

Purification and properties of Sandercyanin, a blue protein secreted in the mucus of blue forms of walleye, *Sander vitreus*

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Abstract A blue protein present in the mucus coating blue forms of walleye, *Sander vitreus*, was purified to homogeneity by a combination of ion exchange and hydrophobic interaction chromatography. The purified protein has a molecular mass of 87,850 and is a homotetramer with a subunit molecular mass of 21,836. Solutions of the protein are deep blue in color and show absorbance maxima at 383 and 633 nm, respectively. Acetone treatment of the protein releases a blue chromophore with the spectral characteristics of biliverdin IX α . The results suggest

that the blue protein is a new biliprotein that we have termed Sandercyanin. The function of Sandercyanin is unknown, but limited amino acid sequence analysis suggests it is a lipocalin that may be involved in the transport of heme degradation products.

Keywords Blue walleye · Biliprotein · Biliverdin · Insecticyanin · Lipocalin · *Tetraodon nigroviridis*

Introduction

Production of color in fish generally is limited to the dermal and epidermal regions of skin and is formed by specialized pigment-producing cells (Fujii 1993). In the case of walleye, *Sander vitreus*, yellow and blue phenotypes have been described, although the nature of the pigments involved have not been reported. Recently, Paradis and Magnan (2005) reported the presence of blue walleye morphotypes in five lakes in central Quebec, Canada. The fish were covered with a blue mucus, a phenomenon previously seen in blue walleye forms present in The Great Lakes and Lake Nipissing, Canada (Regier et al. 1969; Scott and Crossman 1973). The cause and evolutionary significance of the blue color has not been determined. In this communication we describe and name a novel blue protein, Sandercyanin, present in the mucus of blue walleye populations in north-western Ontario.

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Materials and methods

Blue and yellow walleye forms were captured by hook and line from the Papaonga River system in northwestern Ontario. Mucus was carefully removed from the dorsal side of the fish with a knife and mixed with approximately three volumes of lake water. Samples were stored at 4°C until analyzed.

Protein purification

All chromatographic procedures were performed in a cold room at 4°C using an automated fast protein chromatography system (Bio-Rad Laboratories, Hercules, CA). Chromatography columns and column supports were from Amersham Biosciences, Piscataway, NJ, except for ceramic hydroxyapatite, which was obtained from Bio-Rad Laboratories.

The diluted mucus solution (110 ml) from blue walleye forms was centrifuged at 15,800g for 15 min and the residue discarded. Ammonium sulfate (32 g) was slowly added to the supernatant until the mixture became turbid. After 1 h at 4°C a white precipitate formed which was removed by centrifugation at 13,000g for 10 min. Ammonium sulfate (18 g) was added to the resulting clear blue supernatant solution followed by centrifugation at 13,000g for 15 min. The blue precipitate was dissolved in 35 ml of Tris buffer (50 mM Tris–HCl buffer, pH 7.0). Ammonium sulfate was removed by ultrafiltration. The concentrated solution (168 mg protein) was applied to a Q-Sepharose column (2.6 × 40 cm²) that had previously been equilibrated with Tris buffer. The column was washed with 200 ml of the same buffer at a flow rate of 1.0 ml per min. Bound protein was eluted with a 600 ml linear gradient (0.0–0.5 M) of KCl in Tris buffer. Blue-colored fractions, eluting at ~0.3 M KCl, were pooled and concentrated to 4.8 ml (10.1 mg of protein) by ultrafiltration. Ammonium sulfate (4.0 M) was added to the concentrated solution to give a final concentration of 1.0 M. The solution was clarified by centrifugation at 13,000g for 10 min and applied to the top of a Phenyl Sepharose High-Performance Column (5.0 × 1.0 ml²) that had previously been equilibrated with Tris buffer containing 1.0 M (NH₄)₂SO₄. The column was washed with the same buffer at a flow rate of 1.0 ml per min until protein was no longer detected in the eluate. Bound protein was eluted with a linear gradient of

(NH₄)₂SO₄ (1.0–0.0 M) in the same buffer. Blue-colored fractions were pooled and concentrated by ultrafiltration. Ammonium sulfate was removed by buffer exchange with 5.0 mM KH₂PO₄, pH 7.0. This preparation (5.0 mg of protein) was applied to a ceramic hydroxyapatite XK16/70 column (80 × 1.0 ml²) that had been equilibrated with 5.0 mM KH₂PO₄ buffer, pH 7.0. Unbound protein was eluted with 100 ml of the same buffer at a flow rate of 1.0 ml per min before initiating a linear gradient (5–200 mM) of KH₂PO₄ buffer, pH 7.0. Dark blue fractions (1.0 ml) eluting from the column were pooled, concentrated by ultrafiltration and exchanged into 10 mM KH₂PO₄ buffer, pH 7.0, before adding in 30 µl aliquots to liquid nitrogen. The resulting pellets were stored at –70°C.

Protein determination

Protein concentrations were determined by the method of Bradford (Bradford 1976) using bovine serum albumin as the standard.

Electrophoresis

Protein purity and subunit molecular weights were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5% gels (Laemmli 1970). Samples were boiled for 6 min prior to loading onto the gels. Protein standards used for the estimation of subunit molecular masses were Low-Range Molecular Weight SDS-PAGE Standards (Bio-Rad Laboratories). Protein bands were stained with Coomassie Brilliant Blue R250.

Molecular mass

The molecular mass and subunit molecular mass of the blue protein was determined by matrix-assisted laser desorption ionization-time of flight/mass spectrometry (MALDI-TOF/MS) at The University of Iowa Molecular Analysis Facility, using α -cyano-4-hydroxycinnamic acid (CHCA) in 50% acetonitrile: 50% 0.1% trifluoroacetic acid as matrix.

Cyanogen bromide cleavage

A small crystal of cyanogen bromide was added to 50 µl of formic acid (70%) containing purified blue

protein (15 µg). The reaction mixture was incubated in the dark at 25°C for 24 h and then taken to dryness.

Proteolytic digestion

In separate experiments, purified blue protein was digested with endoproteinases Glu-C, Arg-C, Lys-C, and Asp-C, at ratios of endoproteinase to blue protein recommended by the supplier (Sigma Chemical Co., St. Louis, MO). Reaction mixtures were incubated at 37°C for 2–18 h and then taken to dryness.

Amino acid sequence determination

Peptides produced by cyanogen bromide cleavage and proteolytic digestion were separated by SDS-PAGE, electroblotted onto polyvinylidene membranes and their respective amino acid sequences determined by Edman degradation on an automated amino acid sequencer (Applied Biosystems, Foster City, CA) at The University of Iowa Molecular Analysis Facility.

Transition metals

Transition metals were investigated by inductively coupled plasma mass spectrometry (ICP-MS) on a VG PlasmaQuad II ICP-MS machine at the University of Iowa Hygienic Laboratory, Des Moines, IA.

Results

Distribution of blue mucus in walleye

Blue walleye forms taken from the upper reaches of the Papaonga River system near Ear Falls, in northwestern Ontario, were coated with a blue mucus (Fig. 1A). These fish were albino for yellow skin pigment and, after removal of the mucus layer, appeared steel gray in color. The lack of yellow pigment in the membranes of the dorsal and caudal fins made the structures translucent and allowed clear observation of the blue mucus on the fins giving the fish a blue appearance. In each specimen, blue mucus was located only on the dorsal side of the body and was particularly abundant in the rays and interspinous membranes of the two dorsal fins and the caudal fin.

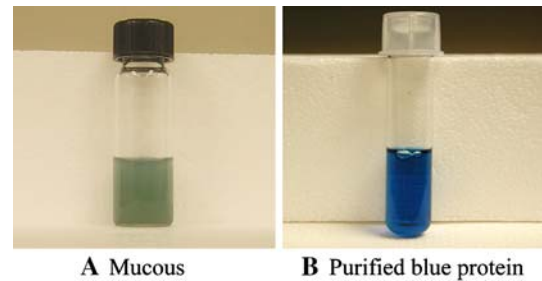


Fig. 1 (A) Mucus from blue walleye forms prior to purification. (B) Solution of purified blue protein obtained after hydroxyapatite chromatography (Table 1)

Subsequent research in the same location showed blue mucus was also present on yellow walleye forms and apparent hybrids that were mottled for yellow color. Nuclear and mitochondrial DNA analyses showed that the blue and yellow forms were not genetically distinguishable. Moreover, the morphology of the blue form did not match any of the morphological characters that distinguish the blue ‘pike’, *Sander vitreus glaucus*, and instead matched the morphology of the walleye, *Sander vitreus vitreus* (C. Stepien, personal communication).

As the study progressed we established a web site¹ where fishermen could report sightings of blue walleye forms. To date, blue forms have been identified throughout northwestern Ontario, central Quebec and northern Saskatchewan and have also been identified in several lakes in Minnesota, Wisconsin, and Michigan. In addition, we have taken blue forms of yellow perch, *Perca flavescens*, from the Papaonga River in Ontario and Winnibigoshish Lake in northern Minnesota.

Purification and characterization of the blue pigment

A blue protein was subsequently purified from the mucus of blue walleye forms collected in the summer of 2003 from the Papaonga River system. The procedure involved a combination of ion exchange and hydrophobic interaction chromatography, and yielded 2.8 mg of a blue protein (Fig. 1B) with a recovery of 19.5% (Table 1). The purified protein was very stable and could be stored at 4°C for up to 8 weeks with no apparent degradation.

¹ www.bluewalleye.com.

Table 1 Purification of blue protein from mucus of blue walleye

Purification step	Protein (mg)	Units ^a	Units/mg	Purification (fold)	Yield (%)
Mucus	264				
(NH ₄) ₂ SO ₄ fraction	168	308	1.93	1	100
Q-Sepharose	10.1	99	9.8	5	32.1
Phenyl-Sepharose	5.0	81	16.2	9	26.3
Hydroxyapatite	2.8	60	21.4	12	19.5

Details of the purification procedure are described in the text

^a One unit of the blue protein is defined as the amount giving an absorbance measurement of 0.1 at 633 nm

Properties of the blue protein

When analyzed by SDS-PAGE, the protein migrated as a single band with a molecular mass of ~21,500 (Fig. 2A, lanes 5, 6). A single band was observed on non-denaturing gels (Fig. 2B, lanes 2, 3). The molecular masses of the native and SDS-treated protein, determined by MALDI-TOF/MS, were 87,850 and 21,836, respectively. The results show that the purified protein is a homotetramer.

The ICP/MS analyses showed that the blue protein did not contain transition metals, and tests for glycosylation were negative (data not shown).

At pH 7.0, solutions of the blue protein showed absorbance maxima at 280, 378, and 633 nm. The color changed to green upon acidification to pH 3.0 with the appearance of new absorbance maxima at 360 and 670 nm with a shoulder at 703 nm (Fig. 3). The original spectrum and blue color was restored when the pH was adjusted back to 7.0. Extraction of the blue protein with cold 90% (v/v) acetone, followed by removal of the solvent, yielded a blue product, which was dissolved in methanol. The absorbance spectrum of the resulting solution showed maxima at 380 nm and a broad peak between 640 and 680 nm and was identical to an authentic sample of biliverdin IX α .

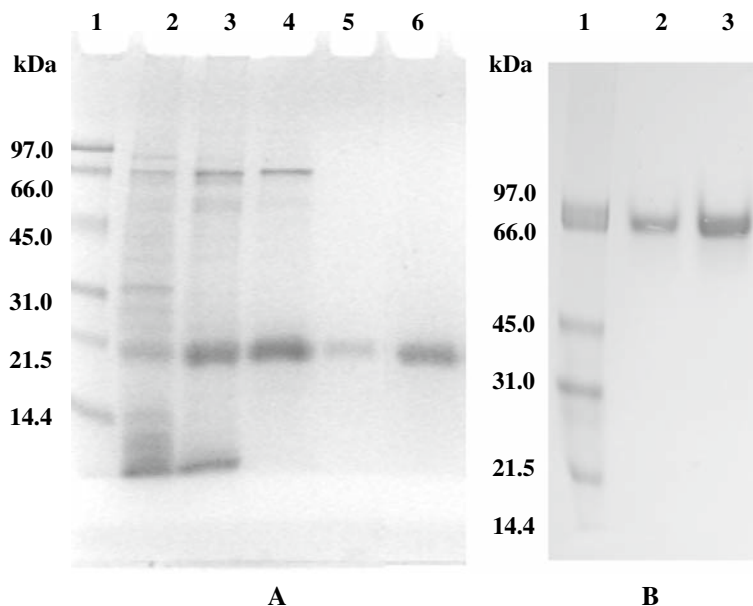


Fig. 2 (A) SDS-PAGE analysis of samples taken during purification of the blue protein. Lane 1, molecular mass standards (numbers at the left are molecular masses in kDa); lane 2, (NH₄)₂SO₄ fraction; lane 3, Q-Sepharose fraction; lane 4, Phenyl-sepharose fraction; lanes 5 and 6, hydroxyapatite

fractions containing 3 and 6 μ g of protein, respectively. (B) Native PAGE of purified blue protein. Lane 1, molecular mass standards; lanes 2 and 3, hydroxyapatite fraction containing 4 and 8 μ g of protein, respectively. Proteins were stained with Coomassie Brilliant Blue R250

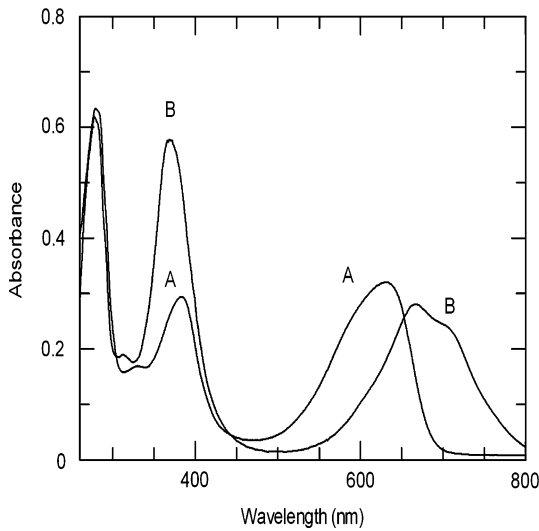


Fig. 3 (A) Absorbance spectrum of purified blue protein (12.5 µg of protein/ml) in 50 mM Tris–HCl buffer, pH 7.0. (B) In 50 mM citrate buffer, pH 3.0

The amino acid composition of the blue protein is shown in Table 2, where it is compared to the amino acid compositions of butterfly, *Pieris brassicae*, bilin binding protein (BBP) and tobacco hornworm, *Manduca sexta*, insecticyanin. The blue protein differs from the two insecticyanins primarily in terms

of the percentage of glutamic acid/glutamine, alanine, arginine, and tyrosine residues.

Attempts to determine the amino acid sequence of the blue protein were hindered due to a blocked N-terminus. In separate experiments, cyanogen bromide cleavage and endoproteinase digestion yielded seven internal peptides, which were separated by SDS-PAGE. The amino acid sequence of each peptide was determined and combination of overlapping sequences resulted in the identification of three different larger peptides. Standard NCBI Protein–Protein BLAST analysis of short peptide sequences showed the three peptides, designated A, B, and C (totaling 77 amino acids), to have the highest sequence identity to an unannotated protein, containing 181 amino acids, in the genome of the green spotted pufferfish, *Tetraodon nigroviridis*. The amino acid sequences of peptides A, B, and C are located at positions 24–53, 88–105, and 111–139, respectively (Fig. 4), and show 73% sequence identity to the *T. nigroviridis* protein. Table 4 shows alignments of the amino acid sequences of the three peptides with analogous regions in apolipoproteins D from zebrafish. *Danio rerio* (53%), guinea pig, *Cavia porcellus* (53% identity), mouse, *Mus muscalus* (49% identity), human, *Homo sapiens* (48% identity).

Table 2 Comparison of amino acid composition for purified blue protein and biliproteins from *Pieris brassicae* and *Manduca sexta*

Residue	Mole (%)		
	Blue protein	<i>P. brassicae</i> ^a	<i>M. Sexta</i> ^b
Asx	10.7	5.8	13.7
Glx	10.5	6.9	7.4
Ser	7.1	6.9	6.9
Gly	6.8	8.7	5.8
His	1.3	3.5	3.2
Arg	3.9	0.6	1.0
Thr	5.8	4.0	4.7
Ala	13.3	4.6	7.9
Pro	5.4	3.5	4.2
Tyr	3.4	9.2	7.4
Val	7.4	11.6	7.4
Met	1.2	0.0	1.0
Cys	1.1	2.3	2.1
Ile	4.7	4.0	4.8
Leu	7.5	3.5	5.8
Phe	5.0	2.9	4.8
Lys	4.9	11.6	10.0

^a Suter et al. 1988

^b Riley et al. 1984

Unnamed protein	1	MNAVQVLP LALLAALAAHAQVIK	PGRCP RPAVQ QEFDAARYLGTWYEIQRLPHRFQTGQC	60
Blue protein			(A) PGRCP+PAVQ++FDAARYLG WY+IQRLP+	
			(A) PGRCPKPAVQEDFDAARYLGVWYDIQRLPN	
Unnamed protein	61	STASYSLKSPGVVGLNREL RADGTVY	SISGTA VAEDPSEPAKLAVSFYENSPPAPYVWL	120
Blue protein			(B) S G+A+AEDPSEPAKL N+ P PY VL	
			(B) SQIGSAIAEDPSEPAKLQ (C) NAAPVPYCVL	
Unnamed protein	121	ATDYDNYTLVYSCTNLLVAHAEF AWILSR RPTLAQESVEELRRALSSVGV DVDKLLSTNQD		181
Blue protein			TDYDNY LVYSC NL	
			LTDYDNYALVYSCINLGAS	

Fig. 4 Alignments of amino acid sequences in peptides (A), (B), and (C) from the blue protein with predicted amino acid sequences at positions 24–53, 88–105, and 113–139, in an unnamed protein (NCBI Accession No. CAF98955) in the

genome of the green spotted pufferfish, *Tetraodon nigroviridis*. Identical amino acids are shaded. Conservative amino acid substitutions are designated by +

Discussion

Several naturally occurring blue proteins have been described. They include copper proteins such as plastocyanin (Freeman and Guss 2001) and azurin (Kolczak et al. 2001); crustacyanin, a carotenoprotein complex found in the lobster, *Homarus gammarus*, carapace (Chayen et al. 2003); and BBPs found in insects (Riley et al. 1984; Huber et al. 1987; Saito 1998). The blue color in BBPs is due to the presence of biliverdin IX γ which is formed by the enzymatic cleavage of heme by heme oxygenase.

The blue protein purified in the present study is the first to be specifically associated with walleye. Initial studies showed the protein does not contain copper or any other transition metal. Its physical properties are different to those reported for crustacyanin. They are

however, remarkably similar to several insect BBPs in terms of subunit composition, absorbance spectrum, and the presence of a biliverdin chromophore (Table 3). Although further studies are necessary to unequivocally establish that the chromophore is the biliverdin IX α isomer. These properties suggest that the blue protein is a biliprotein secreted by the fish into its skin mucus. We propose the name Sandercyanin for this new pigment.

The specific function of Sandercyanin is unknown. Blue walleye forms were known to occupy the deeper waters of the Great Lakes, leading Paradis and Magnan (2005) to suggest the blue color may provide an advantage to walleye in their search for forage fish. This seems reasonable in an environment that would be dominated by blue to blue–green light. A camouflage role has also been

Table 3 Physical properties of blue protein and insect bilin-binding proteins

Protein	Molecular mass (kDa)	Subunit composition	Absorbance spectrum (λ_{max} , nm)	Chromophore
Blue protein (<i>S. vitreus</i>)	87.8	α_4	383,633	Biliverdin IX α
Insecticyanin (<i>M. sexta</i>) ^a	76.6	$\alpha_3(\alpha_4)^b$	378,673	Biliverdin IX γ
Bilin-binding protein II (<i>R. fugax</i>) ^c	48.0	α_2	385,663	Biliverdin IX γ
Biliverdin-binding protein (<i>P. xuthus</i>) ^d	24.0	α	378,648	Biliverdin IX γ

^a Goodman et al. 1985

^b Riley et al. 1984

^c Saito 1998

^d Yamanaka et al. 2000

Table 4 Alignments of amino acid sequences in peptides A, B, and C from Sandercyanin with predicted amino acid sequences in related proteins

Proteins	Peptide A (30 amino acids) ^a	Peptide B (18 amino acids)	Peptide C (29 amino acids)
1. Sandercyanin (walleye)	PGRCPKPAVQEDF DAARYLGVVYDIQRLPN	SQIGSAIAEDP SEPAKLQ	NAAPVPYCVLL TDYDNYALVYSCINL GAS
2. Unnamed (spotted puffer fish)	PGRCPRP AV QQEFD AARYLGTWYEQRLPH	SISGTAVAEDP SEPAKLA	NSPPAPYVWVLA TDYDNYTLVYSCTNLL -V
3. ApoD (zebra fish)	SGKCPQP VPV KDFD PTRVMGRWHEIMKFFS	FIETAKIVD ASEPAKLE	DAPSPYVWVLA TDYDDYTLVYSC TFD FGNL
4. ApoD (guinea pig)	FGKCPNP PV QENF DLNLYLGRWYEIEKIPV	QIEGEATHSN TEPAKLG	LMPSAPYVWVLA TDYDNYALVYSC TNI IWL
5. ApoD (mouse)	LGKCPSP PV QENF DKKYLGRWYEIEKIPA	QVKGEAKQSN VSEPAKLE	LMPAPYVWVLA TDYENYALVYSC TT FFWL
6. ApoD (human)	LGKCPNP PV QENF DVNKYLGRWYEIEKIPT	QIEGEATPVN LTEPAKLE	FMPSPYVWVLA TDYENYALVYSC TC IIGL

1. Sandercyanin, blue walleye, *Sander vitreus*; 2. Unnamed protein, green spotted pufferfish, *Tetraodon nigroviridis* (NCBI Accession No. CAF98955, positions 24–53, 88–105, and 113–139); 3. ApoD, Zebra fish, *Danio rerio* (NCBI Accession No. XP_693007, positions 24–53, 87–104, and 110–138); 4. ApoD, Guinea pig, *Cavia porcellus* (NCBI Accession No. AAB35199, positions 25–54, 89–106, and 112–140); 5. ApoD, Mouse, *Mus musculus* (NCBI Accession No. NP_031496, positions 25–54, 89–106, and 112–140); 6. ApoD, Human, *Homo sapiens* (NCBI Accession No. NP_001638, positions 25–54, 89–106, and 112–140)

^a Identical amino acids are shown in bold type

suggested for insect biliproteins when they are present in larval hemolymph. In this case the green color in the caterpillars is due to a combination of a biliprotein and a yellow carotenoid (Saito 1998). However, the amino acid sequences of the three sandercyanin internal peptides are surprisingly, similar to the predicted analogous sequences of an unannotated protein in the genome of the green spotted pufferfish and also to apolipoproteins D in zebra fish, guinea pig, mouse, and human (Table 4). These observations suggest a possible broader function for Sandercyanin. Apolipoproteins D are small secreted proteins which are characterized by their ability to bind and transport small hydrophobic molecules (Rassart et al. 2000). They, together with biliproteins, are members of the large lipocalin protein family (Flower 1996). The chromophore in Sandercyanin is a hydrophobic biliverdin derivative and transport of biliverdin to the mucus may be an indication of increased heme metabolism in blue walleye forms. In this context, it is of interest to note that chemical stress leads to increased biliverdin excretion in channel catfish, *Ictalurus punctatus* (van den Hurk 2006), although there was no evidence of pollution in the sites sampled in the present study. An alternative suggestion focuses on the ability of biliverdin to quench reactive oxygen radicals and the possible function of biliproteins in photoprotection (McDonagh 1979). Clearly, the physiological function of Sandercyanin awaits more detailed structure/function analyses. Such studies are ongoing in our laboratory.

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