Biochemistry (Moscow), Vol. 67, No. 8, 2002, pp. 927-932. Translated from Biokhimiya, Vol. 67, No. 8, 2002, pp. 1116-1121.

Purification and Characterization of Biliverdin IX_α from Atlantic Salmon (*Salmo salar*) Bile

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Received November 18, 2001
Revision received January 14, 2002

Abstract—Biliverdin IX_α was purified from the bile of Atlantic salmon (*Salmo salar*) using a silica gel (Wakogel C-200) column. The yield was 49.5 mg per 100 ml of fresh bile and purity 95.3%. The biliverdin IX_α in the bile was quite stable when the bile was frozen at −80°C for a period of 40 days. However, 7.1% of the biliverdin IX_α was lost when the bile was stored at 4°C for 20 days. The purified biliverdin IX_α appeared as a single spot with R_f value of 0.25-0.27 on thin layer chromatography (TLC) and one main peak on high performance liquid chromatography (HPLC) at 436 or 650 nm. When the biliverdin IX_α was subjected to enzymic reduction with highly purified biliverdin reductase, two clear isobestic points were seen, at 384 and 670 nm. When the products of the reaction with biliverdin IX_α were extracted in butanol after completion of the reaction, one absorbance peak was observed at 468 nm. The time course of the reduction of biliverdin IX_α to bilirubin IX_α catalyzed by biliverdin reductase depended on reduced pyridine nucleotide. The time course of the NADPH-dependent reaction is different from that of the reaction with NADH. In the reduction of biliverdin IX_α, per mole of biliverdin IX_α reduced or per mole of bilirubin IX_α formed 1 mole of reduced pyridine nucleotide was consumed in both the NADH and NADPH systems.

Key words: biliverdin, heme degradation, biliverdin reductase, bilirubin, salmon

Biliverdin is the final metabolic product of physiological heme degradation and is directly excreted in fish, amphibia, reptiles, and birds [1-4]. Although biliverdin is also the final product of heme degradation in mammals, biliverdin must be reduced to bilirubin by biliverdin reductase and is then excreted [5-8]; biliverdin can pass the placenta only after it is reduced to bilirubin [9]. Biliverdin can be used as a metabolic substance and reagent as well as reduced to bilirubin for making novel medicines [10-12].

Until recently, biliverdin was prepared mainly by the degradation of heme [13] or oxidation of crystalline bilirubin [14]. In the former method, four isomers, biliverdin IX_α, IX_β, IX_γ, and IX_δ, were produced by the coupled oxidation of pyridine hemin with ascorbic acid as the dimethyl esters. Each isomer was further purified by thin layer chromatography (TLC) according to the methods of O'Carra and Colleran [15]. While in the later way, the pure biliverdin IX_α was isolated from the mixture of four oxidation products of bilirubin by silica gel column chromatography. The purification of biliverdin IX_α was affected by four isomers produced in the above two methods.

However, there is only biliverdin IX_α in the bile of fish because heme is cleaved by heme oxygenase of the fish [16-18]. Therefore, biliverdin IX_α can be simply purified from the bile of fish. The present paper reports the purification and characterization of biliverdin IX_α from the bile of Atlantic salmon (*Salmo salar*).

MATERIALS AND METHODS

Materials. Live Atlantic salmon (*Salmo salar*) with weight about 2 kg each were bought from the supermarket. Wakogel C-200, Wakogel B-10, pyridine nucleotides, standard biliverdin IX_α, and bovine serum albumin (BSA) were bought from Sigma (USA). Biliverdin reductase was prepared in our laboratory and conformed to reported standards [1]. All other reagents were analytic or HPLC grade.

Collection of the bile of salmon. Fifty salmon were washed and sacrificed by cutting heads or anaesthetized using tricaine methanesulfonate (MS 222, 0.42 g/liter).

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The gallbladder was exposed through a mid ventral incision. The contents of the gallbladder were aspirated and transferred to ice-cold tubes in the dark. The bile was used immediately or stored for use later at −80°C.

Isolation and purification of biliverdin IX_α. The biliverdin IX_α in the salmon bile was isolated and purified according to the modified methods of Ding and Xu [1, 2, 19]. Bile (100 ml) was applied to a silica gel (Wakogel C-200) column (2.5 × 15 cm) equilibrated with $CHCl_3$–CH_3COOH (97 : 3 v/v) in a dark cold room at flow rate 0.5 ml/min. The column was washed with $CHCl_3$–CH_3COOH (97 : 3 v/v) to completely elute unoxidized bilirubin and unidentified brown and reddish purple pigments. Then biliverdin IX_α (clear dark green color) was eluted at a flow rate of 0.5 ml/min with a slightly more polar eluent, $CHCl_3$–CH_3OH–CH_3COOH (92 : 5 : 3 v/v), and fractions which gave only one spot on analytic TLC were collected.

The solvent in the biliverdin IX_α collection was evaporated under reduced pressure at a freeze-dryer. The pigment was re-dissolved in 1% NaOH (w/v) and precipitated by adding 1% CH_3COOH (v/v). The precipitate was collected by centrifugation and washed several times with distilled water to remove salts, then freeze-dried. The more times re-dissolved and precipitated, the higher the purity of the biliverdin IX_α obtained. The biliverdin IX_α powder obtained was stored in the dark at −80°C.

Protein determination. Protein was determined according to the method of Bradford using BSA as a standard [20].

Assay of biliverdin IX_α by TLC. The biliverdin IX_α powder was dissolved in CH_3OH and analyzed by TLC. TLC was carried out on 5 × 20 cm plates pre-coated with 0.25 mm layers of Wakogel B-10 activated at 150°C for 3 h. The sample was developed with $CHCl_3$–CH_3OH–CH_3COOH (94 : 5 : 1 v/v).

Analysis of biliverdin IX_α by HPLC. The biliverdin IX_α powder obtained was dissolved in CH_3OH and filtered through a 0.2 μM filter (Acro LC13, Gelman Sciences, USA) and then analyzed by HPLC. Analytical conditions were as follows. The column was Water μ-Bondapak C_{18} (0.39 × 30 cm) and flow rate at 1 ml/min. Initial mobile phase was CH_3OH–5% CH_3COOH (60 : 40 v/v), then the amount of CH_3OH was linearly increased to 90% over 20 min and held at 90% for 15 min. Detection wavelengths were 436 and 650 nm and the column temperature was 40°C.

Reductive reaction of biliverdin IX_α. The reductive reaction of biliverdin IX_α was conducted according to the modified method of Ding and Xu [1, 2]. Briefly, the reaction was conducted in a cuvette that was kept in a constant 37°C chamber attached to a Hitachi 200-20 double-team spectrophotometer (Japan). The reaction mixture with a final volume of 2 ml contained 100 mM potassium phosphate buffer (pH 7.4), 10 μM biliverdin IX_α, 1 mg/ml BSA, and 2 μg biliverdin reductase. After the above reaction mixture was pre-incubated for 5 min at 37°C, the reaction was started by the addition of reduced pyridine nucleotide (1.8 mM NADH or 100 μM NADPH, but omitted in the control). The enzymic conversion of biliverdin IX_α to bilirubin IX_α was monitored in terms of the increase in absorbance at 468 nm and the difference spectra in the whole range of 340-700 nm were recorded at 20, 40, and 60 min. The amounts of bilirubin IX_α formed were calculated by assuming that the difference in the millimolar extinction coefficients of bilirubin and biliverdin at 468 nm is 46.0 $mM^{-1}\cdot cm^{-1}$ since the millimolar extinction coefficients of bilirubin and biliverdin at 468 nm were 52.0 and 6.0 $mM^{-1}\cdot cm^{-1}$, respectively, under the experimental conditions employed. The amounts of biliverdin IX_α lost were calculated from the decrease in absorbance at 670 nm, assuming the millimolar extinction coefficient for the decrement at 670 nm to be 13.0 $mM^{-1}\cdot cm^{-1}$, since the millimolar extinction coefficient of biliverdin IX_α at 670 nm was 15.0 $mM^{-1}\cdot cm^{-1}$, bilirubin IX_α also absorbed at 670 nm, giving a millimolar extinction coefficient of 2.0 $mM^{-1}\cdot cm^{-1}$. The amounts of NADH and NADPH consumed were calculated from the decrease in the absorbance at 340 nm, employing a millimolar extinction coefficient of 23.2 $mM^{-1}\cdot cm^{-1}$ instead of 6.2 $mM^{-1}\cdot cm^{-1}$ for the decrement at 340 nm, since the millimolar extinction coefficients of biliverdin and bilirubin at 340 nm were 25.0 and 8.0 $mM^{-1}\cdot cm^{-1}$, respectively. The following formula was used to calculate the amount of consumed NAD(P)H:

$$NAD(P)H_{consumed} = \varepsilon_k \times \Delta E,$$

where ε_k = 6,220 $M^{-1}\cdot cm^{-1}$ at 340 nm, and $\Delta E = E_0 - E_t$ (E_0 and E_t is NAD(P)H absorption at 340 nm at zero and t moments of time, respectively).

Measurement of biliverdin IX_α concentration. Purified biliverdin IX_α (3 mg) was dissolved in 0.3 ml of 100 mM KOH and diluted with 100 mM potassium phosphate buffer (pH 7.4) to 0.2 mM. The precise concentration of biliverdin IX_α in the solution was measured from the absorbance at 670 nm.

RESULTS AND DISCUSSION

Chromatograms of biliverdin IX_α. The TLC pattern of biliverdin IX_α is shown in Fig. 1 and only one single spot with an R_f value of 0.25-0.27 appeared in the purified sample. The result was similar to that of the standard on TLC. Figures 2 and 3 show the HPLC chromatograms of the purified and standard biliverdin IX_α, respectively. Only one main peak appeared in both of them and the retention times of the main peaks were equal at the detection wavelengths. These facts suggest that the purified and standard biliverdin IX_α have the same HPLC properties.

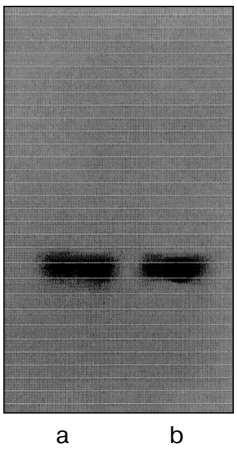

Fig. 1. Thin layer chromatography (TLC) chromatogram of biliverdin IX_α (enlarged 300%): a) prepared biliverdin IX_α; b) standard biliverdin IX_α.

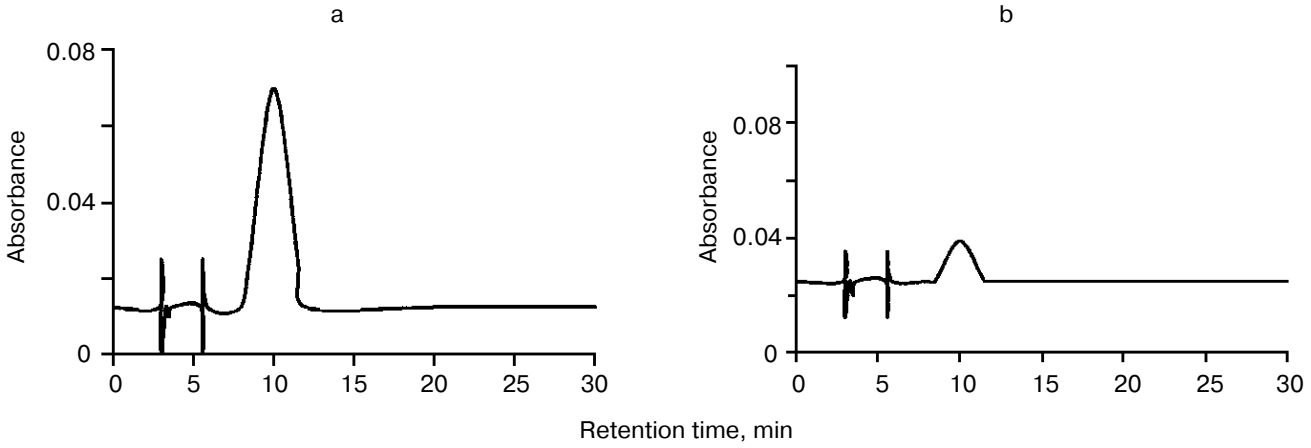

Fig. 2. High performance liquid chromatography (HPLC) chromatogram of prepared biliverdin IX_α: detection at 650 (a) and 436 nm (b).

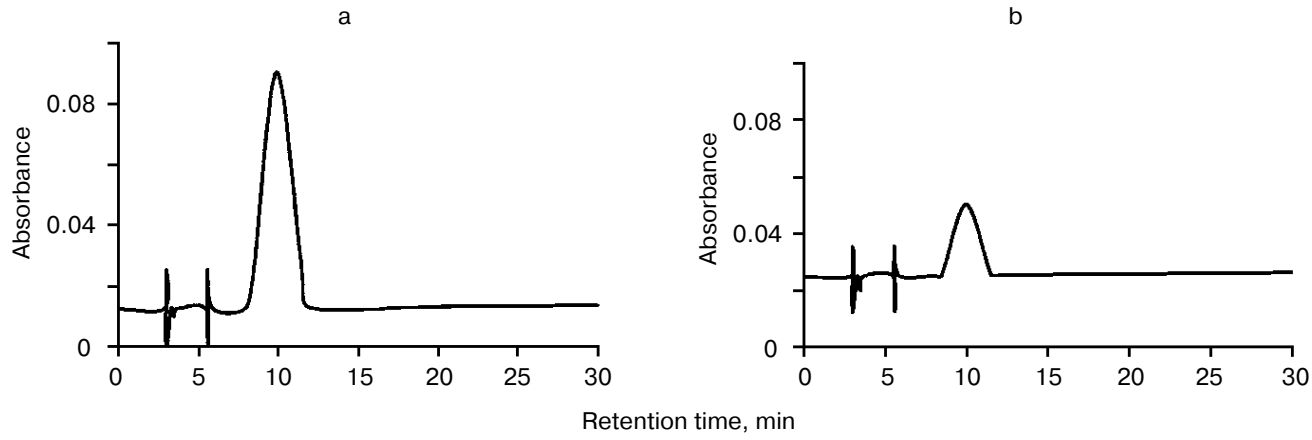

Fig. 3. High performance liquid chromatography (HPLC) chromatogram of standard biliverdin IX_α: detection at 650 (a) and 436 nm (b).

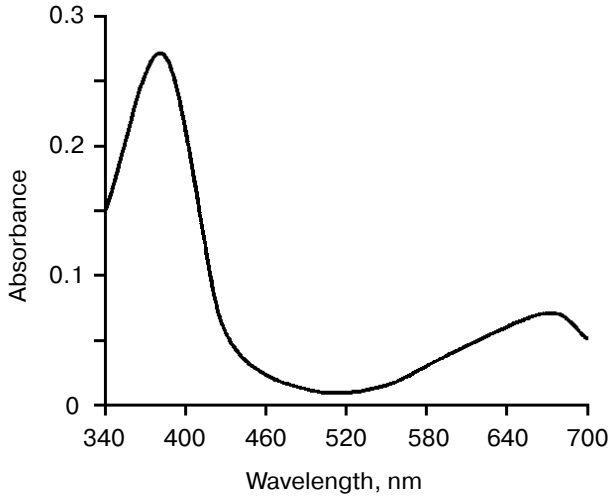

Fig. 4. Absorption spectrum of prepared biliverdin IX_α in assay mixture for biliverdin IX_α reduction but without pyridine nucleotides.

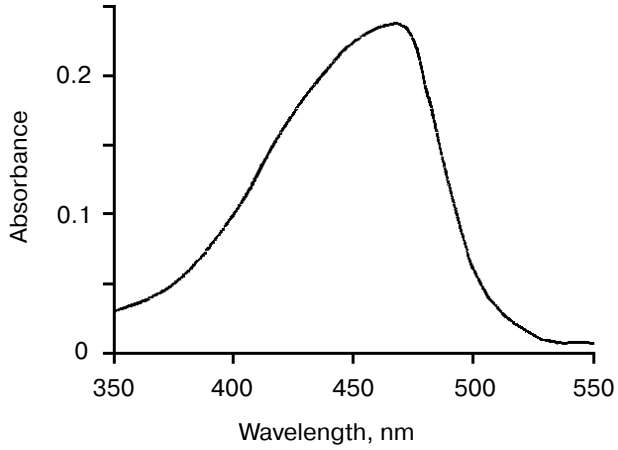

Fig. 5. The absorption spectrum of prepared biliverdin IX_α reaction products extracted into 3 ml of butanol from the reaction mixture after completion of the reaction. The compositions of the reductive reaction mixture are described in "Materials and Methods".

Spectra of biliverdin IX_α. When the biliverdin IX_α was subjected to enzymic reduction with highly purified biliverdin reductase, its absorption spectrum is shown in Fig. 4; two adsorption maxima were observed, at 384 and 670 nm. When the products of the reaction with biliverdin IX_α were extracted in butanol after completion of the reaction, one absorbance peak occurred at 468 nm as shown in Fig. 5. The spectral properties shown in Figs. 4 and 5 are similar to those described by Blanckeart et al. [21].

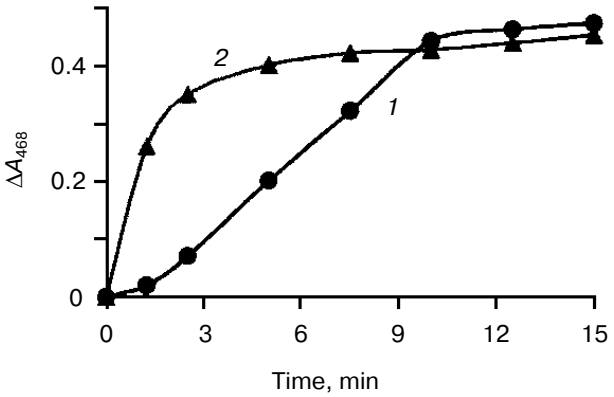

Fig. 6. Time course of biliverdin IX_α reduction in NADPH- (*1*) and NADH-dependent systems (*2*). The reaction mixture is described in "Materials and Methods". The increase of absorbance was recorded at 486 nm.

Time course of biliverdin IX_α reduction. The time course of the reduction of prepared biliverdin IX_α to bilirubin IX_α catalyzed by biliverdin reductase is shown in Fig. 6 and the shapes depended on reduced pyridine nucleotide. The time course of the NADPH-dependent reaction is different from that of the reaction with NADH. The results are consistent with those of previous report [1].

Stoichiometry of the reduction of biliverdin IX_α to bilirubin IX_α. As shown in Table 1, in the reduction of biliverdin IX_α catalyzed by biliverdin reductase, per mole of biliverdin IX_α reduced or per mole of bilirubin IX_α

Table 1. Stoichiometry of the reduction of biliverdin IX_α to bilirubin IX_α

Reaction time, min	Bilirubin formed, nmol	Biliverdin lost, nmol	NADH/NADPH consumed, nmol
	with NADH		
20	2.42	2.98	2.69
40	5.56	5.85	5.25
60	8.38	8.41	8.73
	with NADPH		
20	6.95	6.85	6.68
40	15.05	14.56	14.66
60	23.88	21.38	23.36

Note: The conditions for the reductive reaction of biliverdin IX_α and the calculation of biliverdin IX_α lost, bilirubin IX_α formed, NADH and NADPH consumed are described in "Materials and Methods".

Table 2. Effects of the treatment of salmon bile on the purification of biliverdin IX_α

Bile storage conditions	Bile, ml	Biliverdin IX_α, mg	Biliverdin IX_α/bile, %	Purity, %	Decline rate, %	
					output	purity
Fresh bile	100	49.5	0.0495	95.3	0	0
40 days at −80°C	100	49.2	0.0491	95.2	1.0	0.1
10 days at 4°C	100	48.8	0.0488	94.0	1.0	1.3
20 days at 4°C	100	46.0	0.0460	91.9	7.1	3.4
30 days at 4°C	100	46.1	0.0461	91.6	6.9	3.7
40 days at 4°C	100	45.9	0.0459	91.5	7.3	3.8

Table 3. Effects of isolation methods on the purification of biliverdin IX_α

Purification method	Bile, ml	Biliverdin IX_α, mg	Biliverdin IX_α/bile, %	Purity, %	Output of biliverdin IX_α, mg
Wakogel	100	49.5	0.0495	95.3	47.2
Deposition-Wakogel	100	46.0	0.0460	95.8	44.1
TLC	100	48.8	0.0488	88.7	42.6

formed, 1 mole of reduced pyridine nucleotide is consumed in both the NADH and NADPH systems.

Effects of treatment of salmon bile on the purification of biliverdin IX_α. When heme is cleaved nonenzymatically, all biliverdin isomers, IX_α, IX_β, IX_γ, and IX_δ, are produced. However, only biliverdin IX_α is produced when heme is cleaved by heme oxygenase [16]. The natural animal biliverdin is IX_α produced by heme oxygenase except for one case of biliverdin IX_γ [22-25]. Although the biliverdin IX_α in the bile of salmon is a natural product, its purification yield and purity depended on the treatment of the bile. Table 2 shows the effects of the bile treatment on the purification of biliverdin IX_α. The results show that the best was to isolate biliverdin IX_α using fresh salmon bile that gave the highest output and purity, 0.0495 and 95.3%, respectively. The biliverdin IX_α in the bile was quite stable when the bile was frozen at −80°C for a period of 40 days. However, 7.1% of the biliverdin IX_α in the bile was lost when the bile was stored at 4°C for 20 days. The results imply that biliverdin IX_α in cold-stored bile could be oxidized and decomposed or form biliverdin–iron complex and an unknown 688 nm absorbing material [26]. The biliverdin IX_α was impossible to reduced to bilirubin because biliverdin IX_α was in the bile and stored in the refrigerator that contained oxygen and was exposed to light from time to time.

Effects of isolation method on the purification of biliverdin IX_α. Table 3 shows the effects of isolation methods on the purification of biliverdin IX_α. The results show that the purification yield and purity of biliverdin IX_α depended on the isolation procedure. For yield only, the Wakogel method was the best and the deposition-Wakogel method the worst. For purity only, the deposition-Wakogel method was the best and the TLC method the worst. The Wakogel method produced the highest yield and better purity.

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