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Short communication

# Determination of free and bound riboflavin in cow's milk using a novel flavin-binding protein

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ABSTRACT

A recently described putative protease from the gut bacterium *Bacteroides thetaiotaomicron* (termed ppBat) exhibits two tryptophan residues in the interface which enable specific binding of the isoalloxazine heterocycle of riboflavin and its two cofactor forms, FMN and FAD. Recombinant ppBat was used to capture riboflavin from bovine milk directly without any prior preparation steps. The flavin-loaded protein was then re-isolated by means of affinity chromatography to identify and quantify the captured flavins. Free riboflavin concentrations were determined to 197 and 151 μg/l for milk with 3.5% and 0.5% fat content, respectively. Total riboflavin concentrations were also determined after acid-treatment of milk and were 4–5 times higher than for free riboflavin. Free FMN and FAD were not detectable and only trace amounts of FMN were found in milk following acid treatment. The method appears to be amenable to develop a direct assay for free riboflavin in milk and other foods.



## 1. Introduction

Milk is a very complex biological fluid generated by the mammary glands that serves as the sole nutrient for the newborn. It contains carbohydrates, lipids, proteins and micronutrients such as the vitamins. Among these, vitamin $B_2$ or riboflavin, is present in up to 1 mg/l and thus milk and dairy products supply a substantial amount of the daily recommended allowance of 1.1 and 1.3 mg for adult females and males, respectively (Food and Nutrition Board, 2000). The standard method for determination of vitamin $B_2$ in food is based on acid hydrolysis followed by extraction and HPLC coupled fluorescence detection of riboflavin (EN 14152). This routine method provides reliable data on riboflavin content but does not distinguish between free and bound forms of riboflavin nor does it allow to determine the occurrence of its derivatives, FMN and FAD. However, this information could be valuable to assess the composition and quality of certain foods such as milk. Therefore we have chosen a different approach to determine the flavin content by taking advantage of a recently discovered flavin-binding protein (Knaus et al., 2012). In contrast to riboflavin binding protein (RBP) from chicken egg white the recombinant protein from *Bacteroides thetaiotaomicron* (ppBat) binds riboflavin ($K_d$ = 70 nM), FMN ($K_d$ = 400 nM) and FAD ($K_d$ = 800 nM) and hence can be used to scavenge all three naturally occurring forms of the vitamin. In addition, the recombinant protein is equipped with an N-terminal hexa-histidine tag enabling rapid separation, spectral characterisation and quantification of protein-bound flavins. Using this protein, we could quantify and distinguish free and bound flavins in cow's milk based on absorption properties of the isolated flavin-protein complex and subsequent HPLC analysis.

## 2. Experimental procedures

### 2.1. Reagents and proteins

All chemicals were of the highest grade commercially available from Sigma–Aldrich (St. Louis, MO, USA), Fluka (Buchs, Switzerland) or Merck (Darmstadt, Germany). Nickel sepharose (1 and 5 ml prepacked HisTrap™ HP columns) was from GE Healthcare, Bio-Sciences AB (Uppsala, Sweden). Cow's milk (3.5% and 0.5% fat content) was from *Stainzer Milch* cooperation, Stainz, Styria. Raw milk was obtained from a local farm.

The protein used to scavenge flavins was prepared by recombinant expression in *Escherichia coli* host cells as described previously (Knaus et al., 2012). Purification of the protein was performed as follows: resuspended cells were disrupted by 0.5 s sonication pulses for 10 min while cooling on ice. The cell debris was removed by centrifugation at 18,000*g* for 30 min at 4 °C. The

*Abbreviations:* FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; HPLC, high performance liquid chromatography; ppBat, putative protease from *Bacteroides thetaiotaomicron*.

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hexa-histidine tagged protein was purified by Ni sepharose affinity chromatography as described below. Fractions containing ppBat were combined, dialysed against 20 mM Tris/HCl buffer containing 100 mM NaCl (pH 8.0) over night and concentrated using Centripreps (Merck Millipore, Darmstadt, Germany). The final concentration was determined at 280 nm using an $\varepsilon_{280}$ of 13,075 $M^{-1}$ $cm^{-1}$ which was calculated from the protein sequence using the ProtParam tool on the ExPASy Bioinformatics Resource Portal (http://web.expasy.org/protparam/). The protein was flash-frozen with liquid nitrogen and stored at −20 °C.

### 2.2. Ni sepharose affinity chromatography

After loading of the supernatant onto a Ni sepharose HisTrap™ HP column (5 ml bed volume for protein purification and 1 ml bed volume for extraction of flavins from buffer solution and milk samples, respectively) preliminary equilibrated with lysis buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, 10 mM imidazole, pH 8.0), the column was washed with ten column volumes of wash buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, 20 mM imidazole, pH 8.0) and bound protein was recovered with elution buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, 150 mM imidazole, pH 8.0). The elution of protein was monitored by the method of Bradford (Bradford, 1976) whereby 490 µl BioRad Bradford solution were mixed with 10 µl of the eluted fractions. Thereby a blue coloration of the Bradford solution indicated the presence of protein. The purity of the protein containing elution fractions was afterwards assessed by SDS/PAGE as described by Laemmli, 1970. Each batch of expressed and purified ppBat was tested for its flavin binding affinity to ensure that the protein retained its full binding capacity. In the course of our study we found no changes between protein batches and hence we conclude that our preparations have reliable and reproducible flavin binding properties.

### 2.3. Extraction of flavins from buffer solutions

15 ml of lysis buffer containing 50 nmol riboflavin, FMN and FAD was incubated with 560 nmol ppBat for 30 min. In a second experiment 30 nmol riboflavin and 60 nmol of each FMN and FAD were used. After incubation, ppBat was isolated by Ni sepharose affinity chromatography (1 ml HisTrap™ HP column) as described above. Protein containing fractions were combined and concentrated to 1 ml by microfiltration (Centripreps). An UV/Vis-absorption spectrum was then recorded to determine the concentration of bound flavin using a molar absorption coefficient of ppBat bound riboflavin of 9800 $M^{-1}$ $cm^{-1}$ at 449 nm (Knaus et al., 2012). The molar extinction coefficient of free riboflavin is 12,500 $M^{-1}$ $cm^{-1}$ at 445 nm (Cha & Meyerhoff, 1988). Heat denaturation at 95 °C for 10 min was employed to release bound flavins from ppBat followed by HPLC analysis.

### 2.4. Extraction of flavins from cow's milk

Extraction of flavins from cow's milk was performed as follows: To 100 ml milk 360 nmol of ppBat were added and incubated for 30 min at room temperature. The milk sample was then loaded onto a 1 ml HisTrap™ HP column and ppBat was isolated by Ni sepharose affinity chromatography (see above). The protein containing fractions were combined and concentrated to 1 ml by microfiltration (Centripreps). Protein-bound flavins were analysed by UV/Vis-absorption spectroscopy. In addition, the flow through was collected and precipitated by adding 5 ml 100% trifluoroacetic acid at 4 °C. The precipitate was removed by centrifugation (30 min at 18,000*g*) and the supernatant was brought to pH 8 by slowly adding 1 M NaOH. 25 ml of the supernatant were diluted with an equal volume of lysis buffer and 460 nmol ppBat was added. Following 30 min incubation ppBat was isolated as described before. This two-step isolation of flavins was complemented by a single extraction experiment. In this case, 100 ml of milk was precipitated with 5 ml trifluoroacetic acid and the total flavin was determined using the isolation procedure described above.

### 2.5. UV/Vis absorption spectroscopy

UV/visible absorption spectra were recorded with a Specord 210 spectrophotometer (Analytik Jena, Jena, Germany).

### 2.6. HPLC determination of flavins and lumichrome

Bound flavins were released by thermal precipitation of ppBat at 95 °C for 10 min, cooling on ice for 5 min and centrifugation for 5 min (15,000*g*). The flavin extracts were loaded onto an Atlantis® dC18 8 µm 4.6 × 250 mm column (Waters) and eluted with a water/acetonitril multi-step gradient (0–1.5 min 0–5% acetonitrile; 1.5–20 min 5–60% acetonitrile; 20–22 min 95% acetonitrile) at a flow rate of 1 ml/min. The elution was monitored by UV-absorption at 370 nm and cofactors were identified according to their elution time (FAD = 10.7 min, FMN = 11.6 min, riboflavin = 12.4 min, lumichrome = 15.7 min) and UV/Vis absorption spectrum.

## 3. Results and discussion

The flavin-binding site of ppBat is unusual because it does not strictly distinguish flavins according to the N(10) side chain attached to the isoalloxazine ring system as is typical for flavoproteins. This is due to the formation of a sandwich complex where the isoalloxazine ring system is stacked by two indole side chains of tryptophan residues in the interface of the protein dimer. Fig. 1 shows a cartoon representation of the re-determined crystal structure of ppBat in the presence of riboflavin (compare with pdb code 3cne). Except for the π-stacking interaction the crystal structure of the protein does not show any further binding contacts. This rationalises the finding that all three naturally occurring flavins bind to the protein. To proof that ppBat scavenges riboflavin, FMN and FAD, we incubated a cocktail containing these compounds with excess protein and then isolated the His-tagged protein by affinity chromatography. Bound flavins were released

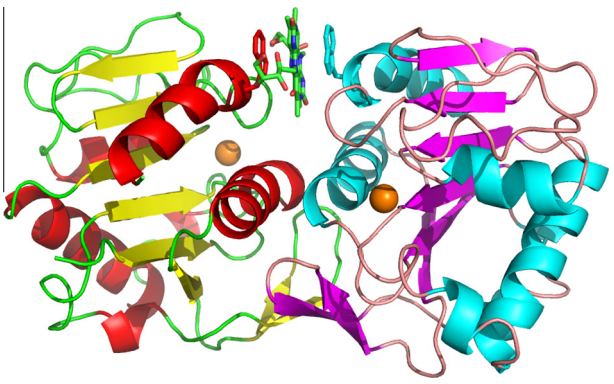

**Fig. 1.** Structural representation of the flavin-binding protein from *Bacteroides thetaiotaomicron* (ppBat). The dimeric protein is shown in a cartoon representation with α-helices shown in red (left protomer) and blue (right protomer) and β-sheets in yellow (left protomer) and magenta (right protomer). The putative zinc atoms in each of the protomers are shown as orange spheres. Riboflavin (top center) is sandwiched between two tryptophans, one from each protomer, and is shown as a stick model. Riboflavin is bound in two orientations with the N(10)-side chain pointing either into the plane or out of plane corresponding to the C2 axis of the dimer. The figure was prepared using the program PyMol (Delano, 2002).