer (Pharmacia Biotech Ltd., Cambridge, England). Similar binding experiments were also carried out using depolymerized actin as well. The molar extinction coefficient of astaxanthin was determined experimentally.

2.3.4.2 Fluorometry method

One hundred μL of polymerized actin (1 mg mL^{-1}) was added to a microcuvette. Actin sample was excited at wavelength of 284 nm (band pass 4 nm) and the fluorescence intensity was measured at emission wavelength of 320 nm (band pass 4 nm) using an Aminco-Bowman Series 2 luminescence spectrometer (Spectronic Instruments, Rochester, NY) at 16 °C. Astaxanthin dissolved in tetrahydrofuran at a concentration of 0.168 mM was gradually added, 2 μL of astaxanthin solution at a time, to 100 μL of actin and mixed it gently by inverting the cuvette several times. Fluorescence intensity of actin was measured after each addition of astaxanthin. Identical experimental conditions were maintained to conduct blank experiment by stepwise addition of tetrahydrofuran to actin.

Fluorescence intensity of actin was measured individually after the gradual addition of a water-miscible organic solvent such as dimethyl formamide, DMSO, acetone or ethanol in order to determine a solvent that would not change the fluorescence of actin upon gradual addition of the solvent.

2.3.4.3 Immobilization of actin

2.3.4.3.1 Immobilization of actin on the ELISA plate

An attempt was made to immobilize actin on the enzyme-linked immunosorbent assay (ELISA) plate (maximum protein binding capacity) in order to perform binding studies between actin and astaxanthin. A series of actin concentrations (0.04-0.2 $\mu g\ \mu L^{-1}$) was prepared by diluting the stock solution of actin with actin extraction buffer and incubated on the ELISA plate at 4 °C for optimizing the concentration of actin to achieve maximum immobilized actin. A 200 µL aliquot from each concentration of actin was pipetted in triplicate wells of the ELISA plate. The incubation period was extended from 20 hr to 72 hr. The protein concentration in the supernatant from each well was determined intermittently at 2.5 hr, 20 hr and 72 hr of incubation by the method of Bradford (1976). The amount of bound actin in the well of the ELISA plate was calculated from the difference between the added and the remained amount of actin in the supernatant of the well.

2.3.4.3.2 Immobilization of actin to Sepharose 4B

Polymerized actin was converted to depolymerized actin by dialyzing against buffer A according to the method of Pardee and Spudich (1982). Depolymerized actin was further dialyzed against the buffer containing 5 mM $NaHCO_3$ and 0.2 mM $CaCl_2$, pH 8.0 (coupling buffer) for 42 hr with changing twice a day to remove Tris. Dialyzed actin was centrifuged at 80,000 *g* for 3 hr at 4 °C and the supernatant protein (actin) concentration was measured by the method of Bradford (1976).

For the coupling experiment, 0.25 g of 6-aminohexanoic acid N-hydroxysuccinimide ester (Sepharose 4B) was subjected to a quick wash with coupling buffer on a sintered glass filter with vacuum to remove additives. The washing step was completed quickly to avoid hydrolysis of the reactive group. Washed Sepharose beads were added to 3 volumes of depolymerized actin (0.6 $\mu g\ \mu L^{-1}$) in a microcentrifuge tube and rotated overnight at 4 °C. Then the mixture was poured off into a poly-prep column to drain the uncoupled protein and washed two times with coupling buffer to remove all free reactant from the beads. The beads were washed again two times with buffer A containing 20 mM Tris-HCl to bind to free reactive groups. Then actin-coupled

Sepharose beads were suspended in buffer A containing 20 mM Tris-HCl (pH 8.0) to prevent protein aggregation and stored at 4 °C. Actin concentration in the drained volume of coupling buffer was determined by the method of Bradford (1976) and the amount of uncoupled actin calculated. The amount of actin coupled to Sepharose beads was calculated from the difference between the added and uncoupled actin. For control, buffer A containing 50 mM Tris-HCl, pH 8.0 was allowed to bind to Sepharose 4B following the procedure mentioned above and then Tris-coupled Sepharose beads were suspended in buffer A containing 20 mM Tris (pH 8.0).

Astaxanthin concentrations ranging from 0.67 μM to 0.168 μM were prepared in DMSO and added to suspended Sepharose 4B (slurry) in a 0.45 μm Durapore filter (Millipore, Bedford, MA, USA). The filter was gently shaken to mix astaxanthin solution with the Sepharose beads and incubated at room temperature for 3 minutes. The percent of each astaxanthin solution to suspended Sepharose beads was maintained at 5% (v/v). The mixture was centrifuged at 7,000 *g* for 9 seconds to separate unbound astaxanthin. After separation, dry Sepharose beads were poured off in a glass vial and bound astaxanthin extracted using organic solvent such as acetone or ethanol.

2.3.4.4 Hydrophobic probe displacement method

The method used for 8-anilino-1-naphthalenesulfonate (ANS) binding to actin was based on the method of Chan et al. (1992). Polymerized actin was buffer-exchanged into 0.6 M NaCl, 50 mM imidazole, pH 6.5, using an Ultrafree Biomax-5 K filter [5,000 nominal molecular weight limit (NMWL); Millipore]. An 8.0 mM stock solution of the hemi-magnesium salt of ANS was made up in the same buffer. An astaxanthin stock solution was prepared in DMSO (ACS reagent grade) at 0.168 mM. The ANS and astaxanthin stock solutions were prepared daily. Ten μL of 8.0 mM ANS was added to 2 mL of actin (0.2 mg mL^{-1}) and mixed thoroughly. The actin-ANS mixture was excited at 372 nm (band pass 4 nm) and fluorescence intensity measured at the emission wavelength of 468 nm (band pass 4 nm) using an Aminco-Bowman Series 2 luminescence spectrometer (Spectronic Instruments) at 16 °C. Astaxanthin stock solution was added stepwise to the actin-ANS mixture, mixed and fluorescence intensity measured after each addition. Identical experimental conditions were maintained to

conduct blank experiment by stepwise addition of DMSO to the actin-ANS mixture. Quenching of ANS fluorescence by astaxanthin was tested by the addition of astaxanthin to 0.8 μM ANS in methanol (HPLC grade).

Another fluorescent hydrophobic dye, Nile Red, which has different excitation and emission properties than ANS, was examined for the displacement strategy. The fluorescence of Nile Red was measured with the excitation and emission wavelengths of 596 and 657, respectively.

2.4 Results and discussion

A single protein band with an apparent molecular weight of 42 kDa was obtained on SDS-PAGE of the purified actin (Fig. 2.2) suggesting that actin isolated from salmon muscle tissue was highly purified.

In the gel filtration chromatography binding assay, separation of bound astaxanthin from free astaxanthin was attempted using the micro Bio-Spin chromatography columns (6 kDa MW exclusion). The eluate of the columns gave inconsistent absorbance readings among the replicates at the same concentration of added astaxanthin. The observed variation in the absorbance of the eluate, which should give a measure of actin-bound astaxanthin, was more than 50% among the replicates of individual binding assay. When astaxanthin solutions (in buffer) were chromatographed in the absence of actin under identical experimental conditions, color was observed in the eluate of the columns suggesting that astaxanthin was present in the form of high molecular weight aggregates. The observation of astaxanthin aggregates in aqueous media is consistent with those of Buchwald and Jencks (1968). Formation of astaxanthin aggregates in aqueous media due to the hydrophobic nature of astaxanthin was a major hindrance in the reliable separation of actin-bound astaxanthin from free astaxanthin on the basis of size. Therefore, the gel filtration chromatography method was abandoned.

An actin fluorescence study was attempted to avoid the problem in separating protein-bound and free astaxanthin. Changes in the fluorescence of actin were measured to assess changes in actin conformation upon binding to astaxanthin. Binding of astaxanthin would be expected to alter the environment surrounding some of the tryptophan, tyrosine and/or phenylalanine residues in the actin structure and hence

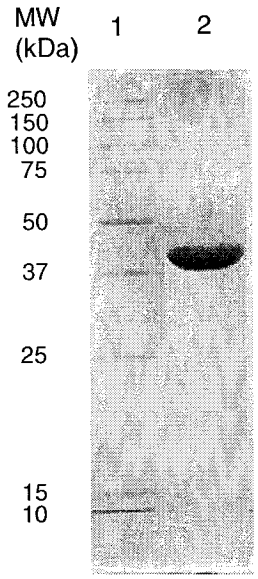

Fig. 2.2 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (12% gel) of actin purified from Atlantic salmon (*Salmo salar* L.) muscle tissue. Lane 1, Precision protein standards. Lane 2, purified salmon muscle actin (approximately 35 μg).

changes in the fluorescence of these amino acids. Separation of bound astaxanthin from free astaxanthin would not be required. However, the changes in actin fluorescence were determined to be caused by the addition of tetrahydrofuran while adding astaxanthin to actin. Similarly, the addition of other solvents such as dimethyl formamide, DMSO or acetone to actin caused similar changes in actin fluorescence. The fluorescence of actin changed more in acetone than in dimethyl formamide or DMSO. Although the fluorescence of actin did not change with the gradual addition of ethanol, however, complete solubilization of astaxanthin was not achieved in ethanol even at very low

concentration of astaxanthin (15 μM). Cogan et al. (1976) dissolved retinol and its related compounds such as retinoic acid, retinyl acetate and retinyl palmitate in ethanol each at a concentration of 0.3-0.03 mM and characterized the binding affinities of these ligands to human-retinol and chicken-retinol binding proteins using the fluorometric method. Similarly, Ong and Chytil (1978) prepared retinol solution in ethanol and determined the binding affinity of cellular retinol-binding protein for retinol. It is apparent that the low solubility of astaxanthin in ethanol compared to retinol or retinoids is contributed by the presence of twice as many conjugated double bonds in astaxanthin than retinol. The low solubility of astaxanthin in ethanol and changes in actin fluorescence due to added solvent precluded further use of this method.

The next approach involved the immobilization of actin on different solid supports (ELISA plate and Sepharose 4B) to conduct actin-astaxanthin binding assays. Insufficient amounts of actin were found to be immobilized on the ELISA plate even after 72 hr of incubation period. Approximately 7.5 μg of actin was found to be immobilized on the ELISA plate at the highest concentration of incubated actin after 72 hr. The amount of immobilized actin was inadequate for the binding assay as actin-bound astaxanthin could not be detected by the spectrophometer.

For coupling, a total of 0.42 mg of actin was added to Sepharose 4B. It was observed that approximately 94% of added actin was coupled to Sepharose beads. However, two problems were recognized in the astaxanthin binding assay with actin-coupled Sepharose beads. The first problem was that astaxanthin bound tightly to the matrix (beads) and was not completely extracted by the solvents. In addition, remarkable binding of astaxanthin to Sepharose beads in the control experiment was also observed which was detected by the intensity of the color of the control beads and actin-coupled beads. Walters (1985) reported that 6-aminohexanoic acid is a commonly used spacer arm in affinity chromatography. In Sepharose 4B, 6-aminohexanoic acid is used as a spacer arm. It has been reported that spacer arms alone caused adsorption (Er-el et al., 1972; Hofstee, 1973). Thus binding of astaxanthin to the control Sepharose beads can probably be explained by binding of astaxanthin to the spacer arm, 6-aminohexanoic acid. The second problem was that despite very careful pipetting, inconsistent volumes of beads were obtained and this led to an error in the quantitative determination of actin-

bound astaxanthin. Coupled beads settled down quickly in a slurry and consequently it was hard to achieve a consistent volume of beads during pipetting. After consideration of these problems, the strategy of using immobilized actin for the binding assay was abandoned.

The ANS displacement method was attempted to examine the affinity of actin for astaxanthin without separating bound astaxanthin from free astaxanthin. It has been reported that astaxanthin binds to the hydrophobic sites on actomyosin (Henmi et al., 1989, 1990a). The hydrophobic fluorescent probe, ANS, also binds to the hydrophobic sites on protein and thus it has been used for determination of protein surface hydrophobicity (Li-Chan et al., 1985; Careche and Li-Chan, 1997). In this approach, ANS was allowed to bind to the actin first and then displaced gradually by adding astaxanthin. The ANS became highly fluorescent when bound to actin, though showed very little fluorescence in buffer. Increasing fluorescence intensity of ANS upon binding to actin suggested that ANS bound to the hydrophobic sites on actin. The fluorescence intensity of actin-bound ANS gradually decreased with increasing concentration of astaxanthin. However, after quench correction, ANS fluorescence actually increased rather than decreased with increasing concentrations of astaxanthin. The decrease in ANS fluorescence with increasing concentration of astaxanthin was due to quenching of ANS fluorescence since astaxanthin absorbs light maximally at the emission wavelength of ANS (468 nm). In addition ANS might have bound to the aggregates of astaxanthin and contributed additional ANS fluorescence, which could explain the increased ANS fluorescence. Angelakou et al. (1999) developed an ANS displacement approach for competitive drug-protein binding studies and monitored the displaced free ANS from its binding sites on the protein molecule by gradual addition of drug using a laboratory-made ANS electrode. However, interference in the monitoring of free ANS due to probable interactions of ANS with aggregated astaxanthin would be a limiting factor for the application of this method in protein-astaxanthin binding studies.

Nile Red showed little fluorescence when incubated with actin (excitation and emission wavelengths of 596 and 657 nm, respectively) and, as for ANS, astaxanthin quenched Nile Red fluorescence even at these higher wavelengths.

2.5 Conclusion

A reliable binding assay is necessary for the characterization of binding between astaxanthin and actin in order to determine the role of muscle protein (actin) in astaxanthin retention in salmon flesh. Several methods for binding assays were evaluated to characterize the binding between astaxanthin and actin. Gel filtration chromatography, intrinsic fluorometry, and a binding assay using immobilized actin and displacement of hydrophobic probe (ANS) were evaluated. None of these methods were found suitable for the characterization of binding between astaxanthin and actin. Formation of astaxanthin aggregates under the experimental condition was a major hindrance for reliable separation of actin-bound astaxanthin from free astaxanthin in the gel filtration chromatography method. In the fluorometry method, fluorescence of actin was changed due to solvent while adding astaxanthin to actin. Actin did not bind sufficiently to the ELISA plates with a 20-hr incubation period and even after the extension of the incubation period up to 72 hr, the amounts of immobilized actin was inadequate for the binding assay. Astaxanthin was found to be tightly bound to the beads when the binding assay between astaxanthin and immobilized actin was performed after coupling actin to Sepharose 4B. Quenching of ANS fluorescence by astaxanthin was a constraint for ANS displacement method. After reviewing all the observations, it seems that dispersion of astaxanthin aggregates in experimental condition would be helpful for the separation of free (unbound) astaxanthin from actin-bound astaxanthin by the ultrafiltration method in order to characterize the binding between actin and astaxanthin. Gentle detergents like bile salts having high critical micelle concentration (CMC) and low aggregation number could be used to disperse in astaxanthin in aqueous buffer. This will be explored further in Chapter 3.

CHAPTER 3

Development of a method to assess binding of astaxanthin to Atlantic salmon (*Salmo salar* L.) muscle proteins

3.1 Abstract

It was difficult to characterize the binding between astaxanthin and muscle protein(s) of Atlantic salmon (*Salmo salar* L.) because of the hydrophobic characteristics of astaxanthin. It was essential to develop a reliable method to characterize the binding between astaxanthin and salmon muscle protein(s) to provide tools for evaluation of the role of muscle protein(s) on astaxanthin retention in Atlantic salmon. An ultrafiltration method was developed using 200 mM sodium cholate for dispersion of astaxanthin aggregates. This allowed the separation of unbound (free) astaxanthin from protein-bound astaxanthin using a 30,000 (30 K) nominal molecular weight limit filter. Astaxanthin binding to salmon muscle actin was assessed by the ultrafiltration method. Astaxanthin binding of actin did not exhibit saturable binding kinetics at the astaxanthin concentrations used in the assay. After salmon muscle proteins were solubilized in different fractions by sequential extraction using low ionic strength solutions, the astaxanthin binding of different fractions was assessed using the ultrafiltration method to find other protein(s) that may bind astaxanthin. The significant difference ($P < 0.05$) observed in the astaxanthin binding of the various fractions suggests an application of this assay to detect differences in affinity of proteins for astaxanthin. The results also suggest that proteins other than actomyosin or actin can bind astaxanthin in Atlantic salmon flesh. This method can be used for the identification of astaxanthin-binding proteins in salmon flesh and other tissues.

3.2 Introduction

Salmonids depend entirely on dietary carotenoids for their flesh pigmentation since they are unable to synthesize carotenoids *de novo*. Deposition of carotenoids in the

muscle tissues provides their distinctive flesh coloration. Astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′ dione) and canthaxanthin (β,β-carotene-4,4′ dione) are widely used carotenoids in the diet of farmed salmon. Retention of these carotenoids is very low in Atlantic salmon; approximately 7.5-12.5% of the dietary astaxanthin is retained in their muscle (Wathne et al., 1998). Retention of carotenoids in animal tissues depends on intestinal absorption, transportation, metabolism and excretion and deposition mechanisms for carotenoids in various tissues (Torrissen et al., 1989). The ability of salmon muscle protein to bind carotenoid pigments may be an important factor for carotenoid pigment retention.

Carotenoid-protein complexes have been extensively studied in crustaceans (Cheesman et al., 1967; Zagalsky, 1976; Zagalsky et al., 1990). Most of the carotenoproteins are characterized by a large bathochromic shift of the pigment component (Goodwin, 1984). Strong interaction between carotenoids and apoproteins allows the purification of carotenoproteins since carotenoids remain tightly bound. The specific interactions of carotenoid-protein complex have been examined by reconstitution of carotenoids and apoproteins (Okoh et al., 1993; Zagalsky et al., 1995). In contrast, a weak bathochromic spectral shift has been reported for carotenoid-protein complexes in salmon (Henmi et al., 1989). It has been suggested that carotenoids bind to the hydrophobic sites of actomyosin in salmon flesh (Henmi et al., 1987, 1989). Henmi et al. (1989) combined carotenoid (astaxanthin and canthaxanthin) with salmon actomyosin and reported that bound astaxanthin was 0.7- 0.86 μg mg^{-1} actomyosin. However, quantitative determination of bound astaxanthin by the procedure of Henmi et al. (1989) is difficult because the actomyosin used in their study was insoluble and astaxanthin may have bound to exposed surfaces of insoluble aggregates. As well, astaxanthin may be expected to dissociate from protein during washing since the interaction between actomyosin and astaxanthin is reportedly weak (Henmi et al., 1987, 1989, 1990b). A reliable binding assay is essential to understand the characteristics of carotenoid pigment (i.e., astaxanthin) binding to Atlantic salmon muscle proteins and their role in retention of pigment.

The aim of the present study was to develop a reliable astaxanthin-protein binding assay procedure to measure the ability of salmon muscle protein(s) to bind astaxanthin.

Using a gel filtration chromatography method, actin has been characterized as a carotenoid binding protein in Atlantic salmon flesh by Côté (2000). However, the gel filtration chromatography method for the astaxanthin-actin binding assay examined in Chapter 2 revealed that the hydrophobic nature of astaxanthin was a major hindrance for reliable separation of protein-bound astaxanthin from free astaxanthin on the basis of size.

To avoid the problem of separating actin-bound astaxanthin from free astaxanthin, several other methods such as fluorometry method, binding assay with immobilized actin and the displacement of hydrophobic probe were examined. The limitations of these methods were discussed in Chapter 2. Although ultrafiltration has been used to determine free ligand in pathological research (Viahos et al., 1982; MacMahon et al., 1983) and in ligand binding studies (Sophianopoulos et al., 1983), the application of this approach for a astaxanthin-actin binding assay suffers from the fact that astaxanthin aggregates in aqueous solutions. For the separation of free astaxanthin from protein-bound astaxanthin, it is necessary to disperse astaxanthin aggregates. Bile salts have been used for the solubilization of steroids (Bakatselou et al., 1991) and poorly soluble drugs (Mithani et al., 1996). It has been reported that the bile salt, sodium taurocholate, increased the solubility of retinoids by several orders of magnitude (Li et al., 1996). In the present study, the properties of astaxanthin aggregates in aqueous buffer were examined and different bile salts were tested to disperse astaxanthin aggregates. Sodium cholate was used in the present study to disperse astaxanthin in assay condition for the separation of unbound (free) astaxanthin from protein-bound astaxanthin using the ultrafiltration method. This method was also used to examine whether other proteins solubilized from Atlantic salmon muscle tissue bind astaxanthin *in vitro*.

3.3 Materials and methods

3.3.1 Fish samples

Atlantic salmon (*Salmo salar* L.; Saint John River strain) were reared in seawater and fed a diet supplemented with astaxanthin (70 mg kg^{-1} diet) at Marine Research Station of the Institute for Marine Biosciences, National Research Council, Sandy Cove, Nova Scotia. Salmon were anaesthetised by TMS (tricaine methanesulfonate; 50 mg L^{-1}

water) and transported on ice to the laboratory (30 minutes). Fish were decapitated, bled and gutted immediately after arrival at the laboratory. Muscle tissue excised from the mid-dorsal region of the fish was extracted immediately following sacrifice.

3.3.2 Isolation and purification of salmon muscle actin

Acetone powder was prepared from coarsely ground muscle of 2.4 kg (average weight) Atlantic salmon according to the method reported for rabbit muscle (Pardee and Spudich, 1982). Details of the acetone powder preparation were shown in the materials and methods section of the previous chapter (Chapter 2, Fig. 2.1). Actin was extracted from the acetone powder using a low ionic strength buffer (buffer A - 2 mM Tris-HCl, 0.2 mM $CaCl_2$, 0.2 mM Na_2ATP, 0.5 mM 2-mercaptoethanol and 0.005% NaN_3, pH 8.0) as described by Watabe et al. (1983) with minor modifications of the actin polymerization step. Actin extracted in buffer A was polymerized in the presence of 0.1 M KCl, 1 mM $MgCl_2$ and 1 mM Na_2ATP (actin polymerization buffer) for 2 hr at 4 °C instead of 30-40 minutes at room temperature. Polymerized actin was then stored at 4 °C with 0.02% NaN_3. Protein concentration was determined by the method of Bradford (1976) using bovine γ-globulin (Bio-Rad Laboratories, Mississauga, ON) as a standard.

3.3.3 Solubilization of Atlantic salmon muscle proteins

Atlantic salmon with an average weight of 1.3 kg were used for solubilization of muscle proteins. Solubilization of muscle proteins in low ionic strength, non-denaturing solutions was performed based on the method reported for chickens (Krishnamurthy et al., 1996). Details of the solubilization process are shown in a flow diagram (Fig. 3.1). Protein concentrations for solubilized fractions were determined similarly as mentioned earlier.

3.3.4 Electrophoretic analysis of actin and solubilized muscle proteins

Purity of actin was assessed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) performed on a 12% polyacrylamide slab gel (Laemmli, 1970). The apparent molecular weight of actin was estimated by comparison with Precision protein standards (Bio-Rad Laboratories).

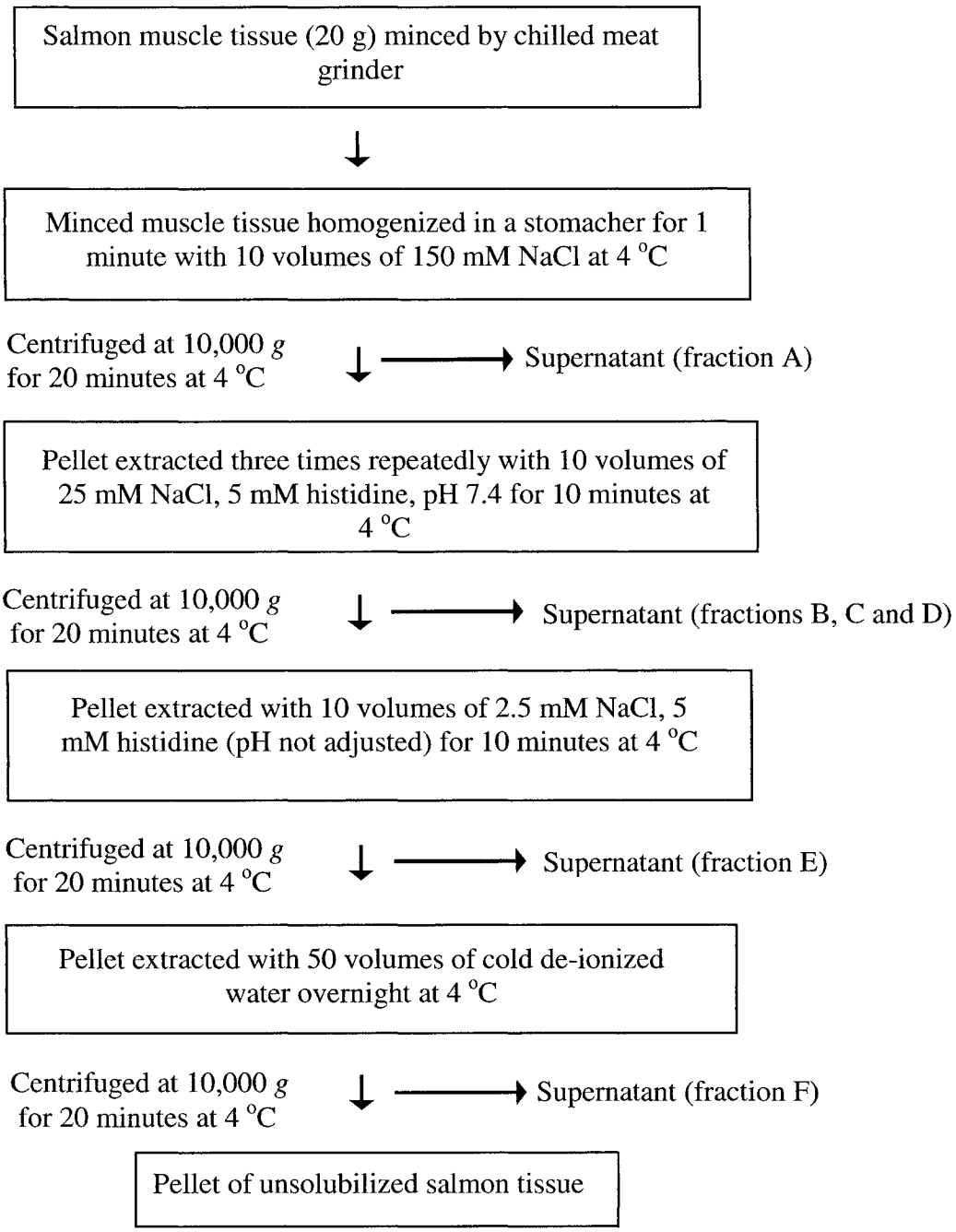

Fig. 3.1 Flow diagram of low ionic solubilization of Atlantic salmon (*Salmo salar* L.) muscle tissue based on the method reported for solubilization of chicken breast muscle by Krishnamurthy et al. (1996).

Solubilized salmon muscle protein fractions were subjected to SDS-PAGE on 5-15% linear gradient slab gel. Broad range protein markers (Bio-Rad Laboratories) were used as molecular weight standards. Gels were stained with 0.1% Coomassie Brilliant Blue G-250 (Bio-Rad Laboratories) in both cases.

3.3.5 Characteristics of astaxanthin aggregates in aqueous buffer

Astaxanthin (F. Hoffmann-La Roche Ltd., Basel, Switzerland) stock solutions were prepared in tetrahydrofuran (HPLC grade) and a 20 μL aliquot was added to each mL of buffer A (actin extraction buffer) to give final concentrations of 2.23 and 6.7 μM astaxanthin. Aliquots of aqueous astaxanthin solutions were filtered through 10,000 (10 K), 30,000 (30 K) and 50,000 (50 K) nominal molecular weight limit (NMWL) ultrafilters and 0.22 and 0.45 μm microporous filters (Ultrafree-MC filter units; Millipore, Bedford, MA). Absorption spectra of the resultant filtrates and non-filtered aliquot of the same sample were obtained in a micro-quartz cell using an Ultrospec 2000 uv/visible spectrophotometer (Pharmacia Biotech Ltd., Cambridge, England). In order to determine the effect of buffer composition on astaxanthin aggregation, Tris-HCl was increased in buffer A to a concentration of 50 mM and the passage of astaxanthin through 0.22 and 0.45 μm filters was determined after adding astaxanthin to final concentrations of 2.23 and 6.7 μM.

3.3.6 Dispersion of astaxanthin aggregates in aqueous buffer

A series of sodium cholate concentrations were examined to disperse astaxanthin aggregates in buffer A. Sodium cholate was dissolved in buffer A at concentrations from 25 to 250 mM. A 20 μL aliquot of astaxanthin in tetrahydrofuran was added to 1 mL of each sodium cholate concentration in a glass vial to give a final concentration of 5.03 μM astaxanthin. The sample was quickly mixed by a brief vortexing and incubated at room temperature for 30 minutes. Two aliquots (400 μL each) of samples were filtered through Ultrafree-MC filters of 10 and 30 K NMWL (regenerated cellulose membrane) at 4,000 *g*. The initial filtrates (about 50 μL) were discarded and the samples were centrifuged at 4,000 *g* for 5-12 minutes to obtain filtrate volumes >100 μL. Absorption

spectra of the resultant filtrates and a non-filtered aliquot of the same sample were obtained similarly as described above. Dispersion of astaxanthin aggregates was assessed by their passage through two different NMWL ultrafilters as determined spectrophotometrically.

In an attempt to assess whether other bile salts would allow the passage of astaxanthin through low molecular weight cut-off filters, the passage of astaxanthin through the 30 K NMWL filter was determined in presence of either sodium taurocholate (200 mM) or sodium taurodeoxycholate (200 mM).

3.3.7 Binding assay using the ultrafiltration method

Astaxanthin solutions were prepared fresh at the time of assay and all assays were performed under yellow light. A Hamilton glass microsyringe was used to measure the required volume of astaxanthin solution for the assays.

Sodium cholate was included in the assay at a final concentration of 200 mM to reduce the molecular weight of astaxanthin aggregates. Polymerized actin and 400 mM sodium cholate solution were combined in a 1:1 ratio in a glass vial with a total volume of 500 μL and mixed with a brief vortex to give final concentrations of actin and sodium cholate of 0.53 mg mL^{-1} and 200 mM, respectively. Ten μL of astaxanthin in tetrahydrofuran were added to the mixed sample to achieve final astaxanthin concentrations of 3.35 to 16.75 μM and incubated for 30 minutes at room temperature followed by a brief vortex. One aliquot of the samples was filtered through Ultrafree-MC filter of 30 K NMWL (regenerated cellulose membrane; Millipore) at 3,500 *g*. After centrifuging for 45 seconds, the initial filtrate was discarded. The samples were centrifuged for an additional 7 minutes. The resultant filtrates and a second aliquot of the samples were diluted in a 1:1 ratio with 200 mM sodium cholate and the absorbance were measured at 485 nm (λ_{max} of astaxanthin determined experimentally) in a micro-quartz cell as described above. Absorbance before and after filtration reflects the total (bound + unbound) and unbound (free) astaxanthin, respectively. Bound astaxanthin absorbance was corrected for protein absorbance by subtracting protein absorbance at 485 nm. Control experiments were conducted using actin polymerization buffer. An astaxanthin standard curve was made in 200 mM sodium cholate. The extinction coefficient of

astaxanthin was calculated to be 1.12×10^5 M^{-1} cm^{-1}. Absorbance values of bound astaxanthin were transformed into molar concentrations.

The binding studies using a single concentration of astaxanthin were carried out on fractions A to F (Fig. 3.1) of solubilized salmon muscle proteins to compare their relative astaxanthin binding ability. All the binding studies were conducted on the day following solubilization of muscle proteins. The protein concentrations of fractions A to F were adjusted to concentrations between 1.2-1.5 mg mL^{-1} by diluting concentrated fractions with extraction buffers and concentrating more diluted fractions with an Ultrafree Biomax-10 K NMWL filter (Millipore). Solubilized fractions of salmon muscle proteins and 400 mM sodium cholate were combined in a 1:1 ratio as mentioned above to give final protein concentrations between 0.64-0.75 mg mL^{-1}. Ten μL of astaxanthin solution dissolved in tetrahydrofuran (HPLC grade) were added to the mixed sample to give a final astaxanthin concentration of 13.4 μM and incubated for 30 minutes at room temperature followed by a brief vortex. Separation and quantification of bound and unbound astaxanthin was performed in the same manner as described above. Control experiments were conducted using each of the different buffers used for muscle protein extraction.

Fractions B, D and F were incubated with different astaxanthin concentrations to compare binding kinetics of these three fractions. Final concentration of protein in the assay was 0.47-0.57 mg mL^{-1} and astaxanthin concentrations ranged from 3.35 to 16.75 μM.

Retention of solubilized muscle proteins in the 30 K NMWL filter was examined once under experimental conditions. After the addition of 400 mM sodium cholate to each fraction of salmon muscle proteins with a ratio of 1:1 in experimental conditions, the samples were mixed and filtered through a 30 K NMWL filter. The resultant filtrates were individually mixed with 4x SDS-PAGE sample buffer in a ratio of 3:1 and subjected to SDS-PAGE on a 15% gel to examine the retention of proteins by the filter. The gel was stained with silver-staining protocol according to the method of Swain and Ross (1995) as shown in Appendix 1.

An attempt was also taken to determine the protein concentration in the filtrate of each fraction following the procedure of Bio-Rad DC protein assay. However, the protein concentration of the various filtrates was not in the detectable range.

3.3.8 Statistical analysis

Binding data of fractions A to F were subjected to one-way analysis of variance (ANOVA). Comparisons of bound astaxanthin means of different fractions (A-F) were carried out using Bonferroni's multiple comparison procedure. Statistical analysis was performed using SYSTAT (version 10 for Windows, SYSTAT Inc. Evanston, IL).

3.4 Results

3.4.1 Electrophoretic analysis of actin and solubilized muscle proteins

SDS-PAGE was used to assess the purity of actin and to provide protein profiles of each of the solubilized muscle proteins fractions. A single protein band with an apparent molecular weight of 42 kDa was obtained on SDS-PAGE for the purified actin (Chapter 2, Fig. 2.2) suggesting that actin isolated from salmon muscle tissue was highly purified. The protein profiles of solubilized muscle protein fractions are shown in Fig. 3.2. The SDS-PAGE patterns of different fractions obtained in three repeated solubilization experiments indicated that the protein profiles were distinct and characteristic for each fraction with a slight variation in protein band intensities in repeated solubilization experiments. Actin was increasingly solubilized in later fractions. A 150 kDa protein was solubilized in fractions C, D and F. Myosin was dominant in fraction F, which also had the highest amount of actin among all the fractions. A trace amount of myosin was observed in fraction E.

3.4.2 Characteristics of astaxanthin aggregates in aqueous buffer

Characteristics of astaxanthin aggregates in buffer A were examined by the passage of astaxanthin aggregates through different cut-off filters in order to assess the dimension of the aggregates in different concentrations of astaxanthin and different buffer composition. Broadening of the spectrum of the non-filtered astaxanthin sample was observed with the increasing astaxanthin concentrations in buffer A. Astaxanthin

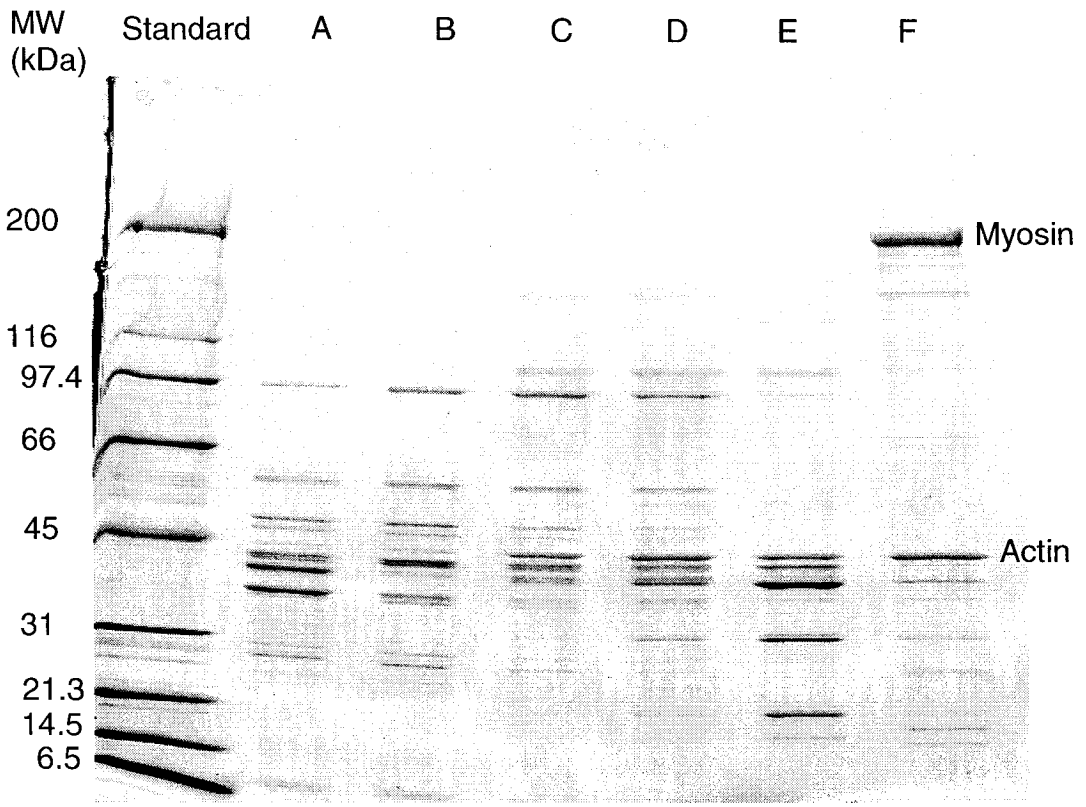

Fig. 3.2 SDS-PAGE gradient gel (5-15%) profiles of solubilized muscle proteins fractions of Atlantic salmon, *Salmo salar*. Molecular weights of standards are indicated at left of the gel. Letters on the top of the lanes indicate different muscle fractions. Fraction A, extraction with 150 mM NaCl; fractions B-D, sequential extractions with 25 mM NaCl and 5 mM histidine, pH 7.4; fraction E, extraction with 2.5 mM NaCl and 5 mM histidine; and fraction F, extraction with cold de-ionized water. Approximately 25 μg of protein from each fraction were loaded in each lane.

aggregates did not pass through 10, 30 and 50 K NMWL filters even at low astaxanthin concentration (2.23 μM) as no astaxanthin spectrum was detected in the filtrates. The passage of astaxanthin through the 0.22 and 0.45 μm filters was 88 and 92%, respectively at a final concentration of 2.23 μM astaxanthin. Conversely, negligible passage of astaxanthin aggregates through these filters was observed at a higher astaxanthin concentration (6.7 μM). There was no astaxanthin absorption in the filtrates of 0.22 and 0.45 μm filters when Tris-HCl was increased in buffer A to a concentration of 50 mM.

3.4.3 Dispersion of astaxanthin aggregates in aqueous buffer

The absorption maximum of astaxanthin in the filtrates was at 485 nm, however, the non-filtered aliquot of the same sample showed absorption maximum at 465 nm in presence of the lowest sodium cholate concentration and it was shifted toward the higher wavelength in presence of higher sodium cholate concentrations. The absorption maximum of astaxanthin both in the filtrates and non-filtered aliquot of the same sample was at 485 nm in the presence of 150 to 250 mM sodium cholate. Astaxanthin in tetrahydrofuran also showed maximum absorption at 485 nm.

The passage of dispersed astaxanthin through the filters was calculated from the maximum absorbance of the samples before and after filtration. The passage of dispersed astaxanthin through 10 and 30 K NMWL filters at different concentrations of sodium cholate are shown in Fig. 3.3. Only 26% of the astaxanthin passed through the 10 K NMWL filter at the highest sodium cholate concentration (250 mM) tested. The passage of astaxanthin through the 30 K NMWL filter was much more efficient than through a 10 K NMWL filter (Fig. 3.3). Astaxanthin passage increased with increasing sodium cholate concentration and 100% astaxanthin passage was obtained through the 30 K NMWL filter in presence of both 200 and 250 mM sodium cholate. Sonicating the sample for 10 minutes in the presence of 100 mM sodium cholate did not improve astaxanthin passage through the10 K filter.

The passage of astaxanthin (5.03 μM) through the 30 K NMWL filter in an equal concentration (200 mM) of sodium cholate, sodium taurocholate and sodium taurodeoxycholate are shown in Fig. 3.4. Approximately 98% of the astaxanthin passed

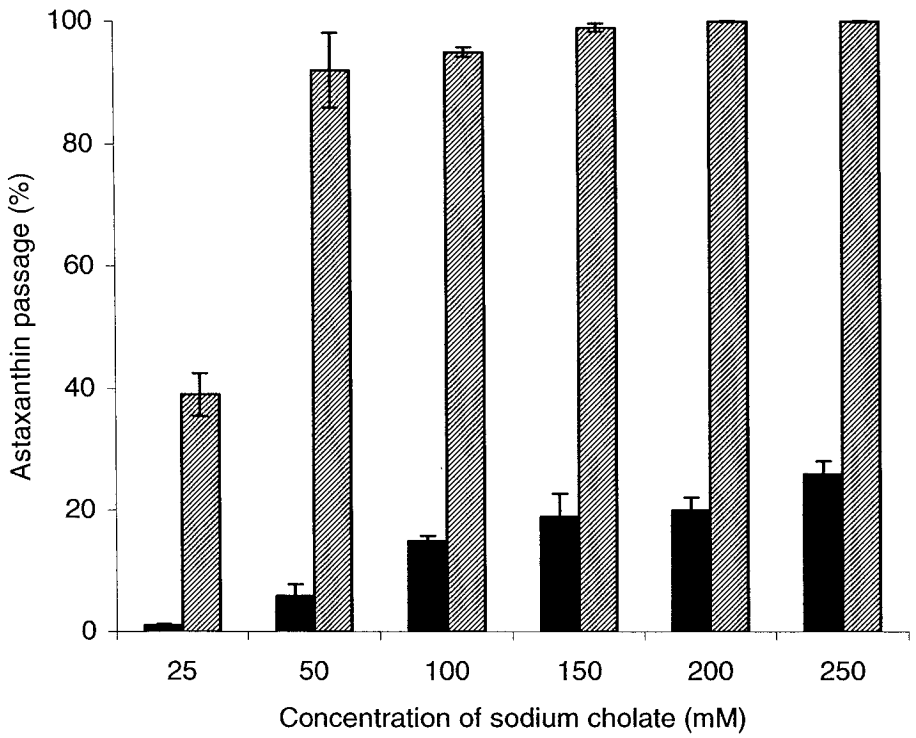

Fig. 3.3 The passage of astaxanthin through two different NMWL spin filters at various concentrations of sodium cholate. Final astaxanthin concentration was 5.03 μM. ■, 10 K NMWL filter; ▨, 30 K NMWL filter. Values are presented as mean ± S.D (n = 3).

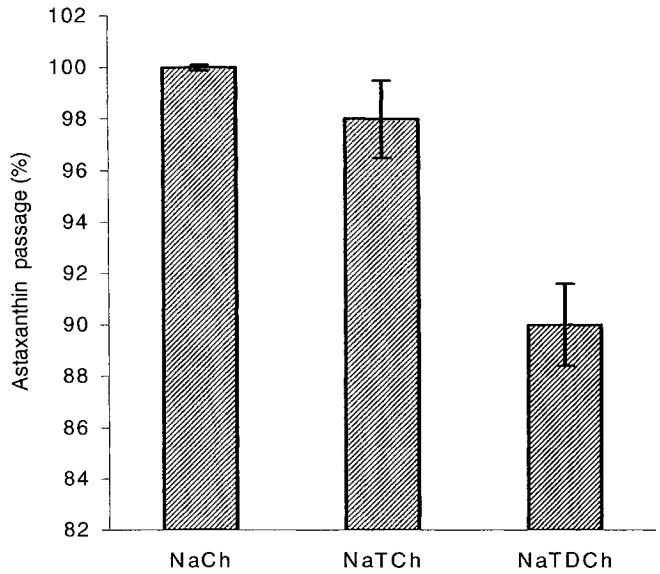

Fig. 3.4 The passage of astaxanthin through the 30 K NMWL filter in presence different bile salts at a concentration of 200 mM. Final astaxanthin concentration was 5.03 μM. NaCh, sodium cholate; NaTCh, sodium taurocholate; and NaTDCh, sodium taurodeoxycholate. Values are presented as mean ± S.D (n = 3).

through the 30 K NMWL filter in the presence of sodium taurocholate, whereas, 90% of the astaxanthin passed in presence of sodium taurodeoxycholate. Again, 100% astaxanthin passage through the 30 K NMWL filter was obtained in 200 mM sodium cholate.

Astaxanthin in buffer A at a concentration up to 10.05 μM freely passed through the 30 K NMWL filter in presence of 200 mM sodium cholate and about 98-99% astaxanthin passed through the filter at the higher astaxanthin concentration.

3.4.4 Binding assay using the ultrafiltration method

The passage of bile salt-dispersed astaxanthin through a 30 K NMWL filter varied with buffer composition. Thus control experiments using identical experimental conditions were carried out in the absence of protein(s) at the time of each binding study to rule out the possibility that astaxanthin aggregates were retained by the filters. Binding assays performed with different concentrations of astaxanthin and purified salmon muscle actin showed that bound astaxanthin was 1.19 mg g^{-1} actin at an astaxanthin concentration of 13.4 μM (Fig. 3.5).

Analysis of data from the binding experiments between solubilized salmon muscle proteins and astaxanthin showed that the protein-bound astaxanthin in the different fractions (Table 3.1) were significantly different ($P< 0.05$). Fraction F showed a significantly higher amount of bound astaxanthin (2.95 mg g^{-1} protein) than that of fractions A, B, C and E. However, no significant difference ($P > 0.05$) was observed between the bound astaxanthin of fractions F and D. Bound astaxanthin was neither significantly different between fractions C and D nor between fractions A and B. Fraction E showed significantly higher amount of bound astaxanthin than that of fractions A and B. The astaxanthin binding curve of actin and fractions B, D and F are shown in Fig. 3.5. Saturation in astaxanthin binding was not observed at the concentrations of astaxanthin used in this study but differences in binding were distinctly evident between fraction B and two other fractions (D and F).

The SDS-PAGE profile of the filtrates of 30 K filter obtained from the filtration of solubilized muscle proteins fractions in presence of 200 mM sodium cholate in experimental conditions are represented in Fig. 3.6. Only one protein band was detected in the filtrates of each fraction as indicated by the electrophoretogram. A protein with an approximate molecular weight of 22 kDa was detected in the filtrate of fractions A to D. However, the approximate molecular weight of the detected protein was 31 kDa in the filtrate of fractions E and F.

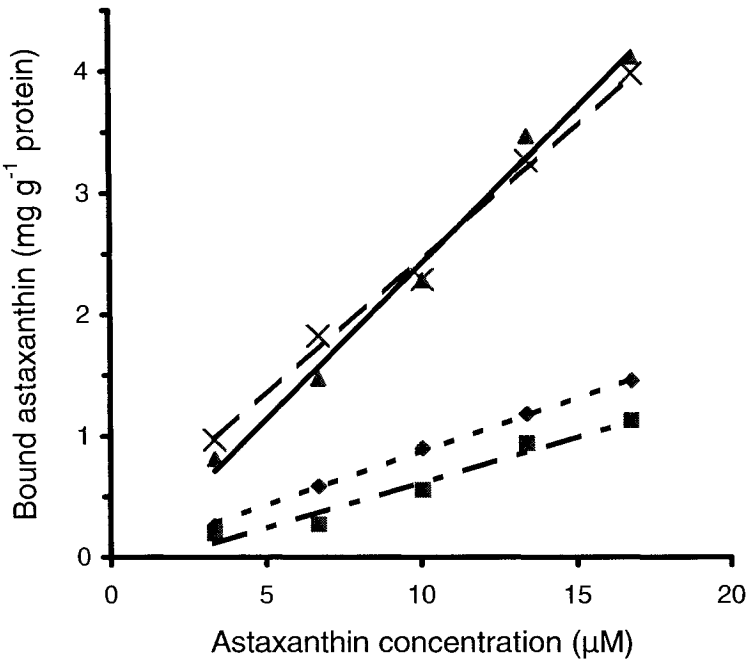

Fig. 3.5 Astaxanthin binding curves of purified salmon muscle actin and three different fractions of solubilized Atlantic salmon muscle proteins.◆ – – ◆, purified salmon muscle actin; ■ – · – ■, fraction B; ▲——▲, fraction D; ×— —×, fraction F.

Table 3.1 Bound astaxanthin in different fractions of solubilized salmon muscle proteins at a final astaxanthin concentration of 13.4 μM as determined by the ultrafiltration method in presence of 200 mM sodium cholate

Fractions	**Bound astaxanthin[a] (mg g^{-1} protein)**
A	1.05 ± 0.19^{d}
B	1.09 ± 0.03^{d}
C	2.38 ± 0.29^{b}
D	2.60 ± 0.30^{ab}
E	1.62 ± 0.53^{c}
F	2.95 ± 0.08^{a}

[a] Mean ± S.D ; n= 3; Values in the column with same superscript are not significantly different at $P < 0.05$

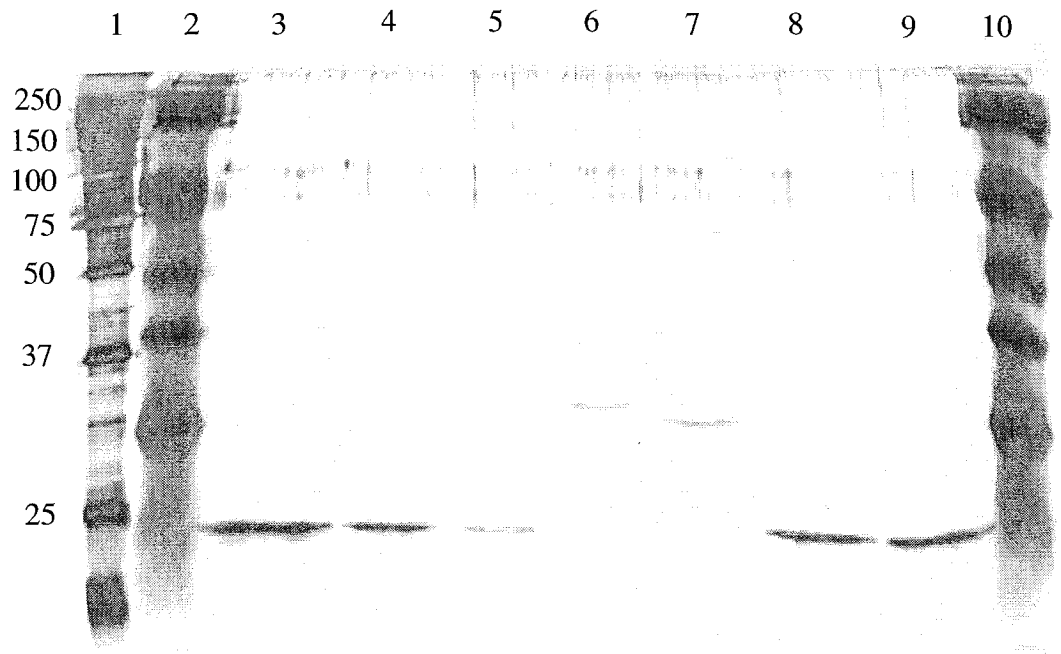

Fig. 3.6 The SDS-PAGE (15% polyacrylamide gel) of filtrates obtained from filtration of muscle protein fractions in the presence of 200 mM sodium cholate through the 30 K NMWL filter in the experimental conditions of the ultrafiltration method. Lane 1, unstained broad range Precision protein standards (Bio-Rad Laboratories); lanes 2 & 10, low range prestained Precision protein standards (Bio-Rad Laboratories); lanes 3 & 9, filtrate of fraction B; lanes 4 -7, filtrate of fractions C, D, E and F, respectively; lane 8, filtrate of fraction A. The molecular masses of broad range Precision protein standards are shown on the left.

3.5 Discussion

Lack of passage of astaxanthin in buffer A through 10, 30 and 50 K NMWL ultrafilters even at a lower concentration of astaxanthin (2.23 μM) further confirmed that astaxanthin aggregates in aqueous buffer. The observation of astaxanthin aggregates in aqueous media is consistent with those of Buchwald and Jencks (1968). The passage of astaxanthin through 0.22 and 0.45 μm filters decreased remarkably at the higher concentration of astaxanthin suggesting an increased size of astaxanthin aggregates at the higher astaxanthin concentration. The changes in astaxanthin passage through the Durapore filter (0.22 and 0.45 μm) in presence of higher concentration of Tris-HCl in buffer A suggests that buffer composition influences astaxanthin aggregation.

The present study then focused on reducing the size of astaxanthin aggregates in aqueous buffer for the separation of free astaxanthin from bound astaxanthin by the ultrafiltration method. This was done to characterize the binding of astaxanthin to muscle proteins of Atlantic salmon. Different bile salts were tested to reduce the size of astaxanthin aggregates by dispersing them in aqueous solution so that dispersed astaxanthin would pass through a low molecular cut-off filter and thus allow the separation of free (unbound) astaxanthin from protein-bound astaxanthin. High critical micelle concentration (CMC) and low aggregation number were key criteria for the selection of bile salts to achieve that target of separation. Bile salts have a low aggregation number and thus will form small micelles which should pass through a low molecular cut-off filter. The aggregation number and micellar molecular weight of sodium cholate was reported to be 2 and 0.9×10^3 respectively, whereas, it was 140 and 90×10^3 for Triton X-100 and 60 and 76×10^3 for Tween 80 (Helenius et al., 1979). It was reported that the aggregation number increased from 2.8 to 4.8 for sodium cholate and 2.2 to 22 for sodium deoxycholate, when NaCl concentration increased from 10 mM to 150 mM (Helenius et al., 1979). Considering the high CMC value [10×10^{-3} mol L^{-1} (Helenius et al., 1979)] and low aggregation number, sodium cholate were selected in order to disperse astaxanthin aggregates in aqueous solutions for use in protein-astaxanthin binding assay condition. Lockwood et al. (2003) reported that the solubility of astaxanthin in water increased 71-fold in presence of 40% Captisol® (sulfobutyl ether β-cyclodextrin sodium) when astaxanthin was presolubilized in methanol. However, the

size of dispersed astaxanthin was in the range of nearly 1 μm since the researchers filtered dispersed astaxanthin solution through the 1 μm filter before the estimation of solubilized astaxanthin. Therefore, dispersion of astaxanthin in aqueous buffer using Captisol® does not have applicability in separating free astaxanthin from protein-bound astaxanthin.

The lower astaxanthin passage obtained in sodium taurodeoxycholate might be caused by the formation of larger micelles since the aggregation number of this bile salt is higher than that of sodium cholate and sodium taurocholate. The aggregation number of sodium taurodeoxycholate and sodium taurocholate is reported to be 8 and 4, respectively in H_20, and 24 and 5, respectively, in presence of 150 mM NaCl (Helenius et al., 1979). Solubility of retinoids increased several orders of magnitude in 10 mM sodium taurocholate, though the size of the solubilized micellar complexes for retinoids was not reported (Li et al., 1996). Further solubilization of retinoids was accomplished in mixed micelles containing sodium taurocholate and egg phosphatidylcholine (Li et al., 1996). For example, etretinate solubility was 1.8 μM in 10 mM sodium taurocholate and increased to 163 μM in mixed micelles of 10 mM sodium taurocholate and 10 mM egg phosphatidylcholine. Mixed micelles were not feasible for the purposes of the dispersion because of their increased size. Hence a higher bile salt concentration was used to achieve further solubility of astaxanthin. Poorly soluble drugs have been shown to have an increased solubility with increased bile salt concentration (Mithani et al., 1996).

Sodium cholate at a concentration of 200 mM was found to reduce the size of astaxanthin aggregates in aqueous solution. The binding assay between astaxanthin and salmon muscle protein in the presence of 200 mM cholate allowed the separation unbound (free) astaxanthin from protein-bound astaxanthin using a 30 K NMWL filter. In the present study, it was observed that astaxanthin binding of actin did not show saturable binding kinetics at the astaxanthin concentrations used in the assay (Fig. 3.6). As a result, solubilized fractions of salmon muscle proteins were used for the binding assay to investigate whether other muscle protein(s) bind astaxanthin. Bound astaxanthin in fractions A and B was 1.05 and 1.09 mg g^{-1} protein, respectively (Table 3.1). Though fractions A and B contained trace amounts of actin (Fig. 3.2), the bound astaxanthin obtained in each fraction was similar to that of purified actin (1.19 mg g^{-1} protein) when

incubated with the same concentration of astaxanthin. This phenomenon suggests that proteins in fractions A and B other than actin are also able to bind astaxanthin. Binding of astaxanthin by all solubilized fractions of salmon muscle proteins suggest that astaxanthin bound not only to actomyosin and/or actin, but to other muscle proteins as well. For example, fractions C, D and E contained similar amounts of actin and fraction E contained very little myosin, yet astaxanthin binding was significantly lower in fraction E. In addition, only fraction F contained a significant amount of myosin, but the similar extent of binding found in fraction F and D further suggest that other proteins are responsible for binding astaxanthin in fraction D. Interestingly, a 150 kDa protein was observed only in fractions C, D and F, the fractions having the highest binding level of astaxanthin. Further characterization of proteins from these fractions is needed to elucidate the major astaxanthin binding protein in salmon flesh.

Astaxanthin-binding curves of three different fractions (B, D and F) did not show saturation kinetics within the working concentrations of astaxanthin used in this study. Non-saturable astaxanthin-binding curves obtained in this study can be explained by two possible hypotheses. The first hypothesis is that the astaxanthin binding is non-specific to salmon muscle proteins. Henmi et al. (1989) proposed that carotenoids bind with one β-ionone ring to a hydrophobic binding site on actomyosin in salmon muscle. Henmi et al. (1991) later reported that binding of salmon muscle actomyosin to carotenoids was non-specific. However, their conclusion was not based on kinetic binding experiments but on the basis of observed correlation between the surface hydrophobicity of actomyosin and combination of astaxanthin and/or canthaxanthin with actomyosin (Henmi et al., 1990a) and on the binding characteristics of carotenoids with bovine serum albumin and egg albumin (Henmi et al., 1991). The second hypothesis is that the affinity of salmon muscle proteins for astaxanthin is low and the Kd may be much higher than the astaxanthin concentrations used in the present study. At higher astaxanthin concentrations, we found that astaxanthin is significantly retained by the filter (30 K NMWL) due to the formation of larger astaxanthin aggregates and this precluded use of higher astaxanthin concentrations. Differential binding is suggested by the slopes observed for astaxanthin binding by actin and the proteins in each of fractions B, D and F. The differences in binding may be due to differences in hydrophobicity of proteins in

these various fractions. From an investigation of different fish species, Henmi et al. (1990a) observed that actomyosins with high surface hydrophobicity bind more astaxanthin and canthaxanthin and inferred that these carotenoids bind non-specifically to the hydrophobic binding sites of actomyosin. In the current study, proteins other than actomyosin showed their ability to bind astaxanthin. In Henmi's studies, only actomyosin was used for the binding studies and among the white-fleshed fish species examined, common mackerel showed higher carotenoid binding than salmonids (Henmi et al., 1990a). However, their work does not account for astaxanthin binding to other muscle proteins, some of which may have a higher affinity for astaxanthin.

One limitation of the current method is that the sodium cholate used to disperse astaxanthin aggregates may interfere with astaxanthin binding to proteins. Due to the hydrophobic nature of astaxanthin, it was impossible to separate bound and unbound astaxanthin without this bile salt.

It was thought that low molecular weight proteins may pass through the 30 K NMWL filter and may underestimate astaxanthin binding by the muscle proteins fractions in the present study. However, the presence of only one protein band at very low levels in the filtrate of all fractions indicates that almost all the proteins of each fraction are retained by the 30 K NMWL filter. It is assumed that very minor amount of protein passed through the filter because the intensities of protein bands were obtained upon mixing 3 volumes of filtrate with 1 volume of SDS-PAGE sample buffer to obtain more protein in the loaded volume. The 1:1 ratio of the filtrate and SDS-PAGE sample buffer would have lowered the intensities of protein bands. No detectable protein concentration in the filtrates also supports that very minor amount of protein passed through the filter. The method developed in the present study is promising for the characterization of astaxanthin binding to purified proteins that would not pass through a 30 K NMWL filter.

Future work using this methodology may also demonstrate the binding affinity of proteins for astaxanthin and other carotenoids in Atlantic salmon muscle. Furthermore, this method can also be used to test the hypothesis that whether astaxanthin binding to muscle proteins is the limiting factor in deposition of astaxanthin in the flesh of juvenile salmon or non-pigmented fish species, such as halibut.

CHAPTER 4

Comparative study of astaxanthin binding to solubilized muscle proteins of Atlantic salmon (*Salmo salar* L.), haddock (*Melanogrammus aeglefinus* L.) and Atlantic halibut (*Hippoglossus hippoglossus* L.)

4.1 Abstract

A study was conducted to compare astaxanthin binding ability of solubilized muscle proteins of Atlantic salmon (*Salmo salar* L.), haddock (*Melanogrammus aeglefinus* L.) and Atlantic halibut (*Hippoglossus hippoglossus* L.). Muscle proteins of juvenile Atlantic salmon, haddock and halibut were solubilized by sequential extraction of muscle tissue using low ionic strength solutions. Electrophoretic protein profiles of the fractions from these species were similar. Each solubilized fraction from the three species was examined for its relative astaxanthin binding capacity. The amount of bound astaxanthin was significantly different ($P<0.05$) among the six fractions of each species. Significant differences in astaxanthin binding were only found for fractions A and E among the species. The amount of bound astaxanthin in various fractions of each species showed a good correlation ($R^2 = 0.80$-0.92) with the ANS (8-anilino-1-naphthalenesulfonate) fluorescence intensity of those fractions. The pattern and extent of astaxanthin binding to the muscle proteins of juvenile salmon, haddock and halibut is comparable to that reported previously for adult Atlantic salmon (Saha et al., 2005). These combined observations suggest that the carotenoid binding capacity of the muscle proteins of salmon is not the limiting factor in the deposition of carotenoid in their flesh.

4.2 Introduction

Fish, like all other animals, are unable to synthesize carotenoids *de novo* and must obtain carotenoids from dietary sources. Among fish species, salmonids have the unique ability to deposit dietary carotenoids in their muscle tissues that provide their distinctive red flesh coloration. In contrast to adult salmonids, juvenile salmonids (< 200 g) have

limited ability to deposit dietary carotenoids in their muscle (Torrissen et al., 1989; Hatlen et al., 1995) and deposit carotenoids mostly in their skin (Torrissen et al., 1989).

Several reviews have been published on carotenoid utilization and metabolism by salmonids (Torrissen et al., 1989; Storebakken and No, 1992; Shahidi et al., 1998; Bjerkeng, 2000). Most of these studies focused on the deposition of dietary carotenoids (astaxanthin and canthaxanthin) in flesh. Meanwhile, very little information is available on the astaxanthin binding ability of the muscle proteins of salmonids. Henmi et al. (1987, 1989) reported that astaxanthin and canthaxanthin bind to actomyosin of salmon muscle. The binding of carotenoids to actomyosin from salmon (Henmi et al., 1989) and from other salmonid and white-fleshed fish species (Henmi et al., 1990a) has been examined. However, Henmi et al. (1989, 1990a) used a very high concentration of actomyosin (800 mg mL^{-1}), which was not in soluble form and hence carotenoids may have bound to exposed surfaces of insoluble actomyosin. In addition, these studies did not include other muscle proteins, which may have higher affinity for astaxanthin. We recently reported the binding of astaxanthin to solubilized muscle proteins of adult Atlantic salmon (*Salmo salar* L.) and the findings suggested that proteins other than actomyosin also bind astaxanthin in Atlantic salmon muscle (Saha et al., 2005).

Carotenoids accumulate in the integument, egg and liver of different species of white-fleshed fish (Katsuyama and Matsuno, 1988; Matsuno and Hirao, 1989; Tsushima et al., 2002). Carotenoids were detected in the plasma, muscle, liver and whole kidney of white-fleshed Atlantic halibut after feeding with a diet supplemented with astaxanthin but astaxanthin deposition in halibut muscle was negligible (Bjerkeng and Berge, 2000).

Binding of astaxanthin to the muscle proteins of juvenile Atlantic salmon has not previously been investigated. Information on the astaxanthin binding capacity of the muscle proteins of juvenile salmon is important to determine whether the lower deposition of dietary carotenoids in muscle of juvenile salmon is related to the carotenoid (astaxanthin) binding ability of their muscle proteins.

The present study was undertaken to investigate the astaxanthin binding capability of solubilized muscle proteins of juvenile Atlantic salmon, haddock (*Melanogrammus aeglefinus* L.) and Atlantic halibut (*Hippoglossus hippoglossus* L.) for comparison with adult salmon in order to examine whether retention of astaxanthin is related to the

astaxanthin binding affinity of soluble muscle proteins. The correlation between the amount of bound astaxanthin and ANS fluorescence intensity of all the solubilized muscle protein fractions of the three species was also tested.

4.3 Materials and methods

4.3.1 Fish samples

Atlantic salmon (Saint John River strain) smolts were reared in seawater at the National Research Council's Marine Research Station (NRC), Sandy Cove, Nova Scotia. Haddock were produced at the marine fish hatchery facility of NRC and reared at the NRC. Atlantic halibut were purchased from R & R Finfish, Digby, Nova Scotia and held at the NRC. Atlantic salmon were fed with Signature Salmon Ration (Shur-Gain Feeds, Truro, Nova Scotia). Haddock and halibut were fed with commercial diets for haddock and halibut (Shur-Gain Feeds, Truro, Nova Scotia), respectively. Salmon, haddock and halibut with average weights of 0.093, 0.45 and 1.6 kg, respectively were used for this study. Fish were anaesthetized with tricaine methanesulfonate (TMS; 50 mg L^{-1}) and transported on ice to the laboratory (30 minutes). Fish were decapitated, bled and gutted immediately after the arrival at the laboratory. Immediately after killing the fish, muscle tissue was excised from the mid-dorsal part of the fish and extracted.

4.3.2 Extraction of muscle proteins

Muscle proteins of juvenile Atlantic salmon, haddock and Atlantic halibut were solubilized into six different fractions (A-F) by sequential extraction of ground muscle using low ionic strength solutions as described elsewhere (Saha et al., 2005). After each extraction, the solubilized protein fraction was separated by centrifugation (10,000 *g*) and the pellet was extracted sequentially with the subsequent buffers. Fraction A was obtained by the homogenization of ground muscle using 150 mM NaCl, whereas fractions B-D were obtained by sequential extraction of the pellet three times with 25 mM NaCl and 5 mM histidine, pH 7.4. Fractions E and F were obtained by extraction of the pellet with 2.5 mM NaCl and 5 mM histidine (pH not adjusted) and deionized water, respectively. Mid-dorsal muscle tissues from two juvenile salmon were pooled together to obtain sufficient ground muscle for the solubilization procedure, whereas muscle

tissues from three haddock were pooled. The solubilization protocol repeated six times for each species. Protein concentration was determined by the method of Bradford (1976) using bovine γ-globulin (Bio-Rad Laboratories, Mississauga, ON) as a standard. To determine the total protein content of the muscle, ground muscle (4 g) was extracted with 50 mL of 0.1 N NaOH and the protein content of the homogenate was determined. The yield of solubilized muscle protein was calculated from the ratio of the total amount of proteins solubilized in six fractions with the total protein content of the muscle.

4.3.3 Electrophoresis

The fractions of solubilized muscle proteins of the three fish species were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% polyacrylamide slab gel according to the method of Laemmli (1970). Precision Plus protein standards (Bio-Rad Laboratories) were used as molecular mass standards. Gels were stained with 0.1% Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories). At the end of the muscle protein extraction procedure, the residual pellets of haddock, halibut and the pellet of adult salmon from a previous study (Saha et al., 2005) stored at – 80 °C were homogenized in deionized water and subjected to SDS-PAGE to compare the proteins remaining in the pellets.

4.3.4 Protein-astaxanthin binding studies

All experiments with astaxanthin were performed under yellow light. The binding studies between astaxanthin (F. Hoffmann-La Roche Ltd., Basel, Switzerland) and various fractions (A-F) of solubilized muscle proteins from salmon, haddock and halibut were conducted according to the ultrafiltration method developed in this laboratory for the separation of free and protein-bound astaxanthin (Saha et al., 2005). The protein concentrations of fractions A to F from different fish were adjusted to concentrations between 1.4-1.5 mg mL^{-1} by diluting concentrated fractions with the respective extraction buffer and concentrating more diluted fractions with an Ultrafree Biomax- 5 K filter [5 K nominal molecular weight limit (NMWL); Millipore, Bedford, MA]. Each of the solubilized muscle protein fractions and 400 mM sodium cholate were mixed in a 1:1 ratio in a glass vial with a total volume of 500 μL to give final protein

concentrations between 0.70-0.75 mg mL^{-1}. Ten μL of astaxanthin dissolved in tetrahydrofuran (HPLC grade) was added to the mixed sample to give a final astaxanthin concentration of 13.4 μM and incubated for 30 minutes at room temperature followed by a brief vortexing. Protein-bound and unbound (free) astaxanthin were separated using Ultrafree-MC filter of 30 K NMWL (regenerated cellulose membrane; Millipore). Control experiments were conducted using each of the extraction buffers used for the solubilization of muscle proteins. Absorbance values of bound astaxanthin were transformed into molar concentrations using the experimentally determined molar extinction coefficient of astaxanthin (1.12×10^5 M^{-1} cm^{-1} at 485 nm) in presence of 200 mM sodium cholate and the amount of astaxanthin bound to each g of protein was calculated. Binding studies were performed on fractions from six solubilization experiments of each species.

4.3.5 ANS fluorescence intensity of solubilized muscle proteins

The protein concentrations of solubilized muscle protein fractions (A-F) from all the three species were adjusted to 0.2 mg mL^{-1} by diluting with the corresponding extraction buffers. Hemimagnesium salt of 8-anilino-1-naphthalenesulfonic acid (ANS) was dissolved in each of the extraction buffers at a concentration of 8.0 mM. The protein fraction (2 mL) was placed in a cuvette and allowed to stand for 5 minutes. Temperature in the cuvette was maintained at 16 °C. Ten μL of ANS was added to the protein fraction and mixed thoroughly. After incubating 2 minutes at 16 °C, the protein-ANS mixture was excited at 388 nm (8 nm band pass) and the fluorescence intensity value was measured at the emission wavelength of 473 nm (8 nm band pass) using an Aminco-Bowman Series 2 luminescence spectrometer (Spectronic Instrument Inc., Rochester, NY). Fluorescence intensity values of the blanks were measured for each protein fraction in absence of ANS and each extraction buffer in presence of ANS.

4.3.6 Statistical analyses

Homogeneity of variance was tested by Bartlett's test and the error residuals by Lillifers test. Data were analysed by two-way analysis of variance with six different fractions of muscle protein and three different fish species (Sokal and Rohlf, 1995) using

Systat 10 (2000). Comparisons of bound astaxanthin means of six different fractions (A-F) were carried out using the Bonferroni multiple comparison procedure. Tukey's multiple comparisons were used to compare the bound astaxanthin means of each fraction among the three species. Confidence level for the mean was 0.95 and differences at $P<0.05$ were considered significant. Results are presented as mean and standard deviation (mean ± SD).

4.4 Results

4.4.1 Solubility of muscle proteins

Similar solubilities of muscle proteins from salmon (71.93 ± 1.29%) and haddock (70.87 ± 2.13%) were achieved in this study. However, significantly higher ($P<0.05$) solubility of muscle proteins was obtained in halibut (76.97 ± 1.55%) than the other two species.

4.4.2 Electrophoresis

The SDS-PAGE profiles of solubilized muscle proteins of juvenile Atlantic salmon, haddock and Atlantic halibut are shown in Figs. 4.1-4.3, respectively. Myosin and actin were the major proteins solubilized in the fraction F of each species. A higher solubility of actin was observed in fractions C-F of each species when compared to fractions A and B. The SDS-PAGE profiles of solubilized muscle proteins of salmon, haddock and halibut were very similar with a few differences in the pattern of the solubility of individual proteins. A 38 kDa protein was solubilized in all fractions of salmon (Fig. 4.1), whereas this protein was not solubilized in fractions E and F of both haddock (Fig. 4.2) and halibut (Fig. 4.3). In halibut, a 35 kDa protein was uniformly solubilized in all fractions (A-F). However, a relatively higher amount of 35 kDa protein was solubilized in the fraction E of haddock and fraction A of salmon than in the other fractions of the same species. Trace amounts of myosin were evident in fractions B-E of salmon (Fig. 4.1) and halibut (Fig. 4.3); however, myosin was barely detectable or absent in the similar fractions of haddock (Fig. 4.2). Relatively higher amounts of 100-115 kDa proteins were solubilized in fractions B-D of haddock than in the similar fractions of juvenile salmon and halibut.

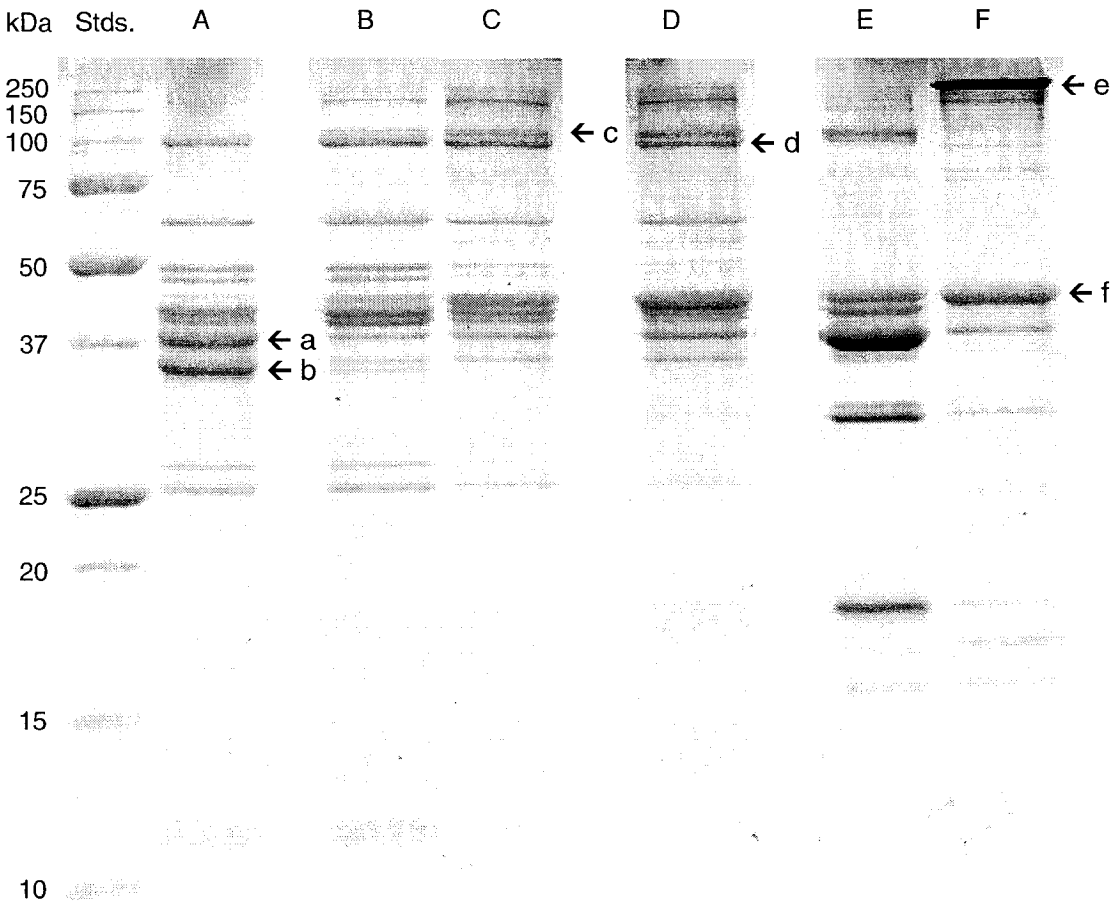

Fig. 4.1 SDS-PAGE (12% gel) protein profiles of solubilized muscle proteins fractions of juvenile Atlantic salmon (*Salmo salar* L.). Molecular weights of standards are shown on the left. Letters on the top of the lanes indicate different muscle fractions. Fraction A, homogenization of muscle with 150 mM NaCl; fractions B-D, sequential extraction of the pellet with 25 mM NaCl, 5 mM histidine (pH 7.4); fraction E, extraction of the pellet with 2.5 mM NaCl, 5 mM histidine; and fraction F, extraction of the pellet with cold de-ionized water. Individual proteins band are marked as a, a 38 kDa protein; b, a 35 kDa protein; c, a 115 kDa protein; d, a 100 kDa protein; e, myosin and f, actin. Total 9.45 µg of protein was loaded in each lane.

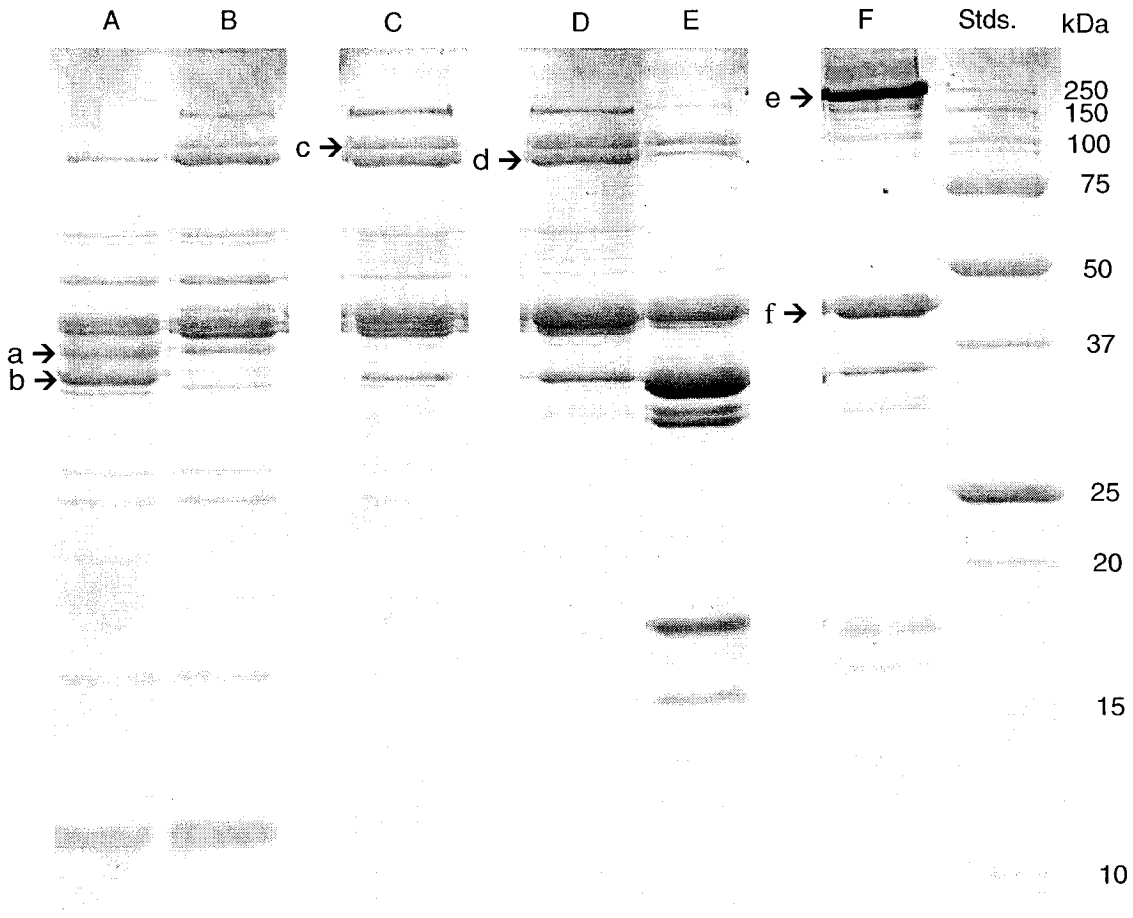

Fig. 4.2 SDS-PAGE (12% gel) protein profiles of solubilized muscle proteins fractions of haddock (*Melanogrammus aeglefinus* L.). Molecular weights of standards are shown on the right. Letters on the top of the lanes indicate different muscle fractions and details of the fractions were mentioned in Fig. 4.1. Individual protein bands are marked similarly as in Fig. 4.1. Total 9.45 μg of protein was loaded in each lane.

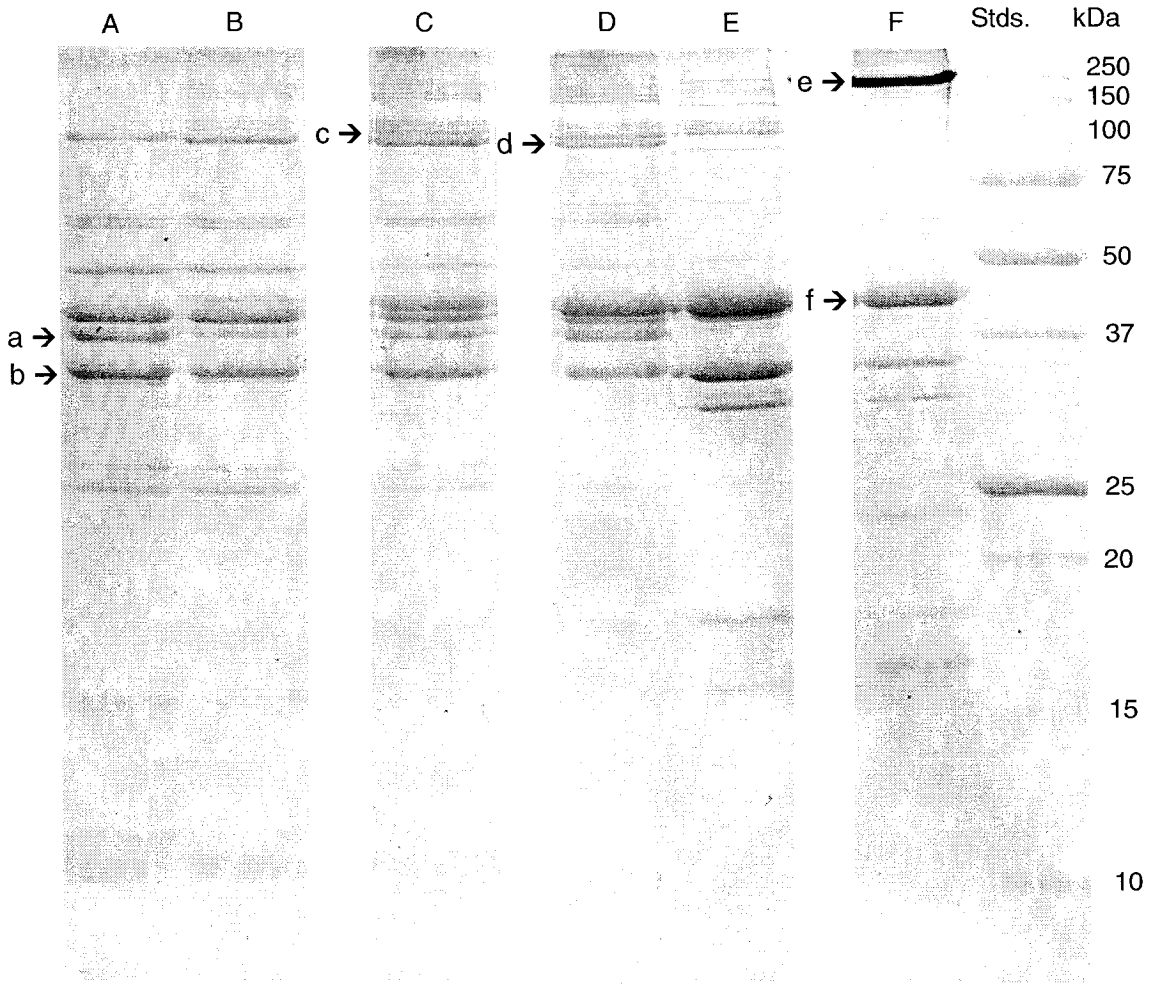

Fig. 4.3 SDS-PAGE (12% gel) protein profiles of solubilized muscle protein fractions of Atlantic halibut (*Hippoglossus hippoglossus* L.). Molecular weights of standards are shown on the right. Letters on the top of the lanes indicate different muscle fractions and details of the fractions were mentioned in Fig. 4.1. Individual protein bands are marked similarly as in Fig. 4.1. Total 9.45 μg of protein was loaded in each lane.

The SDS-PAGE profiles of the pellets (Fig. 4.4) confirmed that actin and myosin were the major proteins remaining in the pellet after the muscle protein extraction procedure. The SDS-PAGE profiles of the pellets of both haddock and halibut were similar to that of fraction F of these species.

4.4.3 Astaxanthin binding to solubilized muscle proteins

The amounts of astaxanthin bound to different solubilized muscle protein fractions from three fish species are presented in Table 4.1. No significant differences ($P>0.05$) in bound astaxanthin of each fraction of B, C, D and F were observed among Atlantic salmon, haddock and halibut. However, astaxanthin bound to both fractions A and E showed significant difference ($P< 0.05$) among the three fish species. Fraction A of Atlantic halibut showed significantly higher level of bound astaxanthin (2.17 ± 0.31 mg astaxanthin g^{-1} protein) than that of salmon and haddock, whereas no significant difference in bound astaxanthin concentration of fraction A was observed between salmon and haddock. Fraction E of halibut also showed a significantly higher ($P<0.05$) amount of bound astaxanthin (2.27 ± 0.52 mg g^{-1} protein) than that of salmon (1.71 ± 0.09 mg g^{-1} protein). However, astaxanthin bound to fraction E was not significantly different ($P>0.05$) between halibut and haddock or between salmon and haddock.

The amount of bound astaxanthin in six different fractions of solubilized muscle proteins of salmon, haddock and halibut (Table 4.1) were significantly different ($P<0.05$). Fraction B of all three different species showed the lowest amount of bound astaxanthin.

No significant difference was observed between the bound astaxanthin of fractions A and B in both Atlantic salmon and haddock. However, in halibut, the amount of bound astaxanthin of fraction A was significantly higher than that of fraction B and there were no significant difference among the bound astaxanthin concentration of fractions A, C and E. An increasing trend in astaxanthin binding was observed from fraction B towards fraction D of all the three species and then decreased in fraction E, which was followed by a rapid increase in astaxanthin binding in fraction F. Bound astaxanthin in fraction F of each of the three species was significantly higher ($P<0.05$) than that of any other fraction. The amounts of bound astaxanthin of fraction F of

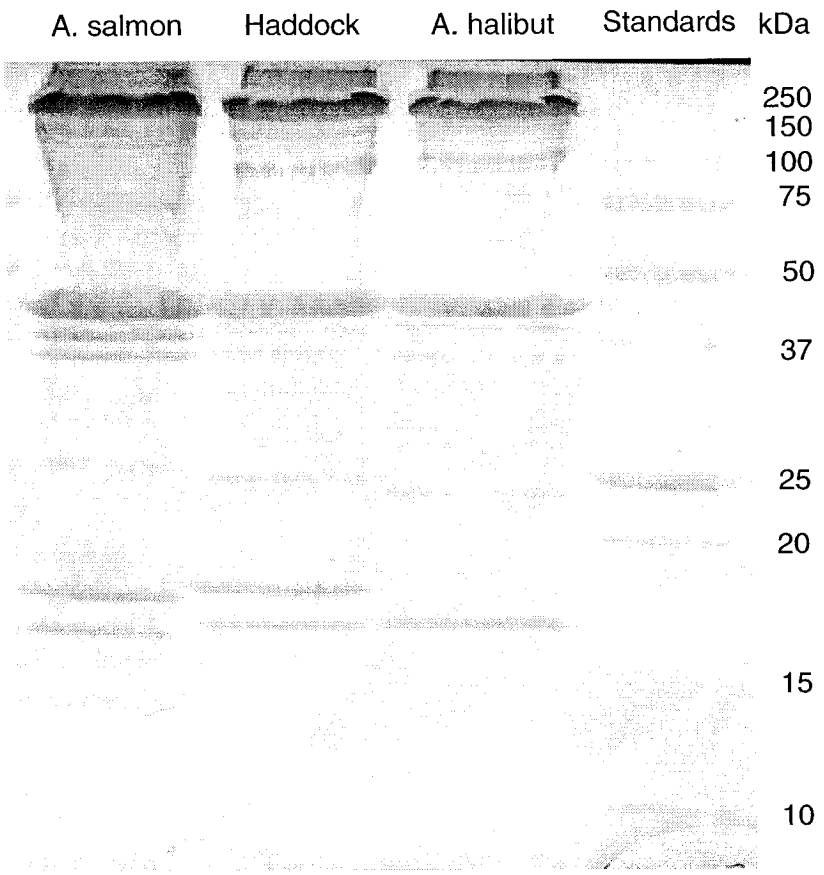

Fig. 4.4 The SDS-PAGE protein profiles on a 12% polyacrylamide gel of the residual pellets of adult Atlantic salmon from the previous study, haddock and halibut at the end of the muscle proteins solubilization procedure. Approximately 20.5 μg of protein was loaded in each lane. Molecular weights of Precision Plus protein standards are shown on the right. Gel was stained with 0.1% Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories) to visualize the protein bands.

Table 4.1 Amount of bound astaxanthin in different fractions of solubilized muscle proteins of juvenile Atlantic salmon, haddock and halibut at a final astaxanthin concentration of 13.4 μM

Protein fractions	Bound astaxanthin* (mg g^{-1} protein)		
	Atlantic salmon	Haddock	Atlantic halibut
A	1.29 ± 0.09^{Ye}	1.56 ± 0.35^{Ycd}	2.17 ± 0.31^{Xc}
B	1.25 ± 0.12^{e}	1.40 ± 0.23^{d}	1.59 ± 0.39^{d}
C	2.33 ± 0.20^{c}	2.43 ± 0.35^{b}	2.55 ± 0.29^{bc}
D	2.85 ± 0.39^{b}	2.71 ± 0.54^{b}	2.85 ± 0.46^{b}
E	1.71 ± 0.09^{Yd}	1.95 ± 0.24^{XYc}	2.27 ± 0.52^{Xc}
F	3.31 ± 0.23^{a}	3.38 ± 0.28^{a}	3.37 ± 0.28^{a}

* Mean ± SD; n = 6

Means with different uppercase superscripts (X, Y) indicate significant differences ($P<0.05$) among the species within a row, while lowercase superscripts (a, b, c, d, e) within a column indicate significant differences among the fractions.

Atlantic salmon, haddock and halibut were 3.31 ± 0.23, 3.38 ± 0.28 and 3.37 ± 0.28 mg g^{-1} protein, respectively. In salmon, the bound astaxanthin levels of fractions E and D were significantly higher than that of fractions A and C, respectively. However, in both haddock and halibut, bound astaxanthin was not significantly different between fractions E and A or between fractions D and C.

4.4.4 ANS fluorescence intensity of solubilized muscle proteins

For each species, the amount of astaxanthin bound to various muscle proteins fractions were plotted against the relative ANS fluorescence intensities of those muscle proteins fractions. A good correlation (R^2= 0.92 for salmon; R^2= 0.87 for haddock; R^2= 0.80 for halibut) was observed between the bound astaxanthin of different fractions of muscle proteins and the relative ANS fluorescence intensities of those fractions (Figs. 4.5-4.7). Fractions A, B and E of all the three species gave lower fluorescence intensity than fractions C, D and F.

4. 5 Discussion

4.5.1 Electrophoresis

The SDS-PAGE profiles of the solubilized muscle proteins of juvenile salmon, haddock and halibut were similar. The protein profiles of the solubilized juvenile salmon muscle proteins were similar to those of muscle proteins solubilized from adult salmon previously (Saha et al., 2005) except for the presence of trace amount of myosin in fractions B-D of juvenile salmon muscle detected in this study. The protein profiles of the pellet remaining after the muscle protein extraction from adult salmon, haddock and halibut were similar as determined by the SDS-PAGE with the profiles of haddock and halibut pellets being similar to those of the fraction F of these species.

4.5.2 Astaxanthin binding to solubilized muscle proteins

The astaxanthin-binding curves of three different fractions (B, D and F) from adult Atlantic salmon were not saturated at astaxanthin concentrations ranged from 3.35 μM to 16.75 μM (Saha et al., 2005). Non-saturated astaxanthin-binding curves can be explained by either the non-specific binding of astaxanthin to muscle protein or the low

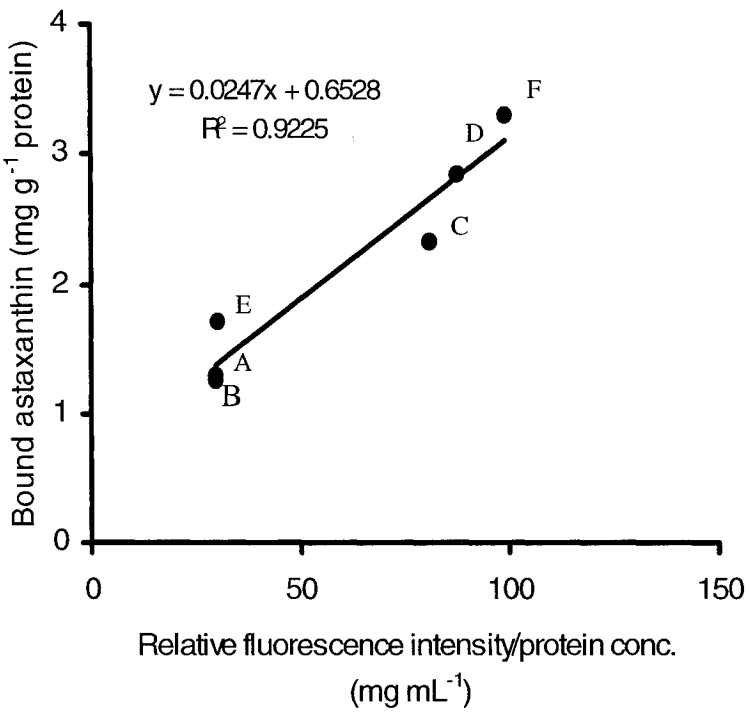

Fig. 4.5 Correlation between relative ANS fluorescence intensity of solubilized muscle protein fractions of juvenile Atlantic salmon and the amount of astaxanthin bound to those muscle proteins fractions. A- F indicates different fractions of solubilized muscle proteins.

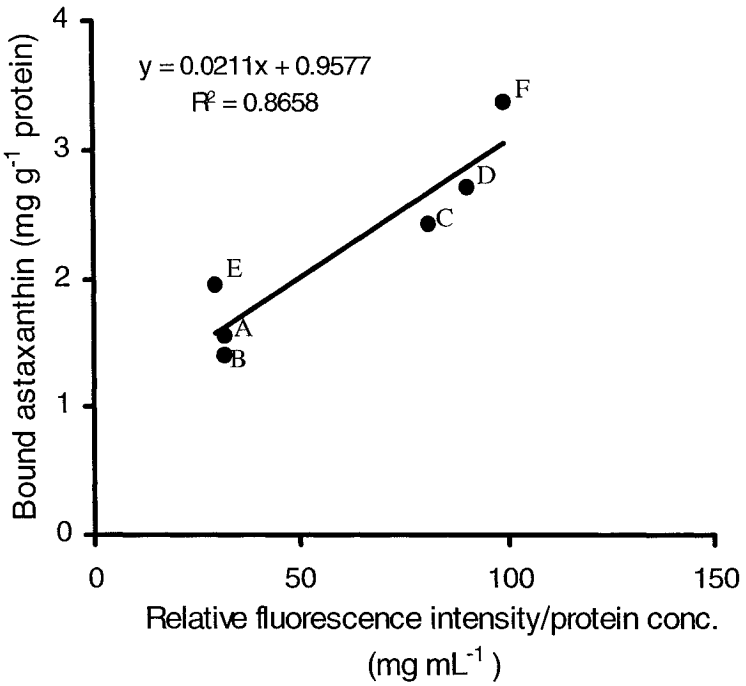

Fig. 4.6 Correlation between relative ANS fluorescence intensity of solubilized muscle protein fractions of haddock and the amount of astaxanthin bound to those muscle proteins fractions. A- F indicates different fractions of solubilized muscle proteins.

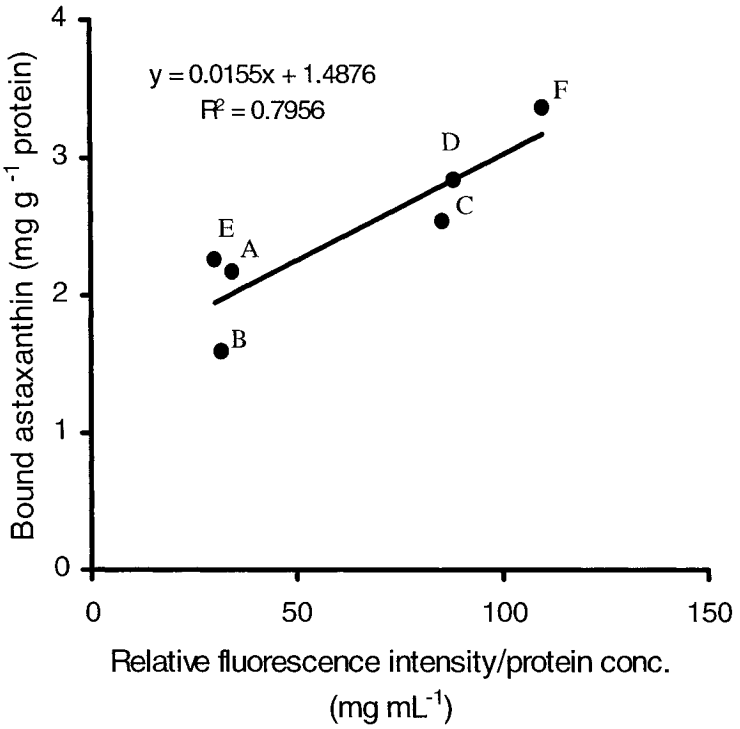

Fig. 4.7 Correlation between relative ANS fluorescence intensity of solubilized muscle protein fractions of Atlantic halibut and the amount of astaxanthin bound to those muscle proteins fractions. A- F indicates different fractions of solubilized muscle proteins.

affinity of muscle protein for astaxanthin and much higher Kd than the astaxanthin concentrations used in the study. Astaxanthin was significantly retained by the filter (30 K NMWL) at higher astaxanthin concentrations due to formation of larger astaxanthin aggregates and thus limited the use of higher astaxanthin concentration for the binding study (Saha et al., 2005). Therefore, a single concentration of astaxanthin (13.4 μM) was used in this study to compare the relative astaxanthin binding of the muscle proteins of juvenile Atlantic salmon, haddock and halibut. The relative pattern of astaxanthin binding among the six different fractions of juvenile salmon muscle proteins observed in the current study are similar to that of adult salmon (average weight of 1.3 kg) muscle proteins reported previously (Saha et al., 2005). Moreover, the level of bound astaxanthin in all the fractions of juvenile salmon muscle proteins was slightly higher than that of adult salmon muscle proteins. The amounts of bound astaxanthin (mean ± S.D) in fractions A to F of adult salmon were 1.05 ± 0.19, 1.09 ± 0.03, 2.38 ± 0.29, 2.60 ± 0.3, 1.62 ± 0.53 and 2.95 ± 0.08 mg g^{-1} protein, respectively (Saha et al., 2005). The similarity in astaxanthin binding to the muscle proteins of adult and juvenile Atlantic salmon contrasts significantly with the reported differences in muscle astaxanthin concentration of juvenile and adult salmon. Storebakken et al. (1987) reported no astaxanthin deposition in the flesh of Atlantic salmon that grew from 62 to 81 g after 8 weeks of feeding with diets supplemented with different levels of astaxanthin. However, a minor deposition of astaxanthin in the flesh was detected at the next sampling following 16 weeks of feeding when salmon reached a body weight about 120 g (Storebakken et al., 1987). Hatlen et al. (1995) observed that Arctic charr (*Salvelinus alpinus*) of 1+ year class were less efficient in flesh pigmentation than the older fish after feeding three year classes of fish with diets containing two dietary astaxanthin levels. Torrissen et al. (1989) suggested that salmonids do not deposit carotenoids in their flesh efficiently until they attain a minimum body weight and this weight varies among salmonids. Accumulation of carotenoids in the skin of small salmonids demonstrates their ability to absorb dietary carotenoids. March et al. (1990) proposed that small salmonids may have limited ability in transport carotenoids from the blood plasma to the muscle or to retain carotenoids in the flesh. Our findings strongly supports this view since the amount of astaxanthin bound to various fractions of solubilized muscle proteins of juvenile salmon

was higher than those of adult salmon as reported previously (Saha et al., 2005). Astaxanthin and canthaxanthin are the main carotenoids in the flesh of salmonids; however, idoxanthin, a metabolite of astaxanthin (Schiedt et al., 1985) accumulates more in the flesh of smaller salmonids than in larger salmonids (Schiedt et al., 1989; Aas et al., 1997). The poor flesh pigmentation of salmonids may be caused by rapid metabolism of absorbed astaxanthin to colorless derivatives (March et al., 1990).

Haddock and Atlantic halibut are white-fleshed fish and do not accumulate carotenoids in their flesh. The findings demonstrated that astaxanthin binding ability of the solubilized muscle proteins of white-fleshed fish species was comparable to that of juvenile and adult salmon. It is well known that deposition of carotenoid is associated with the absorption of dietary carotenoid from the digestive tract and the metabolic transformations of dietary carotenoid. Bjerkeng and Berge (2000) reported that the retention of astaxanthin was 3.9% in the muscle of salmon and nil in the muscle of halibut after feeding a diet containing 66 mg astaxanthin kg^{-1} dry matter. They suggested that the lower retention of astaxanthin in halibut than salmon might be due to higher metabolic transformation of dietary astaxanthin (Bjerkeng and Berge, 2000). Information is not available on the absorption of dietary carotenoid or carotenoid metabolism in haddock. Wild haddock feed on crustaceans, which constituted 10% of the total stomach content of animals ranging in size from 200 g to 6 kg caught from the Scotian Shelf of Atlantic Canada (Fishermen and Scientists Research Society/DFO 2000, unpublished data). In crustacea, a relatively large proportion of astaxanthin is in the esterified form (Torrissen et al., 1989). Therefore, the lack of flesh pigmentation of haddock *in vivo* may be caused by several factors including the limited digestion and absorption of astaxanthin esters, inability to transport astaxanthin to extrahepatic tissues and its metabolic transformation in liver. It has been shown that transport of lipid from the liver to the muscle is limited in haddock due to low levels of serum very low density lipoprotein, VLDL (Nanton, 2002).

4.5.3 ANS fluorescence intensity of solubilized muscle proteins

Astaxanthin and canthaxanthin have been proposed to bind to the hydrophobic binding sites on actomyosin (Henmi et al., 1989). Henmi et al. (1990a) reported a

correlation between the surface hydrophobicity of actomyosin of different fish species and binding of carotenoids with those actomyosins and presumed that carotenoids bind non-specifically to the hydrophobic binding sites on actomyosin. The hydrophobic fluorescent probe ANS has been used to determine the hydrophobicity of different proteins (Li-Chan et al., 1985; Chan et al., 1992; Careche and Li-Chan, 1997). In the current study, the protein fractions showing the highest relative ANS fluorescence intensities were able to bind the highest amount of astaxanthin. The correlation between the relative ANS fluorescence intensities of various fractions of solubilized muscle proteins and the amount of bound astaxanthin further supports the hypothesis that astaxanthin binds non-specifically to the hydrophobic binding sites on proteins.

Interestingly, the SDS-PAGE profiles, ANS fluorescence intensity and astaxanthin binding to the individual fractions from different species were similar suggesting that the muscle proteins of the different species were similarly extracted in this study.

4.6 Conclusion

The pattern of astaxanthin binding among the fractions derived from three species was similar and no striking difference in the astaxanthin binding ability of muscle proteins was observed among juvenile Atlantic salmon, haddock and Atlantic halibut at a final astaxanthin concentration of 13.4 μM. The extent and pattern of astaxanthin binding to muscle proteins of these fish species was similar to that of adult salmon at the same astaxanthin concentration (Saha et al., 2005). It appears that astaxanthin binding to the muscle proteins of these species do not play role in astaxanthin deposition in their flesh. These observations suggest that the lack of flesh pigmentation of these species *in vivo* are not related to the affinity of muscle proteins for carotenoid and may be caused by greater capacity for metabolic transformations or reduced ability to transport of carotenoid to the muscle.

CHAPTER 5

Investigation of astaxanthin transport proteins in muscle membranes of Atlantic salmon (*Salmo salar* L.) using the proteomics approach

5.1 Abstract

Adult Atlantic salmon (*Salmo salar* L.) fed on a diet supplemented with astaxanthin (astaxanthin fed salmon) and a control diet not supplemented with astaxanthin (control group salmon) were used in the present study. Membrane fractions from the muscle of astaxanthin fed and control group salmon were subfractionated on a sucrose density gradient centrifuge in order to identify proteins up-regulated in response to dietary astaxanthin using proteomic analysis. Electrophoretic analyses of various fractions were accomplished by both one- and two-dimensional gel electrophoresis. Two-dimensional gel electrophoresis of various fractions did not show any consistent difference between astaxanthin fed and control group salmon. Only one-dimensional gel electrophoresis of the fraction 1 obtained from the second homogenization (S_2Fr_1) of muscle tissue from both the astaxanthin fed and control group salmon revealed the up-regulation of a few proteins (24-25 kDa, 23-24 kDa and 16-17 kDa) in astaxanthin fed salmon. The fraction 1 of the second homogenization (S_2Fr_1) of salmon muscle tissue was relatively enriched in plasma membrane vesicles as characterized by specific enzyme markers such as Na^+K^+-ATPase, Ca^{2+}-ATPase and acetylcholinesterase activity. Mass spectrometry analysis revealed that the up-regulated protein of molecular weight between 24-25 kDa in astaxanthin fed salmon showed extensive homology with myelin protein zero (P0), a membrane glycoprotein in the peripheral nervous system. Further study is necessary to elucidate the transport mechanism of carotenoid from the blood into the muscle of Atlantic salmon.

5.2 Introduction

Astaxanthin binding capability of the muscle proteins extracted from juvenile Atlantic salmon, halibut and haddock (Saha et al., manuscript submitted) was found to be

comparable to that of adult salmon (Saha et al., 2005), even though the flesh of these fish have different ability to take up astaxanthin *in vivo*. Several studies documented that small salmonids do not accumulate carotenoids in their flesh as efficiently as adult salmonids (Storebakken et al., 1987; Torrissen et al., 1989; Hatlen et al., 1995). Halibut and haddock do not accumulate carotenoids in their flesh and are thus considered as white-fleshed fish. Similar astaxanthin binding capability of solubilized muscle proteins of adult and juvenile salmon and halibut suggested that astaxanthin binding capability of salmon muscle proteins do not play role in astaxanthin deposition in their flesh (Saha et al., manuscript submitted).

The findings of Bjerkeng and Berge (2000) showed that Atlantic halibut were able to absorb dietary supplemented astaxanthin. Comparable astaxanthin binding capability of the solubilized muscle proteins of adult salmon, juvenile salmon and halibut along with the findings of Bjerkeng and Berge (2000) suggest that the blood/muscle interface may be the key factor limiting the deposition of astaxanthin in muscle. If this is the case, then a membrane bound transport or receptor protein may be involved in transporting astaxanthin from the blood into the muscle. Differences in protein profiles of membrane fractions of muscle tissue may provide information on whether a transport or receptor protein is up-regulated in response to dietary astaxanthin in adult salmon. The present study was undertaken to identify proteins up-regulated in the membrane fractions of astaxanthin fed adult salmon muscle using a proteomics approach.

5.3 Materials and methods

5.3.1 Fish samples

Atlantic salmon (*Salmo salar* L.; Saint John River strain) were reared in the facilities of National Research Council's Marine Research Station, Sandy Cove, Nova Scotia. Fish were fed two experimental diets, one supplemented with astaxanthin (Carophyll pink; F. Hoffmann-La Roche Ltd., Basel, Switzerland) and the other one not supplemented with astaxanthin (control diet). The composition of the control diet is presented in Table 5.1. Astaxanthin was supplemented in the control diet at a rate of 70 mg kg^{-1} diet. Salmon with average weight of 2.0-2.4 kg were used for this study. Fish

Table 5.1. Composition of the control diet

Dietary ingredients	Amount (%)
Herring meal[1]	46.0
Herring oil[2]	14.0
Gelatin[3]	2.0
Soybean meal[1]	10.0
CPSP-G[4]	4.0
Dried whey[5]	5.0
Wheat[6]	12.8
Corn starch, pre-gelatinized[7]	4.0
Vitamin premix[8]	1.2
Mineral premix[9]	1.0

[1] Corey Feed Mills Ltd., Fredericton, NB, Canada.
[2] Stabilized with 0.06% ethoxyquin, Comeau Seafood, Saulnierville, NS, Canada.
[3] US Biochemical, Cleveland, OH, USA.
[4] Hydrolyzed fish meal, Sopropêche, France.
[5] Farmers Co-operative Dairy Ltd., Truro, NS, Canada.
[6] Dover Mills Ltd., Halifax, NS, Canada.
[7] National Starch and Chemical Co., Bridgewater, NJ, USA.
[8] Vitamin added to supply the following (per kg diet): vitamin A (retinyl acetate), 6000 IU; vitamin D_3, 3000 IU, vitamin E, 150 IU, vitamin K_3, 30 mg; vitamin B_1 (thiamin HCl), 40 mg; vitamin B_2 (riboflavin), 50 mg; *d*-calcium pantothenate, 150 mg; biotin, 1 mg; folic acid, 15 mg; vitamin B_{12}, 0.1 mg; niacin, 200 mg; pyridoxine HCl, 20 mg; ascorbic acid, 200 mg; inositol, 100 mg; butylated hydroxytoluene (BHT), 15 mg; butylated hydroxyanisole (BHA), 15 mg; 0.2% choline chloride added directly to the main ingredient mixture.
[9] Mineral added to supply the following (per kg diet); manganous sulphate (32.5% Mn), 40 mg; ferrous sulphate (20.1% Fe), 30 mg; copper sulphate (25.4% Cu), 5 mg; Zn sulphate (22.7% Zn), 75 mg, cobalt chloride (24.8% Co), 2.5 mg; sodium selenite (45.6% Se), 1 mg; sodium fluoride (45.2% F), 4 mg.
* For preparation of astaxanthin supplemented diet, astaxanthin was supplemented in the control diet at a rate of 70 mg kg^{-1} diet; both diets were prepared by a steam-pelleting process.

were anaesthetised by TMS (tricaine methanesulfonate; 50 mg L^{-1} water) and transported on ice to the laboratory (about 30 minutes). Fish were decapitated, bled and gutted immediately after arrival at the laboratory. White muscle tissue was excised immediately from the mid-dorsal part of the fish. All subsequent steps were performed at 4 °C.

5.3.2 Isolation of membrane fractions

Membrane fractions were isolated from the muscle tissue of Atlantic salmon fed an astaxanthin supplemented diet and control diet according to the procedure described by Saito et al. (1984). All steps were performed at 0-4 °C unless otherwise stated. The muscle tissue was ground using a chilled meat grinder and then ground muscle (50 g) was homogenized in 250 mL of 0.3 M sucrose, 5 mM imidazole-HCl, pH 7.4 (homogenization buffer) using a chilled blender (Waring products Div., New Hartford, CT; model 33BL73) at maximum speed for 1 minute. The homogenate was centrifuged in a JA-14 rotor (Beckman Instruments Inc., Fullerton, CA, model J-21 centrifuge) for 10 minutes at 7,700 *g*. The first supernatant (S_1) was filtered through several layers of cheesecloth to remove free-floating fat and then kept on ice. The pellet was rehomogenized in 250 mL of homogenization buffer, centrifuged and filtered as described above. The second supernatant (S_2) was saved and the pellet was discarded. Both supernatants (S_1 and S_2) were centrifuged at 110,000 *g* for 90 minutes in a Beckman 70 Ti rotor and microsomal pellets were obtained. The microsomal pellets obtained from S_1 and S_2 were pooled accordingly and suspended in 21 mL and 14 mL of homogenization buffer, respectively instead of 11 mL according to Saito et al. (1984) using a Dounce homogenizer. Microsomal solution was layered onto a sucrose step gradient, 7 mL of solution for each gradient tube. The density gradient steps consisted of, 7 mL of each, 45% (w/w) sucrose, 38% (w/w) sucrose, 32% (w/w) sucrose and 27% (w/w) sucrose, all buffered with 5 mM imidazole-HCl, pH 7.4 (Fig. 5.1). All sucrose solutions were stored at 4 °C and used within 24 hr. The gradients were centrifuged overnight (15 hr) in a Beckman SW 28 rotor at 70,000 *g*. The membrane fractions at the interfaces between the gradient steps (Fig. 5.1) were collected and diluted approximately two-fold with 5 mM imidazole-HCl, pH 7.4 and centrifuged for 2 hr at 125,000 *g*. The

pellets were finally resuspended in homogenization buffer and aliquots were immediately stored at –80 °C until use. An aliquot of each fraction was stored in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer at –20 °C for one-dimensional gel electrophoresis. Protein concentrations of various membrane fractions were determined by the method of Bradford (1976) with bovine γ-globulin as a standard.

5.3.3 Enzyme assays

Characterization of membrane fractions obtained from microsomes of the second homogenization was accomplished by assaying specific enzyme markers such as Na^+K^+-ATPase, Ca^{2+}-ATPase and acetylcholinesterase in order to characterize the type of isolated membranes.

5.3.3.1 Na^+K^+-ATPase

Na^+K^+-ATPase activity of different fractions was assayed according to the method described by Seiler and Fleischer (1982) with slight modifications in the preincubation step and the temperature maintained for the enzyme reaction. The sample was preincubated in absence of sodium dodecyl sulphate (SDS). The preincubation medium contained 1 mg mL^{-1} of sample protein, 40 mM imidazole/HEPES, pH 7.3 and 2 mM Tris/EDTA. The sample was incubated at room temperature for 20 minutes. The reaction temperature was maintained at 30 °C instead of 37 °C according to Seiler and Fleischer (1982). An aliquot (10 µL) of incubated sample was subjected to 50-fold dilution into temperature equilibrated (30 °C) assay media containing 120 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 3 mM Na_2ATP, 0.5 mM EGTA, 5 mM NaN_3, 30 mM imidazole/HCl, pH 7.5, with or without 1 mM ouabain. The glasswares, including the glass tubes (screw-capped) used in this assay, were acid-washed and phosphate free detergent was used to clean them. The reaction proceeded for 10 minutes at 30 °C and the volume of the reaction mixture was 0.5 mL. The reaction was stopped by the addition of 0.5 mL of 5% (w/v) SDS, 10 mM Na_2EDTA and immediate placement on ice. The differences in inorganic phosphate released from ATP in the absence and presence of ouabain reflects the ouabain-sensitive Na^+K^+-ATPase activity.

Inorganic phosphate liberated in the reaction was quantified colorimetrically according to Seiler and Fleischer (1982) with the modification that reagent IV contained 12% (w/v) sodium ascorbate, 1% (w/v) ammonium molybdate and 10% (w/v) trichloroacetic acid (TCA) instead of 20% TCA. Reagent IV was always prepared fresh prior to use. For phosphate estimation, 0.5 mL of reagent IV was added to the reaction mixture, mixed by vortexing and incubated on ice for 9 minutes. Then 1.5 mL of reagent III containing 2% (w/v) sodium arsenite, 2% (w/v) tri sodium citrate dihydrate and 2% (v/v) acetic acid was added to the mixture and mixed by vortexing. The mixture was incubated at 37 °C for 10 minutes to develop the color and the absorbance was measured at 850 nm using an Ultrospec 2000 uv/visible spectrophotometer (Pharmacia Biotech Ltd., Cambridge, England). A standard curve for inorganic phosphate was developed in assay media using Na_2HPO_4. Enzyme activity was expressed as μmol inorganic phosphate hr^{-1} mg^{-1} protein.

5.3.3.2 Ca^{2+}-ATPase

The Ca^{2+}-ATPase activity was assayed according to the method described by Seiler and Fleischer (1982). The Ca^{2+}-stimulated ATPase was measured by the release of inorganic phosphate in absence (1 mM Tris/EGTA) and presence (50 μM) of Ca^{2+}. The glassware used in this assay was cleaned in similar way as mentioned in the previous enzyme assay. The assay was performed in screw-capped glass tubes in presence of 100 mM KCl, 3 mM $MgCl_2$, 3 mM Tris-ATP, 30 mM imidazole, pH 7.2, 5 μg mL^{-1} of ionophore A23187, 10 μg of protein sample, and either 50 μM $CaCl_2$ or 1 mM Tris/EGTA. The final reaction volume was 0.5 mL. The reaction proceeded for 5 minutes at 25 °C and then stopped by the addition of 0.5 mL of 5% (w/v) SDS, 10 mM Na_2EDTA and immediate placement on ice. The amount of inorganic phosphate released in this reaction was determined colorimetrically as described above for the Na^+K^+-ATPase assay.

5.3.3.3 Acetylcholinesterase

Acetylthiocholine was used as the substrate for acetylcholinesterase and the activity was measured according to the method of Steck and Kant (1974) with the

modifications that the preincubation of membrane fraction using 5 mM sodium phosphate, pH 8 (5P8) or Triton X-100 was omitted. Acetylthiocholine chloride solution was prepared at a concentration of 12.5 mM and kept refrigerated. Dinitrobenzoic acid (DTNB) solution (10 mL) was prepared fresh at a concentration of 10 mM in 100 mM phosphate buffer, pH 7.0 as the reagent was reported to be more stable in pH 7.0 buffer rather than a pH 8.0 buffer. Fifteen mg of sodium bicarbonate was added to 10 mL of DTNB solution prior to use. Membrane fractions were diluted to 0.4 mg mL^{-1} using 100 mM phosphate buffer pH 7.5. First 0.3 mL of phosphate buffer (100 mM, pH 7.5) was pipetted into a cuvette, then 0.025 mL of diluted membrane fraction was added and the volume was finally made to 0.7 mL by adding 0.375 mL of 100 mM phosphate buffer, pH 7.5. This sequence of pipetting was followed to ensure that protein was well mixed with the phosphate buffer in the cuvette. Fifty µL (i.e. 0.05 mL) each of DTNB and acetylthiocholine chloride solution were then added to the sample in the cuvette and mixed by gently pipetting 2-3 times. The reaction was followed spectrophotometrically by reading the absorbance every minute at 412 nm at room temperature. A blank assay was performed using buffer, substrate and DTNB solutions to correct for non-enzymatic hydrolysis of acetylthiocholine at room temperature. The time dependant change in absorbance at 412 nm was used to calculate the acetylcholinesterase activity as described by Ellman et al. (1961). The extinction coefficient of 1.36×10^4 M^{-1} cm^{-1} for 5-thio-2-nitro-benzoic acid reported by Ellman (1959) was used for this calculation.

5.3.4 Electrophoresis

5.3.4.1 One-dimensional electrophoresis

Aliquots of different membrane fractions obtained from both the first and second homogenization stored in SDS-PAGE sample buffer at –20 °C were subjected to SDS-PAGE on both 12% and 8% polyacrylamide slab gel of 1 mm thickness according to the method of Laemmli (1970). Precision Plus protein standards (Bio-Rad Laboratories, Hercules, CA, USA) were used as molecular mass standards. Gels were stained by silver-staining protocol according to Swain and Ross (1995). However, for mass spectrometry study, a silver-staining procedure based on the method of Shevchenko et al. (1996) was used to stain the gels. Details of both staining procedures are represented in

Appendix 1. After staining, the protein bands of interest were excised for identification of protein by mass spectrometry.

5.3.4.2 Two-dimensional electrophoresis

Membrane fractions obtained from the second homogenate of salmon muscle were diluted 1:10 with rehydration buffer containing 6 M urea, 2 M thiourea, 2% CHAPS, 20 mM dithiothreitol (DTT) and 0.5% carrier ampholytes (IPG buffer, pH 3-10), and applied to 18 cm Immobiline DryStrips pH 3-10 (Amersham Biosciences, Uppsala, Sweden). Approximately 200 μg of protein was used for this electrophoretic study. Fractions that were already diluted were subjected to freeze-drying and resuspended in rehydration buffer to achieve 200 μg of protein. Isoelectric focusing was conducted using the Multiphor II system (Amersham Biosciences) with linear pH gradient (3-10) strips. Focusing was initiated at 30 V for 10 hr and then increased to 8000 V over a 6 hr period, and held at 8000 V for the last 3 hr. The sample strips were then equilibrated for 15 minutes in 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, 2% SDS, 65 mM DTT, then equilibrated for another 15 minutes with 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, 2% SDS, 135 mM iodoacetamide. The second dimension was run on 1 mm thick, 14% T SDS-PAGE gel and run for 2100 Vhr. Precision protein standards (Bio-Rad, Hercules, CA, USA) were used to estimate the molecular weights of proteins. Proteins were visualized using silver staining procedure based on the method of Shevchenko et al. (1996) as described in Appendix 1. Mass spectrometric analysis of the protein spot from two-dimensional gel electrophoresis was not performed because the electrophoretogram of various fractions did not show consistent differences between the astaxanthin fed and control group salmon.

5.3.5 Protein digestion

Protein spots of interest excised from the gel were processed for trypsin digestion using the Investigator ProGest automated digestion unit. Excised protein bands were placed in a 96 well reaction plate. To remove the high concentration of buffers and detergents used in electrophoresis, the gel pieces were subjected to several successive dehydration/ rehydration washes with acetonitrile and ammonium bicarbonate buffer (pH

7.8), respectively. The ProGest reduced the disulphide bonds of protein by incubating the gel pieces with DTT (10 mM in 25 mM ammonium bicarbonate) at 60 °C and alkylated the free sulfhydryl groups with iodoacetamide (100 mM in 50 mM ammonium bicarbonate). Then trypsin stored in acidic buffer to prevent autodigestion was activated by the ProGest by diluting the enzyme with 25 mM ammonium bicarbonate buffer, pH 7.8. The activated trypsin was added to each gel sample and incubated at 37 °C for approximately 1.5 hr. Each well further received a small aliquot of water to replace any solution lost through evaporation and the samples were incubated for a further 1.5 hr. The resulting peptides were extracted from the gel pieces by a series of washes with ammonium bicarbonate buffer (pH 7.8), formic acid (10%) and acetonitrile. After digestion, the peptides were immediately evaporated to dryness on a SpeedVac concentrator.

5.3.6 Mass spectrometry

Dried peptides were resuspended in 5% methanol and 0.5% formic acid. All the samples were analysed by LC-MS/MS using an Eksigent HPLC system equipped with a 10 cm x 150 μm Chromolith Cap Rod C_{18} column. The separation of tryptic peptides was carried out using a linear gradient from 15 to 30% B over 17 minutes (A: 5% acetonitrile, 0.2% formic acid; B: 80% acetonitrile, 0.2% formic acid). The HPLC was interfaced to an Applied Biosystems MDS SCIEX Q TRAP mass spectrometer via a nanoflow source. Data were acquired in the information dependent acquisition mode. In the first quadrupole, peptide masses were obtained. The peptides were then fragmented by argon in the second quadrupole and the fragments (all product ions) were mass analysed in the third quadrupole. The MS/MS spectra were subjected to the database search program Mascot (Matrix Science, London, England) for searching against the NCBI database and Genome Research on Atlantic Salmon Project (GRASP) EST database to identify proteins. Homology searches of the sequences of protein obtained from the Mascot search against the GRASP EST database (Rise et al., 2004) were carried out using the BLAST protein algorithm against the NCBI GenBank non-reductant protein database (Blastp; Altschul et al., 1997).

5.4 Results

5.4.1 Isolation of membrane fractions

Microsomes obtained from the first and second homogenization (S_1 and S_2, respectively) yielded several distinct bands on the sucrose density gradient (Fig. 5.1). Fraction 1 of the second homogenization (S_2) at the sample/27% sucrose interface was enriched with plasma membrane as characterized by enzyme activities. Characterization of the fraction 1 from the first homogenization (S_1) at the sample/27% sucrose interface was not possible as the final pellet (after centrifugation) could not be saved due to its' very small size and flocculence. However, two other fractions from the first homogenization (S_1) were obtained at the 32/38% and 38/45% sucrose interfaces (Fig. 5.1A). Fractions 2, 3 and 4 from the second homogenization (S_2) were found at the 27/32%, 32/38% and 38/45% sucrose interface, respectively (Fig. 5.1B). Fraction 5, which was only observed in the second homogenization (S_2) settled at the bottom of the gradient tube.

5.4.2 Enzymatic characterization of membrane fractions

The enzyme activities of various fractions, fractionated on sucrose gradient centrifuge, from microsomes obtained from the second homogenization of salmon muscle are summarized in Table 5.2. The data of the various enzyme activity studies obtained from the similar fraction of astaxanthin fed salmon and control group salmon were combined together and represented as mean since the specific enzyme activities of similar fraction from both groups were found to be statistically insignificant. Only fractions 1 (S_2Fr_1) and 2 (S_2Fr_2) exhibited the Na^+K^+-ATPase activity with the activity in fraction 1 being higher than fraction 2. Fraction 1 showed the lowest Ca^{2+}-ATPase activity (5.71 $\mu mol\ hr^{-1}\ mg^{-1}$ protein) among the four fractions and a remarkable gradual increment in this enzyme activity was observed in the subsequent fractions. Fraction 4 showed 3-fold higher Ca^{2+}-ATPase activity than fraction 2 (Table 5.2). Acetylcholinesterase activity was found to be distributed in all four fractions.

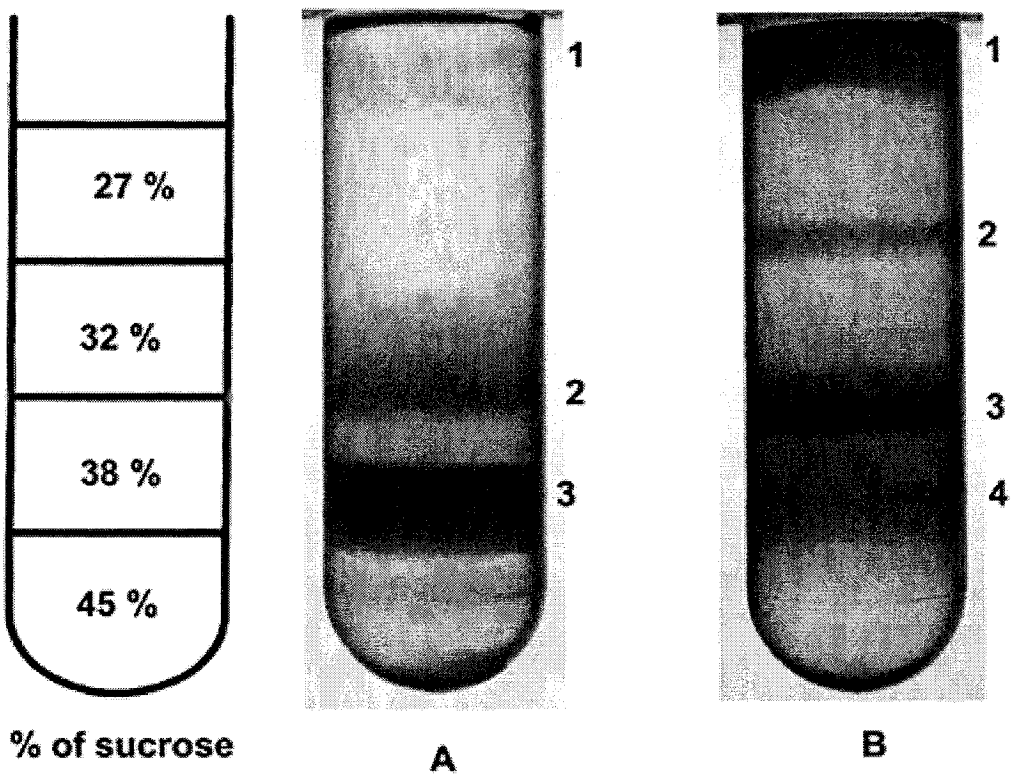

Fig. 5.1 Fractionation of salmon muscle tissue microsomes on discontinuous sucrose gradient. A, fractions (1-3) obtained from the first homogenization (S_1) of Atlantic salmon muscle tissue. B, fractions (1-4) obtained from the second homogenization (S_2) of salmon muscle tissue.

Table 5.2 Enzymatic characterization of membrane fractions, fractionated by sucrose step gradient centrifugation, of microsomes prepared from the second homogenization of Atlantic salmon muscle tissue

Assays	Fractions	Enzyme activity*
Total ATPase[a] (μmol $hr^{-1}mg^{-1}$ protein)	S_2Fr_1	2.70 ± 0.37 (5)
	S_2Fr_2	1.91 ± 0.47 (3)
	S_2Fr_3	N.D
	S_2Fr_4	N.D.
Total ATPase [a] + 1 mM ouabain (μmol hr^{-1} mg^{-1} protein)	S_2Fr_1	1.88 ± 0.37 (5)
	S_2Fr_2	1.50 ± 0.62 (3)
	S_2Fr_3	N.D.
	S_2Fr_4	N.D.
Ouabain-sensitive (Na^+K^+)-ATPase [a,b] (μmol hr^{-1} mg^{-1} protein)	S_2Fr_1	0.82 (5)
	S_2Fr_2	0.41 ± 0.33 (3)
	S_2Fr_3	N.D.
	S_2Fr_4	N.D.
Ca^{2+}-ATPase activity (μmol hr^{-1} mg^{-1} protein)	S_2Fr_1	5.71 ± 1.61 (4)
	S_2Fr_2	32.05 ± 5.27 (2)
	S_2Fr_3	73.64 ± 1.77 (2)
	S_2Fr_4	90.29 (1)
Acetylcholinesterase (nmol min^{-1} mg^{-1} protein)	S_2Fr_1	16.86 ± 3.02 (2)
	S_2Fr_2	16.17 ± 0.42 (2)
	S_2Fr_3	25.00 ± 1.25 (2)
	S_2Fr_4	22.94 (1)

* Mean ± S.D

The numbers in parentheses represent the total number of samples analyzed from astaxanthin fed and control group salmon except (1), which represents sample analysed from astaxanthin fed group.

[a] Activity was measured after pre-incubation without SDS.

[b] Ouabain-sensitive ATPase was obtained by subtracting the values obtained in the presence of ouabain from the total ATPase.

N.D. = Not detected.

5.4.3 Electrophoresis

One-dimensional gel electrophoresis of various fractions obtained from microsomes prepared from both the first and second homogenization (S_1 and S_2, respectively) of muscle tissue of astaxanthin fed and control group salmon were performed on both 12% and 8% polyacrylamide gel (1 mm thick). Electrophoresis was performed on 8% gel to obtain better separation of higher molecular weight proteins. The protein profiles of various fractions obtained from the first homogenization did not show any difference between the control group and astaxanthin fed salmon (data not shown). The one-dimensional gel electrophoretic profiles of fraction 1 obtained from the second homogenization (S_2Fr_1) of salmon muscle tissue on 12% and 8% polyacrylamide gel are represented in Figs. 5.2 and 5.3, respectively. Protein bands with the molecular mass between 22-25 kDa and other bands with molecular weight of 16-17 kDa were found to be up-regulated in astaxanthin fed salmon as indicated by one-dimensional gel electrophoresis of membrane fraction 1 (Fig. 5.2). Up-regulated protein bands marked as A (24-25 kDa), B (22-23 kDa) and C (16-17 kDa) on a mass spectrometry-compatible silver-stained gel are shown in Fig. 5.4.

The two-dimensional gel electrophoresis of fraction 1 of the second homogenization of muscle tissue (S_2Fr_1) both from the astaxanthin fed and control group salmon are shown in Figs. 5.5 and 5.6, respectively. The two-dimensional gel electrophoretic profiles of other fractions obtained from the same homogenization of muscle tissue (S_2Fr_2-S_2Fr_4) of astaxanthin fed and control group salmon are represented in Appendix 2 (Figs. 1-6). The protein profiles obtained from the two-dimensional gel electrophoresis of fraction 1 did not show consistent differences between the control group and astaxanthin group salmon. Similar two-dimensional gel electrophoresis results were obtained for other fractions.

5.4.4 Mass spectrometry

The protein bands of interest marked in Fig. 5.4 were trypsin digested and subjected to mass spectrometry analysis. After chromatographic separation of the digest mixture, a mass spectrometer with MS/MS capability recorded the mass of the individual

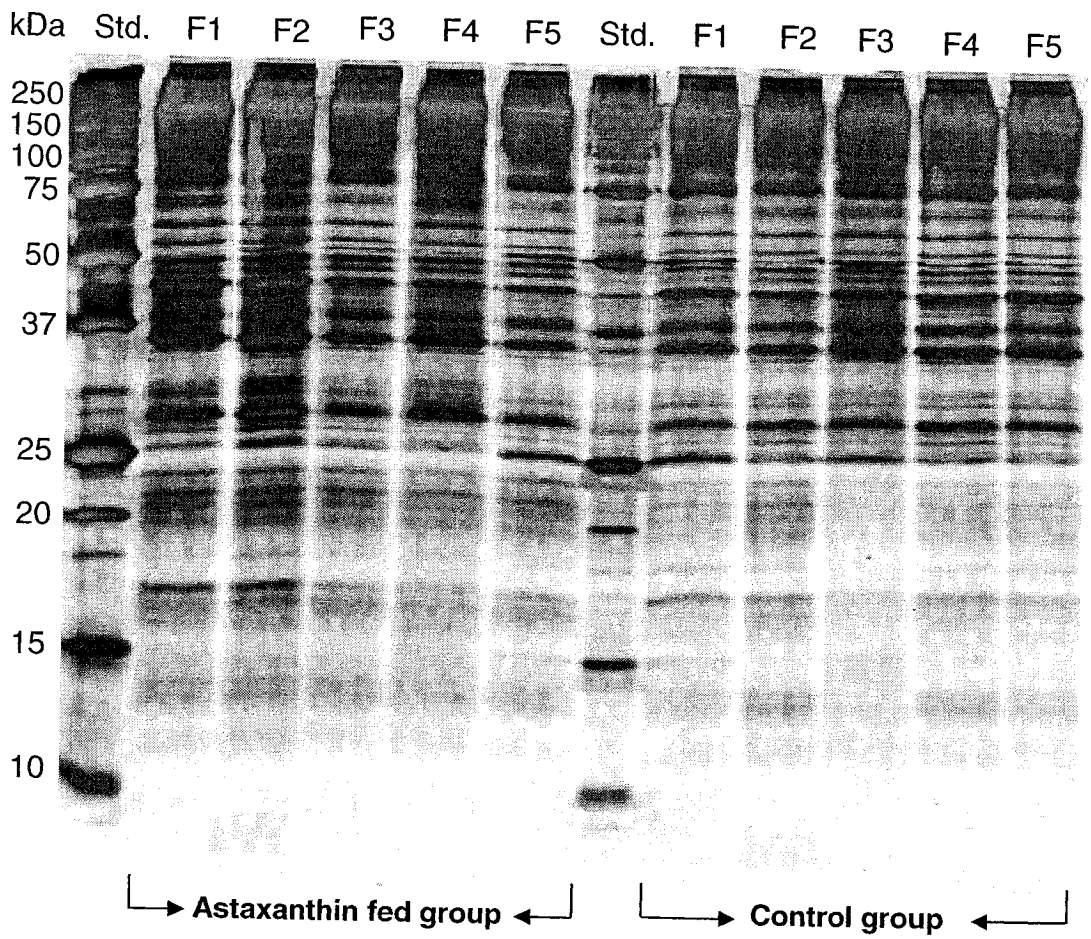

Fig. 5.2 The SDS-PAGE (12% gel) profiles of the fraction 1 of microsomes, fractionated using sucrose gradient centrifugation, obtained from the second homogenization (S_2) of muscle tissue from astaxanthin fed and control group Atlantic salmon. Gel was stained with silver staining according to the method of Swain and Ross (1995). Precision Plus protein standards were used as markers and the molecular weights of standards are shown on the left. Lane F1-F5 indicates individual salmon from both groups. Total 4 μg of protein was loaded in each lane.

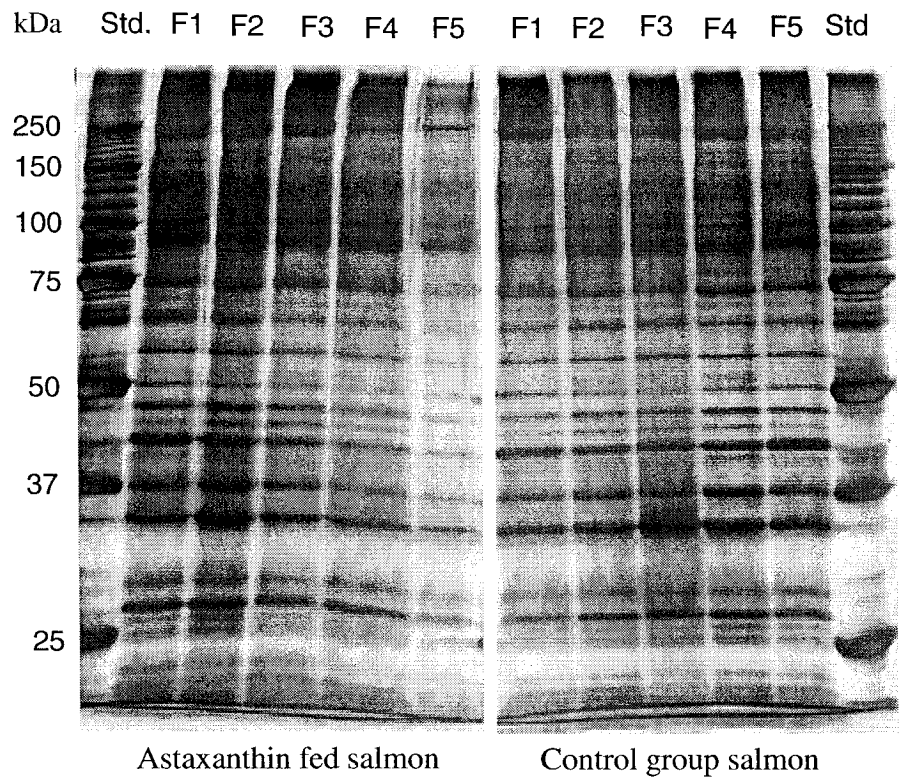

Fig. 5.3 The SDS-PAGE protein profiles of the fraction 1 obtained from microsomes of the second homogenization (S_2) of muscle tissue of both astaxanthin fed and control group salmon on 8% polyacrylamide gel. Gel was stained with silver staining protocol according to Swain and Ross (1995). Molecular weights of standards are shown on the left. Total 4 μg of protein was loaded in each lane.

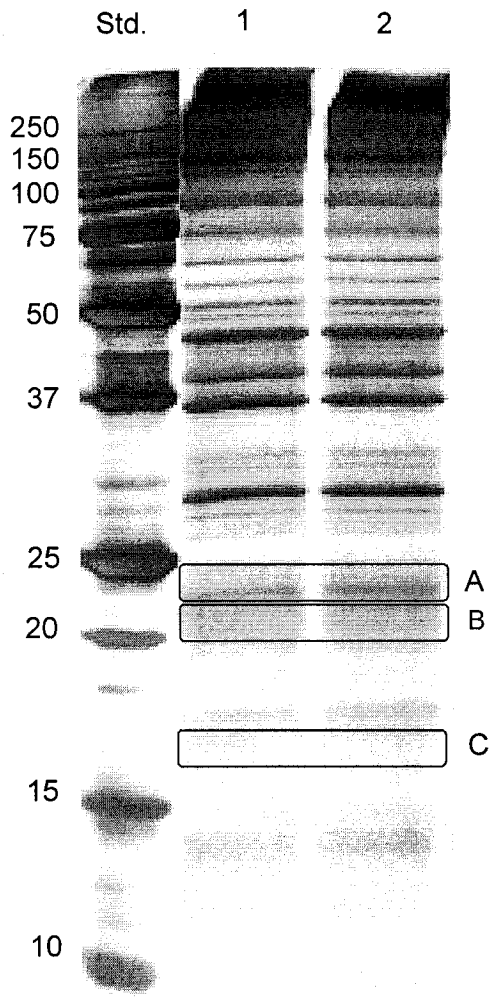

Fig. 5.4 The SDS-PAGE protein profiles of fraction 1 obtained from the second homogenization of astaxanthin fed salmon muscle tissue (S_2Fr_1) on 12% polyacrylamide gel. The up-regulated protein bands are marked and shown in the boxes those were excised for mass spectrometry analysis. A, proteins having molecular weight between 24-25 kDa; B, proteins with molecular weight of 22-23 kDa; C, proteins with molecular weight of 16-17 kDa. Staining protocol based on the method of Shevchenko et al. (1996) were used to visualize the protein bands. Molecular weights of standards are shown on the left. Lanes 1 and 2 both contained 4 μg of protein from an astaxanthin fed salmon.

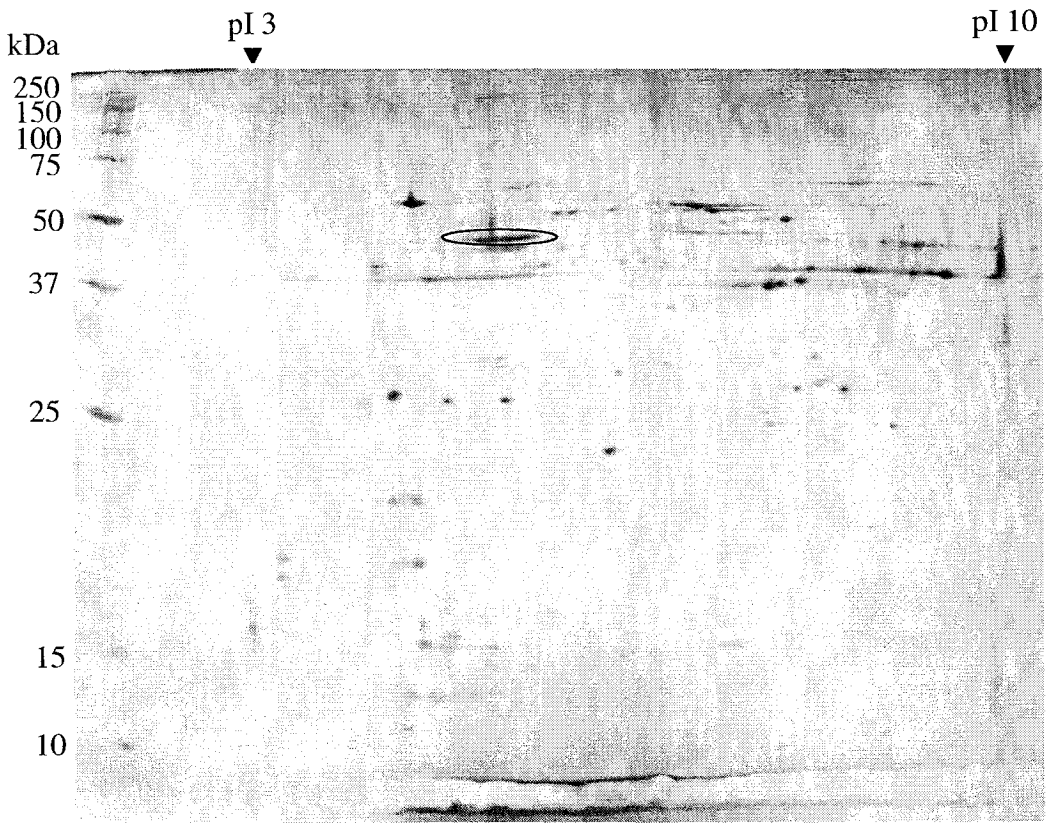

Fig. 5.5 Two-dimensional gel electrophoresis of the fraction 1 obtained from the second homogenization of muscle tissue of astaxanthin fed Atlantic salmon. Molecular masses of Precision protein standards are indicated at left. The numbers at the top of the gel indicate ends and respective pI of isoelectric focusing strips (i.e. IPG strips). Encircled spot on gel indicate shifting of a glycosylated protein compared to similar fraction or other fractions either from astaxanthin fed or control group salmon. Approximately 200 μg of proteins were analyzed on 14% polyacrylamide gel with a thickness of 1 mm.

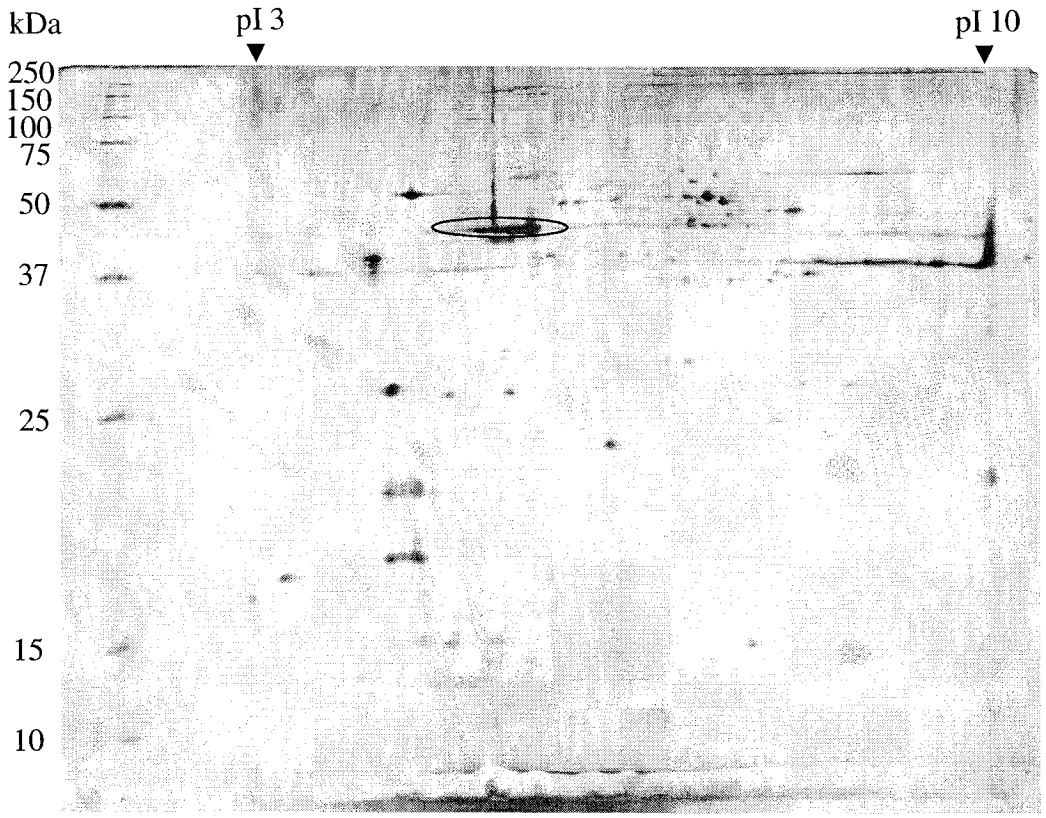

Fig. 5.6 Two-dimensional gel electrophoresis of the fraction 1 obtained from the second homogenization of muscle tissue of control group Atlantic salmon. Molecular masses of markers are indicated at left. The numbers at the top of the gel indicate ends and respective pI of isoelectric focusing strips (i.e. IPG strips). Encircled spot indicate shifting of a glycosylated protein compared to similar fraction or other fractions either from astaxanthin fed or control group salmon. Approximately 200 μg of proteins were analyzed on 14% polyacrylamide gel with a thickness of 1 mm.

peptides, which were then fragmented and fragment ion spectra (MS/MS spectra) were obtained for the respective peptides.

The experimental mass values were compared with calculated peptide mass or fragment ion mass values, obtained by applying appropriate cleavage rules to the entries in a comprehensive primary sequence database. An appropriate scoring algorithm identified the best-matched peptide or protein. The Mascot search of the MS/MS spectra of the tryptic peptides from the protein bands marked as A and B against the GRASP EST database (Rise et al., 2004) displayed significant hits. Five peptides from a non-annotated salmon protein, the top best-matched protein with a score of 299, matched to those of the up-regulated protein (marked as A, Fig 5.4) in astaxanthin fed salmon are shown in bold in Fig. 5.7. However, three peptides (underlined peptides, Fig. 5.7) of the same protein showed matching to those of the up-regulated protein (marked as B, Fig 5.4) in astaxanthin fed salmon with a score of 125. The protein score is the sum of individual peptide scores. In both cases, individual ion scores ≥ 48 indicated identity or extensive homology and are reported to be significant ($P<0.05$). Peptides with a score below the set threshold of the corresponding database were said to have greater than 5% probability of being a random event and the result was discarded. The sequence of the matched salmon protein in 1-letter code is represented in Fig. 5.7.

```
  1 RASLCPISPS LHSGTPPSAT SL_EPKDSGT MLTILALASV ILLGIVPQQS
 51 EAIVIYTGWE RHALVGSDIR LSCSFFSWRW TSDDVTFSWS YRADGARDSI
101 SVFHYTGGAP YVDNKGPFRD RLEFVGNPGR RDGSILLKNL DYGDNGTFTC
151 DAKNPPDIVG RASSVRLLVF EKVPIQAGVI TGAIIGAVLG LLVLVVVIYY
201 LMRFLVARRV FNLSVSKHGK KG
```

Fig. 5.7 The sequence of the matched non-annotated salmon protein of 24-25 kDa and 22-23 kDa (marked as A and B, respectively; Fig. 5.4). Peptides matching with the tryptic peptides of A are shown in bold. Peptides matching with the tryptic peptides of B are shown as underlined.

According to Mascot search results against a non-annotated salmon EST database (GRASP), the nominal molecular mass of the matched protein was found to be 24,159 Da with a calculated pI value of 9.22. The Mascot search results of the MS/MS spectra obtained from the up-regulated protein having a molecular weight between 16-17 kDa (marked as C, Fig. 5.4) are found to be insignificant, that is there was greater than a 5% probability that the match was a random event.

A peptide summary report groups peptide matches into protein hits, derives a protein score from the combined ion scores (Perkins et al., 1999). Table 5.3 shows good matching of predicted peptides from a non-annotated 24,159 Da salmon protein EST sequence to the MS profiles of tryptic peptide from the 24-25 kDa protein (A). Having more peptides identifying the protein added more confidence to the result. High-quality matching of several different peptide sequences within the identified protein with the MS/MS spectra in the data file is an indication of the most reliable protein identification (Leibler, 2002).

In Table 5.3, the first line contains the protein molecular mass, a non-probabilistic protein score, derived from the ions scores, and the number of peptide matches. The score for an MS/MS match is based on the absolute probability (5% or less) that the observed match between the experimental data and the database sequence is a random event (Perkins et al., 1999).

Table 5.3 Peptide summary showing the scores, expectation values and molecular weight of the peptides matched to the tryptic peptides of the 24-25 kDa protein (A) as compared to the sequence of the matched salmon protein

Mass: 24,159 Score: 299 Peptide matched: 5 Frame: 1

No.	Observed[a]	Mr(expt)[b]	Mr(calc)[c]	Delta[d]	Miss[e]	Score[f]	Expect[g]	Peptide sequence
1.	494.92	987.82	987.51	0.30	0	58	0.0065	LEFVGNPGR
2.	595.27	1188.53	1188.54	-0.01	0	52	0.023	LSCSFFSWR
3.	630.79	1259.57	1258.64	0.93	1	51	0.026	DRLEFVGNPGR
4.	657.27	1968.79	1968.92	-0.13	0	56	0.0039	DSISVFHYTGGAPYVDNK
5.	809.26	2424.75	2426.17	-1.41	1	83	5.4e-06	DSISVFHYTGGAPYVDNKGPFR

[a] Experimental m/z (the ratio of mass to charge) value

[b] Experimental m/z value transformed to a relative molecular mass

[c] Calculated relative molecular mass of the matched peptide

[d] Difference between the experimental and calculated masses

[e] Number of missed enzyme cleavage sites

[f] Ion score of the peptide

[g] Expected value of the peptide match. The lower this value, the more significant the result

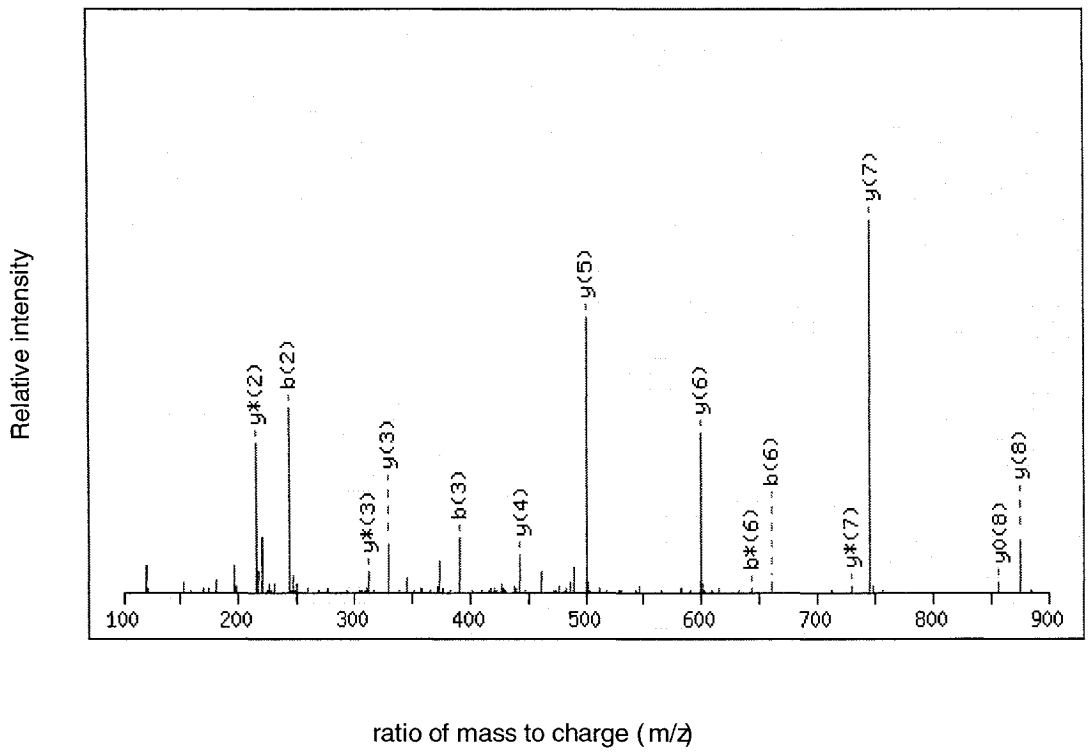

Fig. 5.8 Mass spectrum with the labelling of all matched fragment ions from the matched peptide sequence, LEFVGNPGR.

Fig. 5.8 shows a mass spectrum in which all of the matched fragment ions from the matched peptide sequence, LEFVGNPGR are labelled. The intensity of the peaks is a reflection of the population generated during the ionspray process. The tallest peak is called the basal peak. The matched fragment ions from the matched peptide sequence, LEFVGNPGR are also shown in Table 5.4.

Table 5.4 Ion Results Table: The predicted b- and y-ions from the matched peptide sequence. The sequence is shown in 1-letter code and matched values are in bold typeface

#	b	b^{++}	b*	b^{*++}	b^{0}	b^{0++}	Seq.	Y	y^{++}	y*	y^{*++}	y^{0}	y^{0++}	#
1	114.09	57.55					L							9
2	**243.13**	122.07			225.12	113.07	E	**875.44**	438.22	858.41	429.71	**857.43**	429.22	8
3	**390.20**	195.60			372.19	186.60	F	**746.39**	373.70	**729.37**	365.19			7
4	489.27	245.14			471.26	236.13	V	**599.33**	300.17	582.30	291.65			6
5	546.29	273.65			528.28	264.64	G	**500.26**	250.63	483.23	242.12			5
6	**660.34**	330.67	**643.31**	322.16	642.32	321.67	N	**443.24**	222.12	426.21	213.61			4
7	757.39	379.20	740.36	370.68	739.38	370.19	P	**329.19**	165.10	**312.17**	156.59			3
8	814.41	407.71	797.38	399.20	796.40	398.70	G	232.14	116.57	**215.11**	108.06			2
9							R	175.12	88.06	158.09	79.55			1

Table 5.4 shows the matched fragment ions from the matched peptide, LEFVGNPGR. In the case of peptide fragmentation, the cleavage commonly occurs at the peptide (amide) bond, the bond between the carbonyl group and amide group, resulting in b-ion and y-ion. The b-ion is a fragment in which the positive charge is retained on the N-terminal side whereas the y-ion is a fragment in which the positive charge is retained on the C-terminal side. The tryptic peptides that usually have basic amino acid such as arginine (Arg) or lysine (Lys) at the C-terminus produce y-ions favorably (McCormack et al., 1993). The y- and b-ion both are formed from multiply charged peptides. When a singly charged peptide fragments, either a b- or y-ion is formed; the other part is lost as a neutral molecule since it does not respond to magnetic fields. A neutral loss of ammonia from an ion is indicated with asterisks whereas loss of water is indicated with 0. Ammonia is often lost from b ions and ions containing Arg, whereas water is lost from y ions and ions containing glutamic acid, aspartic acid, serine and threonine (Ballard and Gaskell, 1993).

Homology searches of the sequences of matched protein obtained from the GRASP EST database search were carried out using the BLAST protein algorithm

against the NCBI GenBank non-reductant protein database (Blastp). The sequence alignment of the matched protein with the *Danio rerio* myelin protein zero obtained from the Blast search is shown in Fig. 5.9.

```
M. protein: 31 MLTILALASVILLGIVPQQSEAIVIYTGWERHALVGSDIRLSCSFFSWRWTSDDVTFSWS 90
               ML++LAL SV+LLGI   QS A+V+ T  E+HALVGSD+RLSCSFFSW+WTS +V+F+W
Myelin P0:  1  MLSVLALTSVVLLGIA-SQSTALVVNTDSEKHALVGSDVRLSCSFFSWQWTSPEVSFTWH 59

M. protein: 91 YRADGARDSISVFHYTGGAPYVDNKGPFRDRLEFVGNPGRRDGSILLKNLDYGDNGTFTC 150
               YR DGA+D+IS+ HYTGG  Y  NKGPF++RLEFVGNP RRDGSIL+KNLD+GDNGTFTC
Myelin P0:  60 YRPDGAKDAISILHYTGGEAYPANKGPFQNRLEFVGNPSRRDGSILIKNLDFGDNGTFTC 119

M. protein:151 DAKNPPDIVGRASSVRLLVFEKVPIQXXXXXXXXXXXXXXXXXXXXXXYYLMRFLVARRV 210
               DAKNPPDI G  S++RLLVFEKVP+Q                      YYLMRFLVARRV
Myelin P0: 120 DAKNPPDIGGHPSTIRLLVFEKVPVQAGVITGSIIGVVLGLLILVVAIYYLMRFLVARRV 179

M. protein:211 FNLSVS 216
               F+LS+S
Myelin P0: 180 FSLSMS 185
```

Fig. 5.9 Alignment of the sequence of the matched non-annotated salmon protein with the myelin P0 from *Danio rerio* as obtained from the Blast search against the NCBI. M. protein stands for matched protein. Amino acids that are identical in both sequences are shown in the middle.

The Blast results of the protein sequence showed numerous amino acid sequence matches to the published sequences of the myelin protein zero (P0) from *Danio rerio* (Schweitzer et al., 2003) with the E-value (expectation value) of 9e-67. The sequence of another protein (not shown) that showed two predicted peptides matching with tryptic peptides of the 24-25 kDa protein with a score of 68 was also subjected to Blast search in a similar way. However, neither of these two matched peptides indicated identity

according to their scores. The Blast results of this protein showed numerous matches to the amino acid sequence of the ATP synthase H^+ transporting mitochondrial F0 complex from *Danio rerio* with the E-value of 2e-65 (alignment not shown).

5.5 Discussion

The present experiment was conducted to investigate whether a membrane bound transport or receptor protein is up-regulated in response to dietary astaxanthin in Atlantic salmon.

5.5.1 Enzymatic characterization of membrane fractions

It was observed that most of the microsomal proteins migrated to the highest density fraction and contained predominantly sarcoplasmic reticulum as determined by the protein concentration of the various fractions and the activity of specific enzyme markers. This observation is in agreement with the observation of other studies (Jones et al., 1979; Seiler and Fleischer, 1982). The activity of specific enzyme markers observed in the present study indicated that fraction 1 obtained from the second homogenization of salmon muscle tissue was more enriched with plasma membrane vesicles than the other fractions since fraction 1 showed higher Na^+K^+-ATPase activity and lower Ca^{2+}-ATPase activity than the other fractions. Saito et al. (1984) performed electron microscopic examination of a similar fraction, fraction 1 obtained from the second homogenization of rabbit skeletal muscle using sucrose step density gradient centrifugation and reported that plasma membranes were found in fraction 1.

Seiler and Fleischer (1982) observed higher Na^+K^+-ATPase activity in plasma membrane fraction after the preincubation with SDS and reported that the plasma membrane fraction in their study consisted largely of sealed vesicles. The examination of plasma membrane fraction using electron microscopy by these researchers also confirmed the existence of sealed vesicles. Seiler and Fleischer (1982) reported that addition of 0.4 mg mL^{-1} SDS in the preincubation medium for 1 mg mL^{-1} protein exhibited the maximal Na^+K^+-ATPase activity of the plasma membrane fraction. However, to optimize the SDS concentration for the membrane fraction in the present study, the addition of 0.1-0.5 mg mL^{-1} SDS to the preincubation medium for 1 mg mL^{-1} protein did not increase the

Na^+K^+-ATPase activity at all and enzyme activity decreased with higher concentration of SDS. This observation indicates that the plasma membrane fraction obtained in the present study was mostly leaky.

5.5.2 Electrophoresis

In two-dimensional gel electrophoresis, proteins are separated in the first dimension according to the pI (the pH at which the net charge on the protein is zero) and then in the second dimension by their molecular weights. In one-dimensional gel electrophoresis, proteins are separated according to their molecular weights only. Therefore, a single protein band from a one-dimensional gel electrophoretogram could represent more than one protein. However, one-dimensional gel electrophoresis was performed alongside the two-dimensional gel electrophoresis to track the membrane proteins since membrane proteins are hydrophobic and may partially or completely precipitate in the buffer used in the first dimension of two-dimensional gel electrophoresis as a result do not enter in the second dimension. This limitation of two-dimensional gel electrophoresis in separating hydrophobic proteins may explain the lack of observed difference in two-dimensional gel electrophoresis of fraction 1 between the astaxanthin fed and control group salmon, while up-regulated proteins were observed in one-dimensional gel electrophoresis from astaxanthin fed salmon. Fish to fish variation was observed in the electrophoretogram of two-dimensional gel electrophoresis. For example, changes and/or shifting of the train of spots (encircled in Figs. 5.5-5.6 and Figs. 1-6 in Appendix 2) which is usually due to proteins carrying post-translation modifications like glycosylation was observed in different fractions of salmon irrespective to feeding treatment.

5.5.3 Mass spectrometry

Proteins of interest from one-dimensional gels were subjected to mass spectrometry analysis since no consistent difference was observed between astaxanthin fed and control group salmon in two-dimensional gel electrophoresis. From the Mascot search results against the GRASP EST database, it was observed that predicted peptides from a non-annotated salmon protein showed significant matching to the MS/MS profiles

of tryptic peptides from both of the up-regulated protein bands marked as A and B (Fig 5.4). This might be due to contamination of the protein band A with the band B at the time of their manual excision from the gel for mass spectrometry analysis as these protein bands were very closely located on the 12% polyacrylamide slab gel. One-dimensional gel electrophoresis using an appropriate gradient gel may have eliminated this problem. Protein band B may also be proteolytically derived from protein band A since no protease inhibitor was used in the present study.

Mass spectrometry analysis and subsequent Blast analysis revealed that the 24-25 kDa protein (marked as A, Fig 5.4) in astaxanthin fed salmon was homologous to myelin P0. Myelin P0 is the most abundant protein in the peripheral nervous system, accounting for more than 50% of the total protein (Everly et al., 1973; Kitamura et al., 1976; Roomi et al., 1978). The Schwann cells of the peripheral nervous system produce myelin, which is a multilamellar membrane that wraps around axons.

The preliminary findings of this study suggest that a glycoprotein homologous to myelin P0 is present in the plasma membrane enriched muscle membrane fraction of adult Atlantic salmon in response to dietary astaxanthin. How this may be accomplished is proposed below.

Astaxanthin can be metabolized to retinoic acid. Metabolic transformation of astaxanthin into retinol has been reported in salmonids (White et al., 2003). Retinoic acid is synthesized via a two-step oxidative process from retinol to retinaldehyde and then retinaldehyde to retinoic acid (McCaffery and Dräger, 2000). Retinoic acid acts as a regulator of gene expression by binding to nuclear retinoid receptors (Mey and McCaffery, 2004). It has been suggested that retinoic acid regulate gene expression in peripheral nerve regeneration (Johann et al., 2003; Zhelyaznik et al., 2003). Therefore, it is likely that dietary supplemented astaxanthin may contribute to retinoic acid production and the elevated retinoic acid influences the expression of the myelin P0-like glycoprotein in astaxanthin fed salmon.

Another protein sequence that showed matching to two tryptic peptides from the 24-25 kDa protein also showed numerous matches to the amino acid sequences of the ATP synthase H^+ transporting mitochondrial F0 complex from *Danio rerio*. As stated earlier, a single protein band on one-dimensional gel electrophoresis could represent

more than one protein. Now the question arises which protein, a myelin P0-like glycoprotein or ATP synthase H^+ transporting F0 complex, is up-regulated. Considering the density of mitochondria, it is more reasonable to conclude that the up-regulated protein in astaxanthin fed salmon might be a myelin P0-like glycoprotein since the up-regulated protein was found to be equilibrated in the low buoyant density fraction, fraction 1 at the top, on a density gradient centrifuge. However, matching the protein sequence to the ATP synthase H^+ transporting mitochondrial F0 complex indicates minor mitochondrial contamination in the fraction 1 proteins.

5.6 Conclusion

Electrophoretic analysis of plasma membrane enriched fraction (fraction 1) both from astaxanthin fed and control group salmon indicated that a few proteins were up-regulated in Atlantic salmon in response to dietary supplementation of astaxanthin as indicated by one-dimensional gel electrophoresis. Mass spectrometry analysis revealed that the up-regulated protein with the molecular weight between 24-25 kDa showed extensive homology with the myelin protein zero (P0), a membrane glycoprotein in the peripheral nervous system. From the preliminary findings of the present study it is apparent that the role of transport protein or membrane-bound receptor protein (s) in flesh deposition of carotenoids in salmon still remains elusive. However, it is possible that up-regulation of this myelin P0-like glycoprotein in salmon due to dietary supplementation of astaxanthin may be involved in facilitating the transfer of astaxanthin from the blood into the muscle.

CHAPTER 6

General discussion, conclusion and future research direction

6.1 General discussion

The main goal of the researchers investigating the pigmentation of farmed salmonids is to increase the retention of dietary carotenoid, improve flesh pigmentation and reduce the high cost of astaxanthin and canthaxanthin supplementation in commercial salmonid feeds. Dietary supplementation of carotenoid increases the feed cost of the salmon industry by approximately 10-15% (Torrissen, 1995) while less than 20% of the dietary carotenoids are retained in salmonid flesh (Torrissen et al., 1989). Numerous studies have focused on the deposition of dietary carotenoid in flesh and the various factors that influence the absorption and deposition of dietary carotenoids. However, very little information exists on *in vitro* binding of carotenoids to the muscle proteins of salmonids. No published information is available on the kinetic studies between carotenoids (astaxanthin or canthaxanthin) and salmon muscle protein except the carotenoids binding kinetics of salmon muscle actin investigated by Côté (2000). The carotenoid binding capability of salmon muscle protein may be an important factor in the retention of dietary carotenoid in the salmonid muscle. The following observations support this assumption. The retention of carotenoids in the muscle of salmonids decreases with increasing dose of dietary carotenoids (Choubert and Storebakken, 1989), although carotenoid concentration in the plasma increases with increasing concentration of carotenoids in the salmonid diet (Choubert et al., 1994a; Storebakken and Goswami, 1996). Other interacting factors such as absorption of carotenoid from the gastrointestinal tract, carotenoid transport capacity and metabolism of carotenoids may also influence carotenoid retention (Chapter 1). The present studies were designed to investigate the role of muscle proteins in the retention of dietary carotenoid in the muscle of Atlantic salmon, the most extensively farmed fish species in Atlantic Canada. After examination of several methods to assess the interaction between astaxanthin and salmon muscle actin, it was evident that the development of a reliable method for binding assay

between astaxanthin and salmon muscle actin was essential. Dispersion of astaxanthin aggregates in aqueous buffer using various concentrations of sodium cholate was evaluated to determine a suitable concentration of sodium cholate for the separation of free (unbound) astaxanthin from protein-bound astaxanthin using a low molecular cut-off filter. An ultrafiltration method was developed to assess astaxanthin binding to salmon muscle actin *in vitro*. Binding studies were performed using astaxanthin and various fractions of solubilized muscle proteins of adult salmon to examine whether other muscle protein(s) bind astaxanthin. *In vitro* astaxanthin binding to the muscle proteins of juvenile salmon and white-fleshed fish (haddock and halibut) were examined for comparison with that of adult salmon. To identify a membrane bound transport or receptor protein in salmon using a proteomics approach, membrane fractions were isolated from salmon muscle tissue and analyzed electrophoretically for determination of up-regulated proteins in membrane fractions of salmon in response to dietary astaxanthin. Proteins up-regulated in the plasma membrane enriched fraction were identified by mass spectrometry. These overall findings are discussed below.

Evaluation of gel filtration chromatography, fluorometry, a binding assay using immobilized actin and displacement of hydrophobic probe methods showed that none of these methods were suitable for the characterization of binding between astaxanthin and salmon muscle actin (Chapter 2). The hydrophobic characteristics of astaxanthin created a challenge for reliable separation of actin-bound astaxanthin from free astaxanthin using the gel filtration chromatography method because astaxanthin aggregated in aqueous buffer and the apparent molecular weight of aggregated astaxanthin was in the same range as astaxanthin-actin complexes. The observation of astaxanthin aggregates in aqueous media is consistent with those of Buchwald and Jencks (1968). Astaxanthin solution mixed with actin extraction buffer (buffer A) at a concentration of 2.23 μM was completely retained in the 50,000 (50 K) nominal molecular weight limit (NMWL) filter because of astaxanthin aggregation. The problem of separation of free astaxanthin from bound astaxanthin by the ultrafiltration method due to aggregation of astaxanthin was overcome by dispersing astaxanthin aggregates in aqueous buffer using 200 mM sodium cholate (Chapter 3). Sodium cholate (200 mM) reduced the size of astaxanthin aggregates in aqueous buffer and 100% of the astaxanthin in aqueous buffer (5.03 μM)

passed through the 30,000 (30 K) NMWL filter. However, 100% passage of astaxanthin through the 30 K NMWL filter was not achieved in the presence of either sodium taurocholate (200 mM) or sodium taurodeoxycholate (200 mM). Formation of larger micelles due to higher aggregation numbers of sodium taurocholate and sodium taurodeoxycholate might be a cause of reduced astaxanthin passage in sodium taurocholate or sodium taurodeoxycholate. The aggregation numbers of sodium cholate, sodium taurocholate and sodium taurodeoxycholate are reported to be 2, 4 and 8, respectively in water, and 4.8, 5 and 24, respectively in the presence of 150 mM NaCl (Helenius et al., 1979).

An ultrafiltration method was developed using 200 mM sodium cholate and the separation of unbound (free) astaxanthin from actin-bound astaxanthin was accomplished through a 30 K NMWL filter. Aggregation of astaxanthin is influenced by buffer composition and astaxanthin concentration. Thus control experiments under identical experimental conditions ruled out the possibility that astaxanthin aggregates were retained in the filter and subsequently accounted as bound astaxanthin.

The results of astaxanthin binding studies conducted with solubilized muscle protein fractions of adult salmon suggest that proteins other than actomyosin or actin also bind astaxanthin in Atlantic salmon flesh. Astaxanthin binding curves of purified salmon muscle actin and solubilized muscle protein fractions (B, D and F) of salmon did not exhibit saturation kinetics at astaxanthin concentrations of 3.35 to 16.75 μM. Astaxanthin was progressively retained in the filter with higher concentrations of astaxanthin due to formation of larger astaxanthin aggregates and this precluded use of higher astaxanthin concentrations for the binding assays. The method developed in this thesis can be used to examine binding of astaxanthin to individual muscle proteins of salmonids.

Comparison of *in vitro* astaxanthin binding to the solubilized muscle proteins of juvenile and adult salmon, haddock and halibut revealed that the pattern and extent of astaxanthin binding to the various fractions of muscle proteins of these species were similar (Chapter 4). The similarity in astaxanthin binding to the muscle proteins of juvenile and adult salmon contrasts with the findings of *in vivo* studies, which showed that juvenile salmonid do not deposit carotenoid in their flesh as efficiently as adult

salmon. No carotenoid was deposited in the flesh of Atlantic salmon weighing 81 g after feeding 8 weeks with diets containing different levels of astaxanthin (Storebakken et al., 1987). These authors reported that when salmon body weight reached about 120 g after 16 weeks of feeding, a minor amount of carotenoid (0.6 mg kg^{-1}; mean values of three dietary concentrations) was detected in their flesh and the carotenoid content of the flesh was 2.0 mg kg^{-1} in salmon that weighed 406 g at the end of the experiment. After feeding 3 year classes of Arctic charr, *Salvelinus alpinus*, with astaxanthin (63 mg kg^{-1} diet) for a period of 13 weeks, 1+ year class fish weighing about 80 g deposited 2.3 mg kg^{-1} carotenoid in their flesh (Hatlen et al., 1995). Meanwhile, the Arctic charr of 2+ (average weight 379 g) and 3+ (average weight 878 g) year classes deposited 3.8 and 6.6 mg kg^{-1} carotenoid, respectively, in their flesh.

The SDS-PAGE profiles of the solubilized muscle proteins of juvenile salmon, haddock and halibut were similar and comparable to those of adult salmon. The comparable astaxanthin binding to the muscle proteins of juvenile and adult Atlantic salmon and white fleshed fish (haddock and halibut) suggests that the carotenoid binding capacity of the muscle proteins of salmon is not the limiting factor in the deposition of carotenoid in their flesh. A good correlation (R^2 = 0.80-0.92) between the relative ANS fluorescence intensities of various fractions of solubilized muscle proteins from each species and the amount of astaxanthin bound to those fractions further supports the assumption of Henmi et al. (1990a) that astaxanthin binds non-specifically to the hydrophobic binding sites on proteins. Non-saturated astaxanthin binding kinetics of salmon muscle proteins, binding of astaxanthin to the muscle proteins of haddock and halibut in a similar extent of adult salmon and correlation between the relative ANS fluorescence intensities and bound astaxanthin of various fractions together strongly suggest non-specific binding of astaxanthin to muscle proteins.

Electrophoretic analyses of the protein profiles obtained from one-dimensional gel electrophoresis of isolated membrane fractions from both of the astaxanthin fed and control group salmon revealed that a few protein bands were up-regulated in fraction 1 of the second homogenization (S_2Fr_1) of muscle tissue of astaxanthin fed salmon (Chapter 5). The highest Na^+K^+-ATPase activity and the lowest Ca^{2+}-ATPase activity in fraction S_2Fr_1 indicated that this fraction was relatively more enriched in plasma membrane

vesicles than the other fractions. This observation is in line with the observation of Saito et al. (1984). According to Saito et al. (1984), electron microscopic examination of fraction 1 obtained from the second homogenization of rabbit skeletal muscle showed that fraction 1 contained plasma membranes.

For proteomics analysis, separation of proteins in a sample is generally accomplished by two-dimensional gel electrophoresis, where proteins are separated in the first dimension according to their pI and later in the second dimension by their molecular weights. One limitation of two-dimensional gel electrophoresis is that hydrophobic proteins tend to precipitate in the buffer used in the first dimension of two-dimensional gel electrophoresis and as a result, do not enter the second dimension. Two-dimensional gel electrophoresis of various fractions obtained from the second homogenization did not show any consistent difference between astaxanthin fed and control group salmon. However, one-dimensional gel electrophoresis of fraction S_2Fr_1, fraction relatively enriched in plasma membrane vesicles, showed the up-regulated proteins in astaxanthin fed salmon. The lack of observed difference in two-dimensional gel electrophoresis of fraction S_2Fr_1 between the astaxanthin fed and control group salmon may be explained by partial or complete precipitation of membrane proteins in the buffer used in the first dimension of two-dimensional gel since the membrane proteins are very hydrophobic. In the present study, one-dimensional gel electrophoresis was employed alongside the two-dimensional gel electrophoresis to track the membrane proteins.

The Mascot search results of the MS/MS spectra of the tryptic peptides from the up-regulated protein bands against the GRASP EST database (Rise et al., 2004) showed that five peptides from a non-annotated salmon protein matched to those of the 24-25 kDa up-regulated protein band with a score of 299. Three peptides from the same non-annotated salmon protein matched to the tryptic peptides of the 22-23 kDa up-regulated protein band with a score of 125. However, the Mascot search results of the MS/MS spectra of the tryptic peptides obtained from the up-regulated protein band of 16-17 kDa was found to be insignificant ($P>0.05$).

Matching of the tryptic peptides from both of the up-regulated protein bands (24-25 kDa and 22-23 kDa) to the same non-annotated salmon protein might be due to contamination of the protein band of 24-25 kDa with the protein band of 22-23 kDa

during manual excision of these bands from the gel for mass spectrometry analysis. These two bands were found to be very closely located on the 12% polyacrylamide gel. Another possibility is that the protein band of 22-23 kDa may be proteolytically derived from the protein band of 24-25 kDa.

Mass spectrometry analysis identified that the 24-25 kDa protein band up-regulated in astaxanthin fed salmon was homologous to myelin protein zero (P0). The extent of sequence matching between the matched non-annotated salmon protein and a published sequence of the myelin P0 from *Danio rerio* (Schweitzer et al., 2003) indicated extensive homology of the matched protein with the myelin P0.

Two peptides of another non-annotated salmon protein matched to those of the 24-25 kDa protein and the sequence of this non-annotated salmon protein also showed numerous matching to the sequence of the ATP synthase H^+ transporting F0 complex, one of the mitochrondrial components. However, the up-regulated proteins were found to be equilibrated in the lowest density fraction, fraction 1 at the top, on a sucrose density gradient centrifuge. Considering the density of fraction 1 that contained the up-regulated proteins in the present study and the density of mitochondria, it is more reasonable to conclude that the up-regulated protein in astaxanthin fed salmon might be a P0-like glycoprotein. However, matching the protein sequence to the ATP synthase H^+ transporting F0 complex indicates minor mitochondrial contamination in the fraction 1 proteins.

From the preliminary findings of the investigation of astaxanthin transport protein(s) in muscle membranes of salmon, it is apparent that the role of a transport protein or membrane-bound receptor protein has yet to be determined. However, it is possible that up-regulation of a myelin P0-like glycoprotein in the muscle membrane of salmon in response to dietary astaxanthin may be involved in facilitating the transfer of astaxanthin from the blood into the muscle.

The above discussed findings of this thesis work revealed that this work has ruled out the assumption that astaxanthin binding to the muscle proteins of salmon is the limiting factor in astaxanthin deposition in salmon flesh. Comparable *in vitro* astaxanthin binding to the muscle proteins of adult salmon, juvenile salmon and halibut (Chapter 4) and the ability of Atlantic halibut to absorb dietary supplemented astaxanthin (Bjerkeng

and Berge, 2000) together suggest that the blood/muscle interface may be the key factor limiting the deposition of astaxanthin in the muscle and hence a membrane bound receptor or transport protein may be involved in transporting astaxanthin from the blood into the muscle. Transport of astaxanthin from the blood to the muscle cells appears to be of crucial importance. For investigation of astaxanthin transport protein in muscle membranes of adult salmon, electrophoretic analyses of membrane fractions of salmon muscle tissue were performed in this study to achieve information whether a transport or receptor protein is up-regulated in response to dietary astaxanthin in salmon (Chapter 5). Mass spectrometry identified the up-regulated protein in astaxanthin fed salmon as a myelin P0-like glycoprotein. However, the role of the P0-like glycoprotein in transporting astaxanthin is not clear. Further study for identification of astaxanthin transport protein in the membranes fraction of astaxanthin fed salmon using different experimental approach such as an astaxanthin affinity column is necessary to elucidate the role of transport protein or receptor protein in flesh deposition of carotenoids in salmon.

6.2 Conclusions

1. The gel filtration chromatography, fluorometry, binding assay using immobilized protein and the displacement of a hydrophobic fluorescent probe method were evaluated. None of these methods were found suitable as an astaxanthin-actin binding assay.

a) The major hindrance for the application of the gel filtration chromatography method to separate protein-bound astaxanthin from free astaxanthin is the formation of astaxanthin aggregates under the experimental conditions.

b) The fluorometry method was unsuccessful because the solvent used to prepare the astaxanthin solution caused changes in the intrinsic fluorescence of actin while adding astaxanthin solution to actin.

c) Insufficient binding of actin to the enzyme-linked immunosorbent assay (ELISA) plates even after the extension of the incubation period up to 72 hr was observed and thus precluded the use of this approach.

d) In the binding study using immobilized actin on Sepharose 4B beads, adsorption of

astaxanthin by the Sepharose 4B beads and inconsistency in the pipetted volume of slurry were the major problems with the application of this approach.

e) Displacement of fluorescent hydrophobic probe was not effective because astaxanthin quenched the fluorophore preventing detection of the displacement.

2. Complete dispersion of astaxanthin (5.03 μM) in aqueous buffer is achieved in presence of 200 mM sodium cholate as assessed by 100% passage of the dispersed astaxanthin through a 30 K NMWL filter.
3. An ultrafiltration method was developed using 200 mM sodium cholate for an astaxanthin- protein binding assay.
4. Non-saturable astaxanthin binding kinetics of actin or various fractions of muscle proteins was observed.
5. All fractions of Atlantic salmon muscle protein bind astaxanthin *in vitro* and the extent of binding is different among the fractions.
6. Proteins other than actin or actomyosin can bind astaxanthin in Atlantic salmon flesh.
7. The extent of *in vitro* astaxanthin binding to the muscle proteins of juvenile Atlantic salmon, haddock and halibut was comparable to that of adult Atlantic salmon. Carotenoid-binding capacity of salmon muscle proteins does not limit the deposition of carotenoid in their flesh.
8. Protein bands were found to be up-regulated in the plasma membrane enriched fraction of Atlantic salmon in response to dietary astaxanthin as indicated by one dimensional gel electrophoresis.
9. The 24-25 kDa protein band up-regulated in Atlantic salmon in response to dietary astaxanthin was identified as a myelin P0-like glycoprotein by mass spectrometry.

6.3 Future research direction

The present research focused on development of a method for astaxanthin-protein binding assay, *in vitro* astaxanthin binding to the muscle proteins of both Atlantic salmon (juvenile and adult) and white-fleshed fish, and the proteomics approach to investigate membrane bound astaxanthin transport protein(s) in adult salmon. The findings of this thesis work has ruled out that astaxanthin binding to the muscle protein of salmon is the limiting factor for deposition of astaxanthin in salmon flesh. In investigation of

astaxanthin transport protein in muscle membranes of salmon, the protein with the molecular weight between 24-25 kDa up-regulated in plasma membrane enriched fraction of salmon in response to dietary astaxanthin was identified as a myelin P0-like glycoprotein.

It has been suggested that retinoic acid may regulate gene expression in peripheral nerve regeneration (Johann et al., 2003; Zhelyaznik et al., 2003). Retinoic acid regulates the expression of a gene by binding to nuclear retinoid receptors (Mey and McCaffery, 2004). Therefore, it is likely that the amount of retinoic acid may be elevated in salmon because of dietary supplemented astaxanthin and the elevated retinoic acid influences the expression of the myelin P0-like glycoprotein in astaxanthin fed salmon.

The role of the myelin P0-like glycoprotein in transporting astaxanthin from the blood into the muscle is not clear. Further investigation for identification of astaxanthin transport protein in membranes fraction of muscle tissue of astaxanthin fed salmon using an astaxanthin affinity column is necessary to identify a carotenoid transport mechanism in salmonid.

Juvenile salmonids do not deposit carotenoids in their flesh efficiently (Torrissen et al., 1989; Hatlen et al., 1995), although *in vitro* binding studies showed that the extent of astaxanthin binding to the muscle proteins of juvenile Atlantic salmon was comparable to that of adult salmon (Saha et al., manuscript submitted). Isolation of muscle membranes from both astaxanthin fed and control group juvenile salmon muscle tissue and determination of differences in the protein profiles of membrane fractions of juvenile salmon compared to adult salmon may provide information regarding the failure in flesh pigmentation of juvenile salmon. However, the procedure for isolation of membranes fraction from muscle tissue of juvenile salmon may need to be normalized with that of adult salmon to eliminate any artefact that may indicate the difference between juvenile salmon and adult salmon.

Limited information is available on digestion, absorption and metabolism of two oxygenated carotenoids, astaxanthin and canthaxanthin in salmonid fishes. The mechanism of digestion and absorption is also not clear. Carotenoids are hydrophobic compounds and thus absorption depends on micellar solubilization for their dispersion in the aquatic environment of the intestinal lumen. Poor intestinal absorption of dietary

carotenoids appears to be one of the major limitations to carotenoid utilization in salmonids. Higher bioavailability of dietary carotenoids by intraperitoneal injection compared to oral administration [Maltby (née Wieruszewski) et al., 2003] indicates that the gastrointestinal wall is a barrier for efficient carotenoid absorption. Slow intestinal uptake, biotransformation of dietary carotenoids in the intestinal wall and high plasma carotenoid concentration after administration of carotenoid intraperitoneally indicates that either carrier proteins or enzymes in the intestinal wall play a role in the absorption process. The receptors or carrier proteins responsible for the intestinal uptake of carotenoids have not been identified and need to be investigated.

In studies involving labeled astaxanthin, high radioactivity was recovered in the bile and low activity in the liver, suggesting an active metabolism of this carotenoid in the liver (Torrissen and Ingebrigtsen, 1992). The possibility of a significant amount of urinary excretion of carotenoids has been also proposed (Torrissen and Ingebrigtsen, 1992). The lack of induction of liver or kidney xenobiotic-metabolizing enzyme activities by carotenoids in salmonids reported by Page and Davies (2002) and Amcoff et al. (1998) is in contrast to the observed high radioactivity in the bile and posterior kidney and points to a different metabolizing enzyme system in salmonids. Enzyme systems responsible for carotenoid metabolism need to be identified.

References

Aas, G.H., Bjerkeng, B., Hatlen, B., Storebakken, T., 1997. Idoxanthin, a major carotenoid in the flesh of Arctic charr (*Salvelinus alpinus*) fed diets containing astaxanthin. Aquaculture **150**, 135-142.

Aas, G.H., Bjerkeng, B., Storebakken, T., Ruyter, B., 1999. Blood appearance, metabolic transformation and plasma transport proteins of ^{14}C-astaxanthin in Atlantic salmon (*Salmo salar* L.). Fish Physiol. Biochem. **21**, 325-334.

Al-Khalifa, A.S., Simpson, K.L., 1988. Metabolism of astaxanthin in the rainbow trout (*Salmo gairdneri*). Comp. Biochem. Physiol. **91B**, 563-568.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acid Res. **25**, 3389-3402.

Amcoff, P., Börjeson, H., Norrgren, L., Pesonen, M., 1998. Cytochrome P4501A-activity in rainbow trout (*Oncorhynchus mykiss*) hepatocytes after exposure to PCB#126 and astaxanthin: *in vivo* and *in vitro* studies. Mar. Environ. Res. **46**, 1-5.

Ando, S., Hatano, M., 1988a. Isolation of apolipoproteins from carotenoid-carrying lipoprotein in the serum of chum salmon, *Oncorhynchus keta.* J. Lipid Res. **29**, 1264-1271.

Ando, S., Hatano, M., 1988b. Bilirubin-binding protein in the serum of spawning-migrating chum salmon, *Oncorhynchus keta* : Its identity with carotenoid-carrying lipoprotein. Fish Physiol. Biochem. **5**, 69-78.

Ando, S., Takeyama, T., Hatano, M., 1986a. Isolation and characterization of a carotenoid-carrying lipoprotein in the serum of chum salmon (*Oncorhynchus keta*) during spawning migration. Agric. Biol. Chem. **50**, 907-914.

Ando, S., Takeyama, T., Hatano, M., 1986b. Transport associated with serum vitellogenin of carotenoid in chum salmon (*Oncorhynchus keta*). Agric. Biol. Chem. **50**, 557-563.

Ando, S., Osada, K., Hatano, M., Saneyoshi, M., 1989. Comparison of carotenoids in muscle and ovary from four genera of salmonid fishes. Comp. Biochem. Physiol. **93B**, 503-508.

Angelakou, A., Valsami, G., Macheras, P., Koupparis, M., 1999. A displacement approach for competitive drug-protein binding studies using the potentiometric 1-anilino-8-naphthalenesulfonate probe technique. Eur. J. Pharm. Sci. **9**, 123-130.

Astorg, P., Gradelet, S., Leclerc, J., Siess, M.-H., 1997. Effects of provitamin A or non-provitamin A carotenoids on liver xenobiotic-metabolizing enzymes in mice. Nutr. Cancer **27**, 245-249.

Astorg, P., Gradelet, S., Leclerc, J., Canivenc, M.-C., Siess, M.-H., 1994. Effects of β-carotene and canthaxanthin on liver xenobiotic-metabolizing enzymes in the rat. Food Chem. Toxicol. **32**, 735-742.

Bakatselou, V., Oppenheim, R.C., Dressman, J.B., 1991. Solubilization and wetting effects of bile salts on the dissolution of steroids. Pharm. Res. **8**, 1461-1469.

Baker, R.T.M., Pfeiffer, A.-M., Schöner, F.-J., Smith-Lemmon, L., 2002. Pigmenting efficacy of astaxanthin and canthaxanthin in fresh-water reared Atlantic salmon, *Salmo salar*. Animal Feed Sci. Tech. **99**, 97-106.

Ballard, K.D., Gaskell, S.J., 1993. Dehydration of peptide $(M + H)^+$ ions in the gas phase. J. Am. Soc. Mass Spec. **4**, 477-481.

Barbosa, M.J., Morais, R., Choubert, G., 1999. Effect of carotenoid source and dietary lipid content on blood astaxanthin concentration in rainbow trout (*Oncorhynchus mykiss*). Aquaculture **176**, 331-341.

Barua, A.B., Goswami, U., 1977. Formation of vitamin A in a freshwater fish. Isolation of retinoic acid. Biochem. J. **164**, 133-136.

Bjerkeng, B., 2000. Carotenoid pigmentation of salmonid fishes - recent progress. In: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Olvera-Novoa, M.A. y Civera-Cerecedo, R., (Eds.). Avances en Nutrición Acuícola V. Memorias del V Simposium Internacional de Nutrición Acuícola. 19-22 Noviembre, 2000. Mérida, Yucatán.

Bjerkeng, B., Berge, G.M., 2000. Apparent digestibility coefficient and accumulation of astaxanthin *E/Z* isomers in Atlantic salmon (*Salmo salar* L.) and Atlantic halibut (*Hippoglossus hippoglossus* L.). Comp. Biochem. Physiol. **127B**, 423-432.

Bjerkeng, B., Hatlen, B., Jobling, M., 2000. Astaxanthin and its metabolites idoxanthin and crustaxanthin in flesh, skin, and gonads of sexually immature and maturing Arctic charr (*Salvelinus alpinus* L.). Comp. Biochem. Physiol. **125B**, 395-404.

Bjerkeng, B., Hatlen, B., Wathne, E., 1999a. Deposition of astaxanthin in fillets of Atlantic salmon (*Salmo salar*) fed diets with herring, capelin, sandeel, or Peruvian high PUFA oils. Aquaculture **180**, 307-319.

Bjerkeng, B., Storebakken, T., Liaaen-Jensen, S., 1992. Pigmentation of rainbow trout from start feeding to sexual maturation. Aquaculture **108**, 333-346.

Bjerkeng, B., Hamre, K., Hatlen, B., Wathne, E., 1999b. Astaxanthin deposition in the fillets of Atlantic salmon *Salmo salar* L. fed two dietary levels of astaxanthin in combine with three levels of α-tocopheryl acetate. Aquacult. Res. **30**, 637-646.

Bjerkeng, B., Følling, M., Lagocki, S., Storebakken, T., Olli, J.J., Alsted, N., 1997. Bioavailability of all-*E*-astaxanthin and *Z*-isomers of astaxanthin in rainbow trout (*Oncorhynchus mykiss*). Aquaculture **157**, 63-82.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72,** 248-254.

Briton, G., 1985. General carotenoid methods. Methods Enzymol. **111**, 113-149.

Buchwald, M., Jencks, W.P., 1968. Optical properties of astaxanthin solutions and aggregates. Biochemistry **7**, 834-843.

Buttle, L.G., Crampton, V.O., Williams, P.D., 2001. The effect of feed pigment type on flesh pigment deposition and colour in farmed Atlantic salmon, *Salmo salar* L. Aquacult. Res. **32**, 103-111.

Careche, M., Li-Chan, E.C.Y., 1997. Structural changes in cod myosin after modification with formaldehyde or frozen storage. J. Food Sci. **62**, 717-723.

Chan, J.K., Gill, T.A., Paulson, A.T., 1992. The dynamics of thermal denaturation of fish. Food Res. Interl. **25**, 117-123.

Chapman, M.J., Goldstein, S., Mills, G.L., Leger, C., 1978. Distribution and characterization of the serum lipoproteins and their apoproteins in the rainbow trout (*Salmo gairdneri*). Biochemistry **17**, 4455-4464.

Chavez, P.R.G., Rengel, D., Gómez, R. Choubert, G., Milicua, J.-C.G., 1998. Canthaxanthin saturation of serum lipoproteins from immature rainbow trout (*Oncorhynchus mykiss*). Comp. Biochem. Physiol. **121B**, 129-134.

Cheesman, D.F., Lee, W.L., Zagalsky, P.F., 1967. Carotenoproteins in invertebrates. Biol. Rev. **42**, 132-160.

Choubert, G., Blanc, J.-M., 1985. Flesh color of diploid and triploid rainbow trout (*Salmo gairdneri* Rich.) fed canthaxanthin. Aquaculture **47**, 299-304.

Choubert, G., Storebakken, T., 1989. Dose response to astaxanthin and canthaxanthin pigmentation of rainbow trout fed various dietary carotenoid concentrations. Aquaculture **81**, 69-77.

Choubert, G., de la Noüe, J., Blanc, J.-M., 1991. Apparent digestibility of canthaxanthin in rainbow trout: effect of dietary fat level, antibiotics and number of pyloric caeca. Aquaculture **99**, 323-319.

Choubert, G., Gomez, R., Milicua, J.-C.G., 1994a. Response of serum carotenoid level to dietary astaxanthin and canthaxanthin in immature rainbow trout, *Oncorhynchus mykiss*. Comp. Biochem. Physiol. **109A**, 1001-1006.

Choubert, G., Milicua, J-C.G., Gomez, R., 1994b. The transport of astaxanthin in immature rainbow trout, *Oncorhynchus mykiss* serum. Comp. Biochem. Physiol. **108A**, 245-248.

Choubert, G., Milicua, J.-C.G., Martinez, R.G., Sance, S., Pett, H., Negre-Sadargues, G., Castillo, R., Trilles, J.-P., 1992. Distribution of canthaxanthin in immature rainbow trout (*Oncorhynchus mykiss*) serum. Comp. Biochem. Physiol. **103A**, 403-405.

Chytil, F., Ong, D.E., 1987. Intracellular vitamin A-binding proteins. Ann. Rev. Nutr. **7**, 321-335.

Cogan, U., Kopelman, M., Mokady, S., Shinitzky, M., 1976. Binding affinities of retinol and related compounds to retinol binding proteins. Eur. J. Biochem. **65**, 71-78.

Côté, M., 2000. Isolation and characterization of the carotenoid-binding protein in Atlantic salmon (*Salmo salar*). M.Sc. Thesis, Dalhousie University, pp 92.

Crozier, G.F., 1970. Tissue carotenoids in prespawning and spawning sockeye salmon (*Oncorhynchus nerka*). J. Fish. Res. Board Can. **27**, 973-975.

Davies, B.H., 1985. Carotenoid metabolism in animals: a biochemist's view. Pure Appl. Chem. **57**, 679-684.

During, A., Harrison, E.L., 2004. Intestinal absorption and metabolism of carotenoids: insights from cell culture. Arch. Biochem. Biophys. **430**, 77-88.

Ellman, G.L., 1959. Tissue sulfhydryl groups. Arch. Biochem. Biophys. **82**, 70-77.

Ellman, G.L., Courtney, K.D., Anders, V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharma. **7**, 88-95.

Erdman, J.W. Jr., 1988. The physiological chemistry of carotenes in man. Clin. Nutr. **7**, 101-106.

Erdman, J.W. Jr., Bierer, T.L., Gugger, E.T., 1993. Absorption and transport of carotenoids. Ann. N.Y. Acad. Sci. **691**, 76-85.

Er-el, Z., Zaidenzaig, Y., Shaltiel, S., 1972. Hydrocarbon coated Sepharose: Use in the purification of Glycogen phosphate. Biochem. Biophys. Res. Commun. **49**, 383-390.

Essassi, D., Zini, R., Tillement, J-P., 1990. Use of 1-anilino-8-naphthalenesulfonate as a fluorescent probe in the investigation of drug interactions with human alpha-1-acid glycoprotein and serum albumin. J. Pharm. Sci. **79**, 9-13.

Everly, J.I., Brady, R.O., Quarles, R.H., 1973. Evidence that the major protein in rat sciatic nerve myelin is a glycoprotein. J. Neurochem. **21**, 329-334.

Foss, P., Storebakken. T., Austreng, E., Liaaen-Jensen, S., 1987. Carotenoids in diets for salmonids. V. Pigmentation of rainbow trout and sea trout with astaxanthin and astaxanthin dipalmitate in comparison with canthaxanthin. Aquaculture **65**, 293-305.

Foss, P., Storebakken. T., Schiedt, K., Liaaen-Jensen, S., Austreng, E., Streiff, K., 1984. Carotenoids in diets for salmonids. I. Pigmentation of rainbow trout with individual optical isomers of astaxanthin in comparison with canthaxanthin. Aquaculture **41**, 213-226.

Frémont, L., Marion, D., 1982. A comparison of the lipoprotein profiles in male trout (*Salmo gairdneri*) before maturity and during spermiation. Comp. Biochem. Physiol. **73B**, 849-855.

Goodwin, T.W., 1984. Carotenoid-protein complexes. In: *The Biochemistry of the Carotenoids*, Vol. II Animals (ed. T.W. Goodwin) pp. 1-21. Chapman and Hall, London.

Gradelet, S., Astorg, P., Leclerc, J., Chevalier, J., Vernevaut, M.-F., Siess, M.-H., 1996. Effects of canthaxanthin, astaxanthin, lycopene, and lutein on liver xenobiotic-metabolizing enzymes in the rat. Xenobiotica **26**, 49-63.

Gross, J., Budowski, P., 1966. Conversion of carotenoids in to vitamin A_1 and A_2 in two species of freshwater fish. Biochem. J. **101**, 747-754.

Guillou, A., Choubert, G., Noue, J.D.L., 1992. Comparative accumulation of labelled carotenoids (^{14}C-astaxanthin, ^{3}H-canthaxanthin and ^{3}H-zeaxanthin) in mature female rainbow trout (*Oncorhynchus mykiss*). Comp. Biochem. Physiol. **102B**, 61-65.

Hardy, R.W., Torrissen, O.J., Scott, T.M., 1990. Absorption and distribution of ^{14}C-labelled canthaxanthin in rainbow trout (*Oncorhynchus mykiss*). Aquaculture **87**, 331-340.

Hata, M., Hata, M., 1972. Carotenoid pigments in goldfish. IV. Carotenoid metabolism. Bull. Jpn. Soc. Sci. Fish. **38**, 331-337.

Hata, M., Hata, M., 1976. Carotenoid metabolism in fancy red carp, *Cyprinus carpio*. II. Metabolism of ^{14}C-zeaxanthin. Bull. Jpn. Soc. Sci., Fish. **42**, 203-205.

Hatlen, B., Aas, G.H., Jørgensen, E.H., Storebakken, T., Goswami, U.C., 1995. Pigmentation of 1, 2 and 3 year old Arctic charr (*Salvelinus alpinus*) fed two different dietary astaxanthin concentrations. Aquaculture **138**, 303-312.

Helenius, A., McCaslin, D.R., Fries, E., Tanford, C., 1979. Properties of detergents. Methods Enzymol. **56**, 734-749.

Henmi, H., Hata, M., Hata, M., 1989. Astaxanthin and/or canthaxanthin-actomyosin complex in salmon muscle. Nippon Suisan Gakkaishi **55**, 1583-1589.

Henmi, H., Hata, M., Hata, M., 1990a. Combination of astaxanthin and canthaxanthin with fish muscle actomyosins associated with their surface hydrophobicity. Nippon Suisan Gakkaishi **56**, 1821-1823.

Henmi, H., Hata, M., Takeuchi, M., 1990b. Resonance Raman and circular dichroism studies of astaxanthin and/or canthaxanthin in salmon muscle. Nippon Suisan Gakkaishi **56**, 1825-1828.

Henmi, H., Hata, M., Takeuchi, M., 1991. Studies on the carotenoids in the muscle of salmon–V. Combination of astaxanthin and canthaxanthin with bovine serum albumin and egg albumin. Comp. Biochem. Physiol. **99B**, 609-612.

Henmi, H., Iwata, T., Hata M., Hata, M., 1987. Studies on the carotenoids in the muscle of salmons. I. Intracellular distribution of carotenoids in the muscle. Tohoku J. Agric. Res. **37**, 101-111.

Hofstee, B.H.J., 1973. Hydrophobic affinity chromatography of proteins. Anal. Biochem. **52**, 430-448.

Hollander, D., Ruble, P.E., 1978. β-carotene intestinal absorption: bile, fatty acid, pH, and flow rate effects on transport. Am. J. Physiol. **235**, E686-E691.

Jewell, C., O'Brien, N.M., 1999. Effects of dietary supplementation with carotenoids on xenobiotic-metabolizing enzymes in the liver, lung, kidney and small intestine of the rat. Br. J. Nutr. **81**, 235-242.

Johann, V., Jelianzik, N., Schrage, K., Mey, J., 2003. Retinoic acid downregulates the expression of ciliary neurotrophic factor in rat Schwann cells. Neurosci. Lett. **339**, 13-16.

Johnson, E.A., Villa, T.G., Lewis, M.G., 1980. *Phaffia rhodozyma* as an astaxanthin source in salmonid diets. Aquaculture **20**, 123-134.

Jones, L.R., Besch, H.R., Jr., Fleming, J.W., McConnaughey, M.M., Watanabe, A.M., 1979. Separation of vesicles of cardiac sarcolemma from vesicle of cardiac sarcoplasmic reticulum: comparative biochemical analysis of component activities. J. Biol. Chem. **254**, 530-539.

Kanai, M., Raz, A., Goodman, D.S., 1968. Retinol-binding protein: the transport protein for vitamin A in human plasma. J. Clin. Invest. **47**, 2025-2044.

Katayama, T., Shimaya, M., Sameshima, M., 1973a. The biosynthesis of astaxanthin. XII. The conversion of labelled β-carotene-15,15'-$^3H_2$ into body astaxanthin in the lobster, *Panulirus japonicus*. Int. J. Biochem. **4**, 223-226.

Katayama, T., Kamata, T., Shimaya, M., Deshimaru, O., Chichester, C.O., 1972. The biosynthesis of astaxanthin. VIII. The conversion of labelled β-carotene-15,15'-$^3H_2$ into astaxanthin in prawn, *Penaeus japonicus* Bate. Bull. Jpn. Soc. Sci. Fish. **38**, 1171-1175.

Katayama, T., Kunishaki, Y., Shimaya, M., Simpson, K.L., Chichester, C.O., 1973b. The biosynthesis of astaxanthin. XIV. The conversion of labelled β-carotene-15,15'-$^3H_2$ into astaxanthin in the crab, *Portunus trituberculatus*. Comp. Biochem. Physiol. **46B**, 269-272.

Katsuyama, M., Matsuno, T., 1988. Carotenoid and vitamin A, and metabolism of carotenoids, β-carotene, canthaxanthin, astaxanthin, zeaxanthin, lutein and tunaxanthin in Tilapia, *Tilapia nilotica*. Comp. Biochem. Physiol. **90B**, 131-139.

Khare, A. Moss, G.P., Weedon, B.C.L., Matthews, A.D., 1973. Identification of astaxanthin in Scottish salmon. Comp. Biochem. Physiol. **45B**, 971-973.

Kiessling, A., Olsen, R.-E., Buttle, L., 2003. Given the same dietary carotenoid inclusion, Atlantic salmon, *Salmo salar* (L.) display higher blood levels of canthaxanthin than astaxanthin. Aquacult. Nutr. **9**, 253-261.

Kiessling, A., Dosanjh, B., Higgs, D., Deacon, G., Rowshandeli, N., 1995. Dorsal aorta cannulation: a method to monitor changes in blood levels of astaxanthin in voluntary feeding of Atlantic salmon, *Salmo salar* L. Aquacult. Nutr. **1**, 43-50.

Kitahara, T., 1983. Behavior of carotenoids in the chum salmon (*Oncorhynchus keta*) during anadromous migration. Comp. Biochem. Physiol. **76B**, 97-101.

Kitamura, K., Suzuki, M., Uyemura, K., 1976. Purification and partial characterization of two glycoproteins in bovine peripheral nerve myelin membrane. Biochim. Biophys. Acta. **455**, 806-809.

Krishnamurthy, G., Chang, H-S., Hultin, H.O., Feng, Y., Srinivasan, S., Kelleher, S. D., 1996. Solubility of chicken breast muscle proteins in solutions of low ionic strength. J. Agric. Food Chem. **44**, 408-415.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**, 680-685.

Lakshman, M.R., Chansang, H., Olson, J.A., 1972. Purification and properties of carotene 15,15′-dioxygenase of rabbit intestine. J. Lipid Res. **13**, 477-482.

Leibler, D.C., 2002. Introduction to Proteomics: Tools for the New Biology. Humana Press, Inc. Totowa, N.J.

Li, C.-Y., Zimmerman, C.L., Wiedmann, T.S., 1996. Solubilization of retinoids by bile salt/phospholipids aggregates. Pharm. Res. **13**, 907-913.

Li-Chan, E., Nakai, S., Wood, D.F., 1985. Relationship between functional (fat binding, emulsifying) and physiochemical properties of muscle proteins. Effects of heating, freezing, pH and species. J. Food Sci. **50**, 1034-1040.

Lockwood, S.F., O'Malley, S., Mosher, G.L., 2003. Improved aqueous solubility of crystalline astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) by Captisol® (sulfobutyl ether β-cyclodextrin). J. Pharm. Sci. **92**, 922-926.

MacMahon, W., Thompson, J., Bowers, W., Sgoutas, D., 1983. A simplified ultrafiltration method for determination of serum free cortisol. Clin. Chim. Acta **131**, 171-184.

Maltby (née Wieruszewski), J.B., Albright, L.J., Kennedy, C.J., Higgs, D.A., 2003. Effect of route of administration and carrier on bioavailability and kinetics of astaxanthin in Atlantic salmon, *Salmo salar* L. Aquacult. Res. **34**, 829-838.

March, B.E., MacMillan, C., 1996. Muscle pigmentation and plasma concentrations of astaxanthin in rainbow trout, Chinook salmon, and Atlantic salmon in response to different dietary levels of astaxanthin. Prog. Fish-Cult. **58**, 178-186.

March, B.E., Hajen, W.E., Deacon, G., MacMillan, C., Walsh, M.G., 1990. Intestinal absorption of astaxanthin, plasma astaxanthin concentration, body weight, and metabolic rate as determinants of flesh pigmentation in salmonid fish. Aquaculture **90**, 313-322.

Matsuno, T., Hirao, S., 1989. Marine carotenoids. In: Ackman, R.G. (Ed.), Marine Biogenic Lipids, Fats, and Oils, **vol. 1**. CRC Press, Boca Raton, Florida, USA, pp. 251-388.

McCaffery, P., Dräger, U.C., 2000. Regulation of retinoic acid signaling in the embryonic nervous system: a master differentiation factor. Cytokine Growth Factor Rev. **11**, 233-249.

McCallum, I.M., Cheng, K.M., March, B.E., 1987. Carotenoid pigmentation in two strains of Chinook salmon (*Oncorhynchus tshawytscha*) and their crosses. Aquaculture **67**, 291-300.

McCormack, A.L., Somogyi, A., Dongre, A., Wysocki, V., 1993. Fragmentation of protonated peptides: surface-induced dissociation in conjunction with a quantum mechanical approach. Anal. Chem. **65**, 2859-2872.

Metusalach, Synowiecki, J., Brown, J., Shahidi, F., 1996. Deposition and metabolism of dietary canthaxanthin in different organs of Arctic charr (*Salvelinus alpinus* L.). Aquaculture **142**, 99-106.

Mey, J., McCaffery, P., 2004. Retinoic acid signalling in the nervous system of adult vertebrates. Neuroscientist **10**, 409-421.

Mithani, S.D., Bakatselou, V., TenHoor, C.N., Dressman, J.B., 1996. Estimation of the increase in solubility of drugs as a function of bile salt concentration. Pharm. Res. **13**, 163-167.

Muto, Y., Goodman, D.S., 1972. Vitamin A transport in rat plasma. Isolation and characterization of retinol-binding protein. J. Biol. Chem. **247**, 2533-2541.

Muto, Y., Smith, F.R., Goodman, D.S., 1973. Comparative studies of retinol transport in plasma. J. Lipid Res. **14**, 525-532.

Nakamura, K., Hata, M., Hata, M., 1985. A study on astaxanthin in salmon *Oncorhynchus keta* serum. Nippon Suisan Gakkaishi **51**, 979-983.

Nanton, D.A., 2002. Lipid metabolism of haddock (*Melanogrammus aeglefinus*). Ph.D Thesis, University of Prince Edward Island, 182 pp.

Okoh, C., Mychkovsky, I., Lakshman, M.R., 1993. Isolation and some properties of a carotenoid-protein complex from rat liver. J. Nutr. Biochem. **4**, 569-575.

Olsen, R.E., Kiessling, A., Milley, J.E., Ross, N.W., Lall, S.P., 2005. Effect of lipid source and bile salts in diet of Atlantic salmon, *Salmo salar* L., on astaxanthin blood levels. Aquaculture 250, 804-812.

Olson, J.A., 1994. Absorption, transport, and metabolism of carotenoids in humans. Pure Appl. Chem. **66**, 1011-1016.

Ong, D.E., Chytil, F., 1978. Cellular retinol-binding protein from rat liver – Purification and characterization. J. Biol. Chem. **253**, 828-832.

Page, G.I, Davies, S.J., 2002. Astaxanthin and canthaxanthin do not induce liver or kidney xenobiotic-metabolizing enzymes in rainbow trout (*Oncorhynchus mykiss* Walbaum). Comp. Biochem. Physiol. **133C**, 443-451.

Page, G.I, Davies, S.J., 2003. Hepatic carotenoid uptake in rainbow trout (*Oncorhynchus mykiss*) using an isolated organ perfusion model. Aquaculture **225**, 405-419.

Pardee, J.D., Spudich, J.A., 1982. Purification of muscle actin. Methods Enzymol. **85**, 164-181.

Parker, R.S., 1996. Absorption, metabolism and transport of carotenoids. FASEB J. **10**, 542-551.

Perkins, D.N., Pappin, D.J.C., Creasy, D.M., Cottrell, J.S., 1999. Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis **20**, 3551-3567.

Peterson, P.A., 1971. Characteristics of a vitamin A transporting protein complex occurring in human serum. J. Biol. Chem. **246**, 34-43.

Prince, M.R., Frisoli, J.K., 1993. Beta-carotene accumulation in serum and skin. Am. J. Clin. Nutr. **57**, 175-181.

Rao, M.N., Ghosh, P., Lakshman, M.R., 1997. Purification and partial characterization of a cellular carotenoid-binding protein from ferret liver. J. Biol. Chem. **272**, 24455-24460.

Renstrøm, B., Liaaen-Jensen, S., 1981. Optical purity of (3S, 3′S)-astaxanthin from *Haematococcus pluvialis*. Phytochemistry **20**, 2561-2564.

Rise, M.L., von Schalburg, K.R., Brown, G.D., Mawer, M.A., Devlin, R.H., Kuipers, N., Busby, M., Beetz-Sargent, M., Alberto, R., Gibbs, A.R., Hunt, P., Shukin,

R., Zeznik, J.A., Nelson, C., Jones, S.R.M., Smailus, D.E., Jones, S.J.M., Schein, J.E., Marra, M.A., Butterfield, Y.S.N., Stott, J.M., Ng, S.H.S., Davidson, W.S., Koop, B.F., 2004. Development and application of a salmonid EST database and cDNA microarray: data mining and interspecific hybridization characteristics. Genome Res. **14**, 478-490.

Roomi, M.W., Ishaque, A., Khann, N.R., Eylar, F.H., 1978. The P0-protein. The major glycoprotein of peripheral nerve myelin. Biochim. Biophys. Acta. **536**, 112-121.

Saha, M.R., Ross, N.W., Gill, T.A., Olsen, R.E., Lall, S.P., 2005. Development of a method to assess binding of astaxanthin to Atlantic salmon *Salmo salar* L. muscle proteins. Aquacult. Res. **36**, 336-343.

Saito, A., Seiler, S., Chu, A., Fleischer, S., 1984. Preparation and morphology of sarcoplasmic reticulum terminal cisternae from rabbit skeletal muscle. J. Cell Biol. **99**, 875-885.

Scalia, S., Isaksen, M., Francis, G.W., 1989. Carotenoids of the Arctic charr, *Salvelinus alpinus* (L.). J. Fish Biol. **34**, 969-970.

Schiedt, K., Leuenberger, F.J., Vecchi, M., 1981. Natural occurrence of enantiomeric and meso-astaxanthin. 5. Ex wild salmon (*Salmo salar* and *Oncorhynchus*). Helv. Chim. Acta **64**, 449-457.

Schiedt, K., Vecchi, M., Glinz, E., 1986. Astaxanthin and its metabolites in wild rainbow trout (*Salmo gairdneri* R.). Comp. Biochem. Physiol. **83B**, 9-12.

Schiedt, K., Foss, P., Storebakken, T., Liaaen-Jensen, S., 1989. Metabolism of carotenoids in salmonids- I. Idoxanthin, a metabolite of astaxanthin in the flesh of Atlantic salmon (*Salmo salar* L.) under varying external conditions. Comp. Biochem. Physiol. **92B**, 277-281.

Schiedt, K., Leuenberger, F.J., Vecchi, M., Glinz, E., 1985. Absorption, retention and metabolic transformations of carotenoids in rainbow trout, salmon and chicken. Pure Appl. Chem. **57**, 685-692.

Schiedt, K., Vecchi, M., Glinz, E., Storebakken, T., 1988a. Metabolism of carotenoids in salmonids. 3. Metabolites of astaxanthin and canthaxanthin in the skin of Atlantic salmon (*Salmo salar* L.). Helv. Chim. Acta **71**, 887-896.

Schiedt, K., Mayer, H., Vecchi, M., Glinz, E., Storebakken, T., 1988b. Metabolism of carotenoids in salmonids. 2. Distribution and absolute configuration of idoxanthin in various organs and tissues of one Atlantic salmon (*Salmo salar* L.) fed with astaxanthin. Helv. Chim. Acta **71**, 881-886.

Schweitzer, V., Becker, T., Becker, G.C., Schachner, M., 2003. Expression of P0 is increased in lesion axon in the central nervous system (CNS) of adult zebra fish. Glia **41**, 301-317.

Seiler, S., Fleischer, S., 1982. Isolation of plasma membrane vesicles from rabbit skeletal muscle and their use in ion transport studies. J. Biol. Chem. **257**, 13862-13871.

Shahidi, F., Metusalach, Brown, J.A., 1998. Carotenoid pigments in seafoods and aquaculture. Crit. Rev. Food Sci. Nutr. **38**, 1-67.

Shevchenko, A., Wilm, M., Vorm, O., Mann, M., 1996. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal. Chem. **68**, 850-858.

Sivtseva, L.V., 1982. Qualitative composition and distribution of carotenoids and vitamin A in the organs and tissues of rainbow trout, *Salmo gairdneri.* J. Ichthyol. **22**, 96-100.

Sivtseva, L.V., Dubrovin, V.N., 1981. Some patterns in the quantitative distribution of carotenoid pigments in the body of rainbow trout, *Salmo gairdneri.* J. Ichthyol. **21**, 142-146.

Sokal, R.P., Rohlf, F.J., 1995. Two-way analysis of variance. In: Biometry: The principles and practice of statistics in biological research, 3rd edition, Academic Press, New York, pp. 321-363.

Sophianopoulos, J.A., Sophianopoulos, A.J., MacMahon ,W.C., 1983. Manganese, calcium, and saccharide binding to concanavalin A, as studied by ultrafiltration. Arch. Biochem. Biophys. **223**, 350-359.

Steck, T.L., Kant, J.A., 1974. Preparation of impermeable ghosts and inside-out vesicles from human erythrocyte membrane. Methods Enzymol. **31**, 172-180.

Storebakken, T., Goswami, U.C., 1996. Plasma carotenoid concentration indicates the availability of dietary astaxanthin for Atlantic salmon, *Salmo salar.* Aquaculture **146**, 147-153.

Storebakken, T., No, H.K., 1992. Pigmentation of rainbow trout. Aquaculture **100**, 209-229.

Storebakken, T., Foss, P., Schiedt, K., Austreng, E., Liaaen-Jensen, S., Manz, U., 1987. Carotenoids in the diets for salmonids. IV. Pigmentation of Atlantic salmon with astaxanthin, astaxanthin dipalmitate and canthaxanthin. Aquaculture **65**, 279-292.

Swain, M., Ross, N.W., 1995. A silver stain protocol for proteins yielding high resolution and transparent background in sodium dodecyl sulphate-polyacrylamide gels. Electrophoresis **16**, 948-951.

Torrissen, O.J., 1986. Pigmentation of salmonids – a comparison of astaxanthin and canthaxanthin as pigment sources for rainbow trout. Aquaculture **53**, 271-278.

Torrissen, O.J., 1995. Strategies for salmonid pigmentation. J. Appl. Icthyol. **11**, 276-281.

Torrissen, O.J., Braekkan, O.R., 1979. The utilization of astaxanthin-forms by rainbow trout. Proc. world symposium on finfish nutrition and fishfeed technology, Hamburg 20-30 June, 1978. Vol. II. Berlin 1979.

Torrissen, O.J., Ingebrigtsen, K., 1992. Tissue distribution of ^{14}C-astaxanthin in the Atlantic salmon (*Salmo salar*). Aquaculture **108**, 381-386.

Torrissen, O.J., Naevdal, G., 1984. Pigmentation of salmonids – genetical variation in carotenoid deposition in rainbow trout. Aquaculture **38**, 59-66.

Torrissen, O.J., Naevdal, G., 1988. Pigmentation of salmonids – variation in flesh carotenoids of Atlantic salmon. Aquaculture **68**, 305-310.

Torrissen, O.J., Hardy, R.W., Shearer, K.D., 1989. Pigmentation of salmonids – carotenoids deposition and metabolism. CRC Crit. Rev. Aquat. Sci. **1,** 209-225.

Torrissen, O.J., Tidemann, E., Hansen, F., Raa, J., 1981. Ensiling in acid – a method to stabilize astaxanthin in shrimp processing by-products and improve uptake of this pigment by rainbow trout. Aquaculture **26**, 77-83.

Torrissen, O.J., Hardy, R.W., Shearer, K.D., Scott T.M., Stone, F.E., 1990. Effects of dietary canthaxanthin level and lipid level on apparent digestibility coefficients for canthaxanthin in rainbow trout (*Oncorhynchus mykiss*). Aquaculture **88**, 351-362.

Tsushima, M., Ikuno, Y., Nagata, S., Kodama, K., Matsuno, T., 2002. Comparative biochemical studies of carotenoids in catfishes. Comp. Biochem. Physiol. **133B**, 331-336.

Viahos, I., MacMahon, W., Sgoutas, D., Bowers, W., Thompson, J., Trawick, W., 1982. An improved ultrafiltration method for determining free testosterone in serum. Clin. Chem. **28**, 2286-2291.

Walters, R.R., 1985. Affinity Chromatography. Anal. Chem. **57**, 1099A-1114A.

Watabe, S., Itoch, Y., Hashimoto, K., 1983. Isolation and characterization of actin from mackerel dark muscle. Bull. Jpn. Soc. Fish. **49**, 491-497.

Wathne, E., Bjerkeng, B., Storebakken, T., Vassvik, V., Odland, A.B., 1998. Pigmentation of Atlantic salmon (*Salmo salar*) fed astaxanthin in all meals or in alternating meals. Aquaculture **159**, 217-231.

Weedon, B.C.L., Moss, G.P., 1995. Structure and Nomenclature. In: G. Britton, S. Liaaen-Jensen, and H. Pfander (Eds.), Carotenoids Vol. 1A: Isolation and Analysis. Birkhauser Verlag, Basel, Boston, Berlin. pp. 27-69.

White, D.A., Ørnsrud, R., Davies, S.J., 2003a. Determination of carotenoid and vitamin A concentrations in everted salmonid intestine following exposure to solutions of carotenoid *in vitro*. Comp. Biochem. Physiol. **136A**, 683-692.

White, D.A., Page, G.I., Swaile, J., Moody, A.J., Davies, S.J., 2002. Effect of esterification on the absorption of astaxanthin in rainbow trout, *Oncorhynchus mykiss* (Walbaum). Aquacult. Res. **33**, 343-350.

White, D.A., Moody, A.J., Serwata, R.D., Bowen, J., Soutar, C., Young, A.J., Davies, S.J., 2003b. The degree of carotenoid esterification influences the absorption of astaxanthin in rainbow trout, *Oncorhynchus mykiss* (Walbaum). Aquacult. Nutr. **9**, 247-251.

Wieruszewski, J.B., 2000. Astaxanthin bioavailability, retention efficiency and kinetics in Atlantic salmon (*Salmo salar*) as influenced by pigment concentration and method of administration (kinetics only). M.Sc. Thesis, Simon Fraser University, pp. 99.

Withler, R.E., 1986. Genetic variation in carotenoid pigment deposition in the red-fleshed and white-fleshed Chinook salmon (*Oncorhynchus tshawytscha*) of Quesnel River, British Columbia. Can. J. Genet. Cytol. **28**, 587-594.

Wolf, G.W., 1991. The intracellular vitamin A-binding proteins: An overview of their functions. Nutr. Rev. **49**, 1-12.

Ytrestøyl, T., Coral-Hinostroza, G., Hatlen, B., Robb, D.H.F., Bjerkeng, B., 2004. Carotenoid and lipid content in muscle of Atlantic salmon, *Salmo salar*, transferred to seawater as 0+ or 1+ smolts. Comp. Biochem. Physiol. **138B**, 29-40.

Zagalsky, P.F., 1976. Carotenoid-protein complexes. Pure Appl. Chem. **47**, 103-120.

Zagalsky, P.F., Eliopoulos, E.E., Findlay, J.B.C., 1990. The architecture of invertebrate carotenoproteins. Comp. Biochem. Physiol. **97B**, 1-18.

Zagalsky, P.F., Mummery, R.S., Eliopoulos, E.E., Keen, J.N., 1995. Crustacyanin, the lobster carapace astaxanthin-protein: effects of modification of tyrosine residues of apocrustacyanin with tetranitromethane on the ability of the protein to reconstitute astaxanthin. Comp. Biochem. Physiol. **110B**, 393-401.

Zhelyaznik, N., Schrage, K., McCafferey, P., Mey, J., 2003. Activation of retinoic acid signalling after sciatic nerve injury: up-regulation of cellular retinol binding proteins. Eu. J. Neurosci. **18**, 1033-1040.

APPENDIX 1

Silver staining protocols

1. Silver staining protocol (Swain and Ross, 1995)

Prepare all solutions fresh just prior to use. Use at least 100 mL of solution per one-dimensional gel. Always prepare double volume of sodium carbonate solution and add formalin prior to use.

Steps	Solutions	Time of treatment[a]
1. Fix	40% ethanol 10% acetic acid	10 minutes (can be kept overnight)
2. Rinse	De-ionized water	10 minutes
3. Fix/Sensitize	0.05% glutaraldehyde 0.01% formalin 40% ethanol	5 minutes
4. Rinse	40% ethanol	20 minutes
5. Rinse	De-ionized water	20 minutes
6. Sensitize	Sodium thiosulphate (0.2 g L^{-1})	1 minute
7. Rinse	De-ionized water	2 × 1 minute
8. Silver	0.1% silver nitrate	20 minutes
9. Rinse	De-ionized water	1 minute
10. Develop	2.5% anhydrous sodium carbonate 0.04% formalin	1 minute[b]
11. Develop	2.5% anhydrous sodium carbonate 0.04% formalin	5 minutes[c]
12. Stop	5% acetic acid	5 minutes[d]

[a] Carry out steps 1-12 on a shaker at room temperature
[b] Incubate until solution turns yellow and then immediately pour off the solution
[c] Allow to develop until desired level of staining is achieved
[d] Store gels in 0.03% Na_2CO_3

2. **Silver staining protocol suitable for mass spectrometry analysis** (derived from the method of Shevchenko et al., 1996 with some modifications).

Staining protocols using glutaraldehyde can not be used for gels that are to be analysed by mass spectrometry. Prepare all solutions fresh just prior to use. Use at least 500 mL of solution per two-dimensional gel.

Steps	Solutions	Time of treatment[a]
1. Fix	50% methanol 10% acetic acid	30 minutes (can be kept overnight)
2. Fix	50% methanol	15 minutes
3. Rinse	De-ionized water	5 × 5 minutes
4. Sensitize	Sodium thiosulphate (0.2 g L^{-1})	1 minute
5. Rinse	De-ionized water	2 × 1 minute
6. Silver	Silver nitrate (2 g L^{-1}; chilled to 4 °C)	25 minutes
7. Rinse	De-ionized water	2 × 1 minute
8. Develop	Sodium carbonate anhydrous (30 g L^{-1}) 0.025% formalin	~ 1 minute[b]
9. Develop	Sodium carbonate anhydrous (30 g L^{-1}) 0.025% formalin	~ 3-5 minutes[c]
10. Stop	EDTA sodium salt (14 g L^{-1})	10 minutes
11. Rinse	De-ionized water	2 × 1 minute

[a] Carry out steps 1-11 on a shaker at room temperature
[b] Incubate until solution turns yellow
[c] Develop until desired level of staining is achieved

APPENDIX 2

Two-dimensional gel electrophoretic profiles of fractions obtained from second homogenization (S_2Fr_2-S_2Fr_4) of muscle tissue of astaxanthin fed and control group salmon

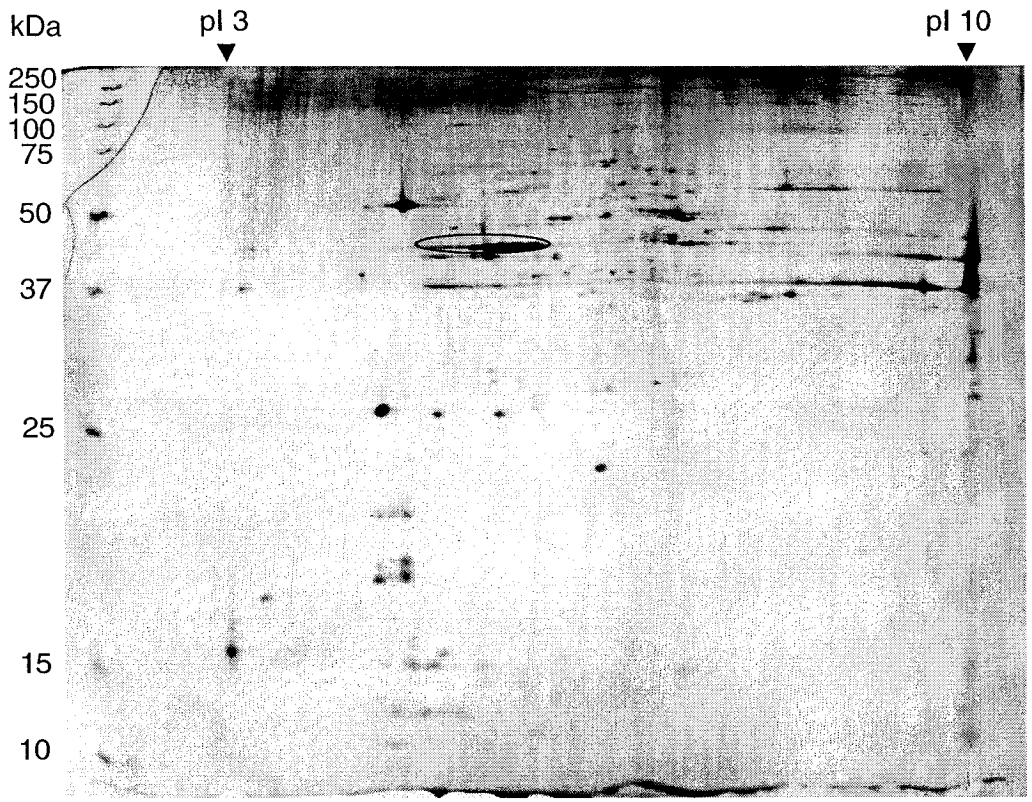

Fig. 1 Two-dimensional gel electrophoresis of fraction 2 obtained from the second homogenization (S_2Fr_2) of muscle tissue of astaxanthin fed Atlantic salmon. Molecular masses of protein standards are indicated at left. The numbers at the top of the gel indicate ends and respective pI of isoelectric focusing strips (i.e. IPG strips). Encircled spots indicate a glycosylated protein. Approximately 200 μg of proteins were analyzed on 14% polyacrylamide gel (1mm thick).

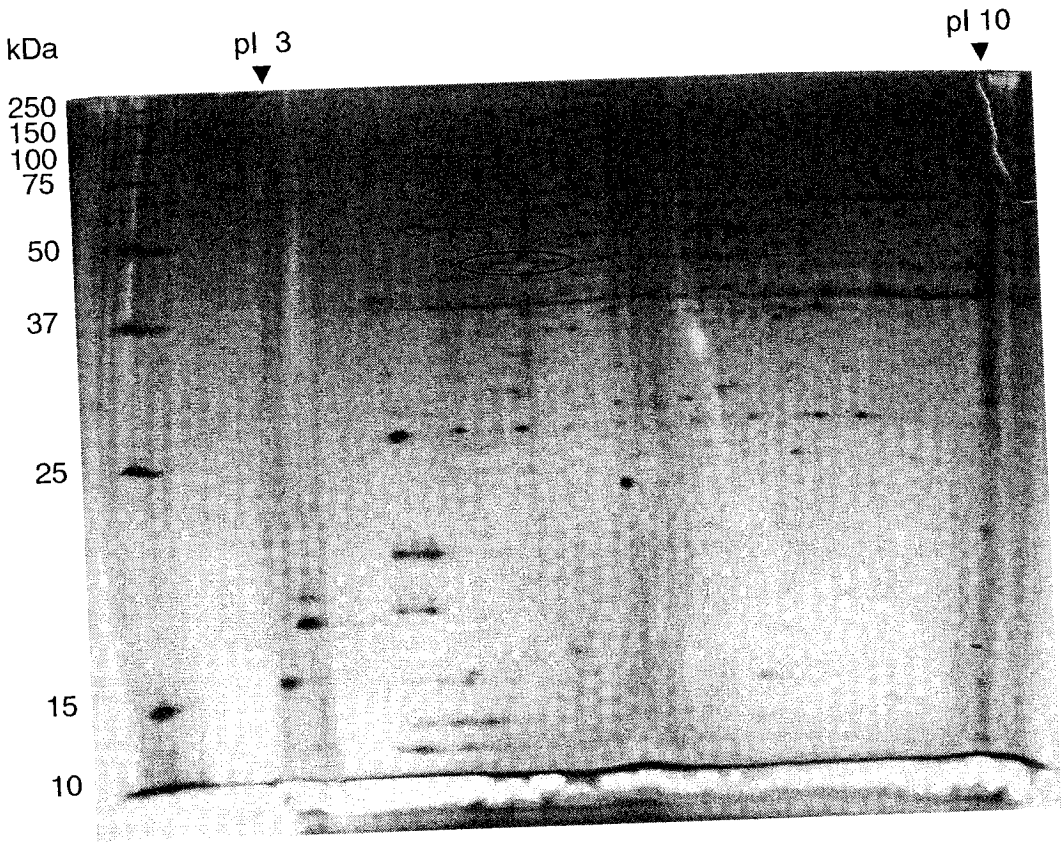

Fig. 2 Two-dimensional gel electrophoresis of the fraction 2 of the second homogenization (S_2Fr_2) of the muscle tissue of control group Atlantic salmon. Molecular masses of protein standards are indicated at left. The numbers at the top of the gel indicate ends and respective pI of isoelectric focusing strips. Encircled spots indicate a glycosylated protein. Approximately 200 μg of proteins were used for electrophoretic study on 14% polyacrylamide gel of 1 mm thickness.

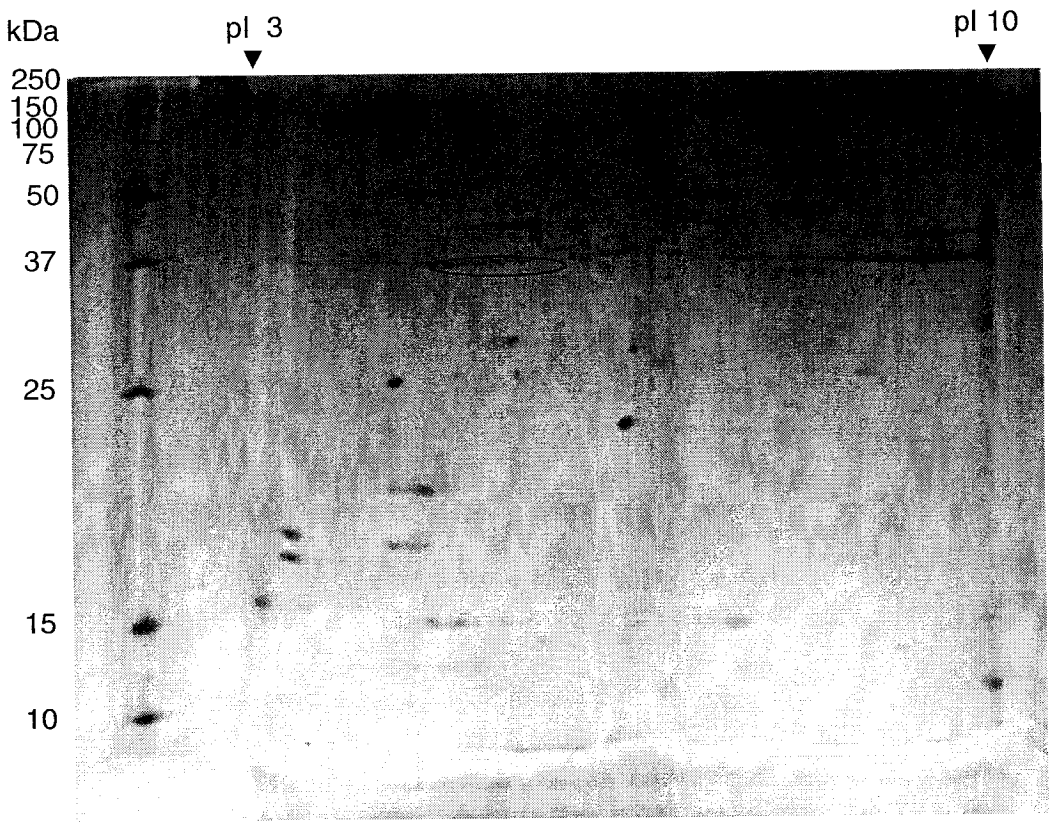

Fig. 3 Two-dimensional gel electrophoresis of the fraction 3 from the second homogenization (S_2Fr_3) of muscle tissue of astaxanthin fed Atlantic salmon. Molecular masses of protein standards are indicated at left. The numbers at the top of the gel indicate ends and respective pI of isoelectric focusing strips. Encircled spots indicate a glycosylated protein. Approximately 200 μg of proteins were analyzed on 14% polyacrylamide gel (1mm thick).

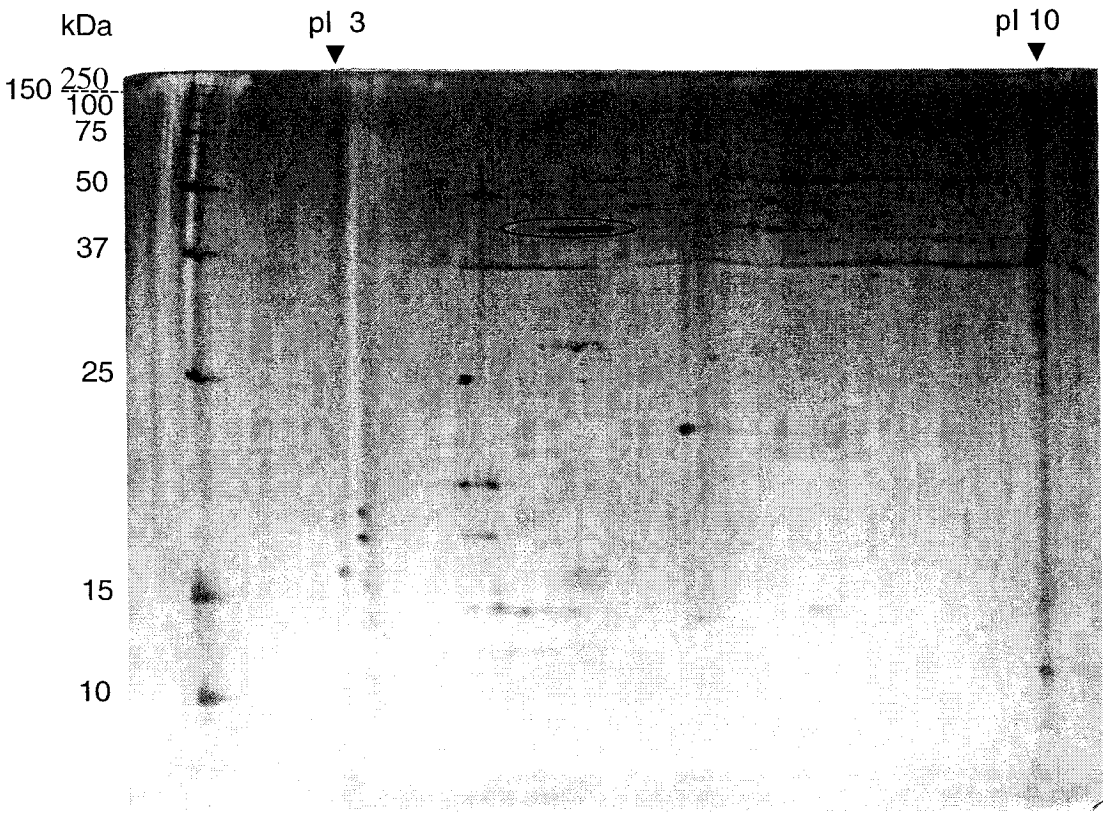

Fig. 4 Two-dimensional gel electrophoresis of fraction 3 obtained from the second homogenization (S_2Fr_3) of muscle tissue of control group Atlantic salmon. Molecular masses of markers are indicated at left. The numbers at the top of the gel indicate ends and respective pI of isoelectric focusing strips. Encircled spots indicate a glycosylated protein. Approximately 200 μg of proteins were analyzed on 1 mm thick 14% polyacrylamide gel.

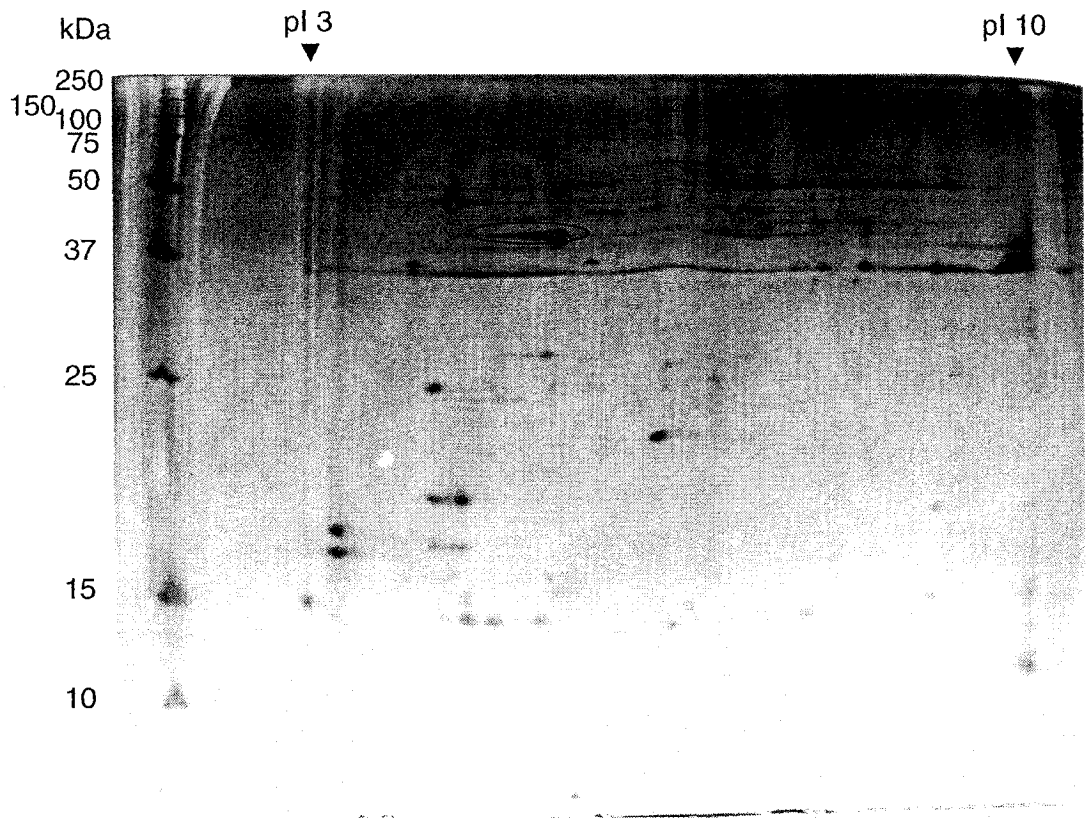

Fig. 5 Two-dimensional gel electrophoresis of fraction 4 prepared from the second homogenization (S_2Fr_4) of muscle tissue of astaxanthin fed Atlantic salmon. Molecular masses of protein standards are indicated at left. The numbers at the top of the gel indicate ends and respective pI of isoelectric focusing strips (i.e. IPG strips). Encircled spots indicate a glycosylated protein. Approximately 200 μg of proteins were analyzed on 14% polyacrylamide gel (1mm thick).

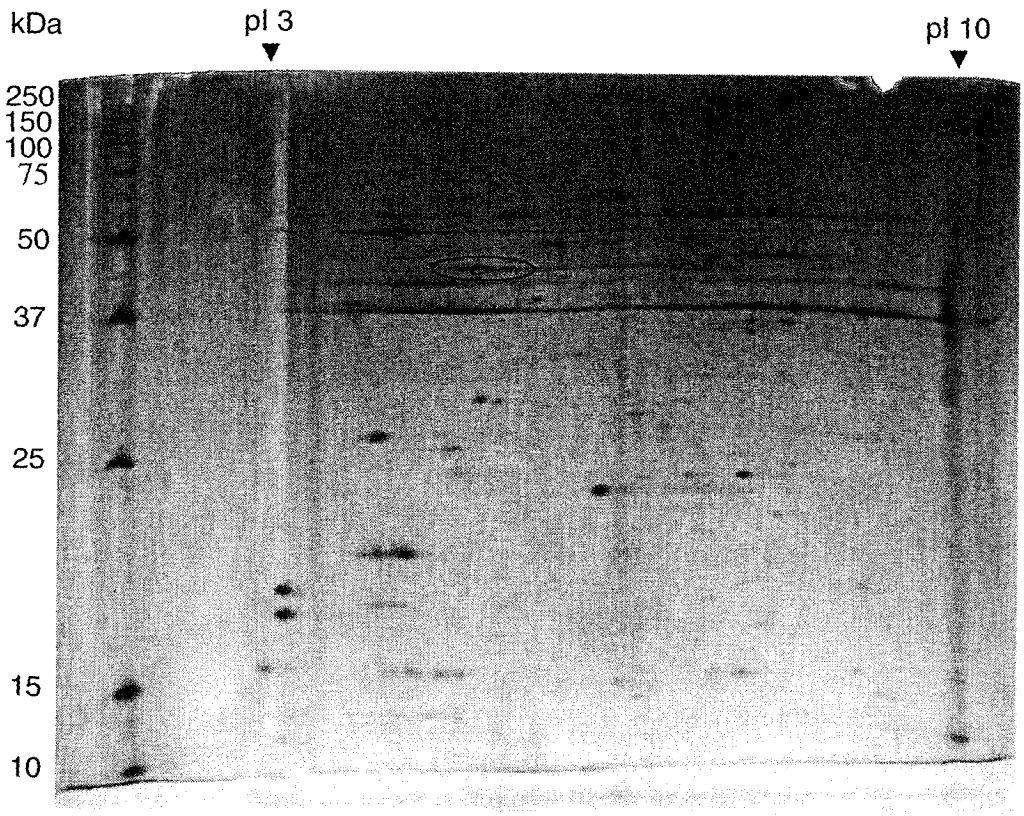

Fig. 6 Two-dimensional gel electrophoresis of fraction 4 prepared from the second homogenization (S_2Fr_4) of muscle tissue of control group Atlantic salmon. Molecular masses of protein standards are indicated at left. The numbers at the top of the gel indicate ends and respective pI of isoelectric focusing strips (i.e. IPG strips). Encircled spots indicate a glycosylated protein. Approximately 200 μg of proteins were analyzed on 14% polyacrylamide gel (1mm thick).