



Notes & Tips

Blue native electrophoresis to study mitochondrial complex I in *C. elegans*

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ABSTRACT

Blue native polyacrylamide gel electrophoresis (BN-PAGE) is an essential tool for investigating mitochondrial respiratory chain complexes. However, with current BN-PAGE protocols for *Caenorhabditis elegans* (*C. elegans*), large worm amounts and high quantities of mitochondrial protein are required to yield clear results. Here, we present an efficient approach to isolate mitochondrial complex I (NADH:ubiquinone oxidoreductase) from *C. elegans*, grown on agar plates. We demonstrate that considerably lower amounts of mitochondrial protein are sufficient to isolate complex I and to display clear in-gel activity results. Moreover, we present the first complex I assembly profile for *C. elegans*, obtained by two-dimensional BN/SDS-PAGE.

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There are a growing number of diseases that are known to be associated with disturbed function of the mitochondrial oxidative phosphorylation system (OXPHOS).¹ This includes certain devastating neurodegenerative disorders with onset in early childhood (e.g., Leigh syndrome) as well as several prevalent diseases of adulthood (e.g., Parkinson's disease, Alzheimer's disease, diabetes mellitus type 2) [1,2]. NADH:ubiquinone oxidoreductase or complex I (CI), the first and largest protein complex of the respiratory chain, is the most frequently affected component of the OXPHOS system. Human CI consists of 45 different subunits, which are assembled via a complicated process that finally results in the functional holo-complex.

In vivo studies are important to gain further insights into OXPHOS disease pathogenesis and to develop treatment strategies. During the last years, *C. elegans* has been established as a model system to study mitochondrial (dys-)function in a wide array of OXPHOS disorders [3–5]. Importantly, there is an extensive evolutionary conservation of mitochondrial composition between mammalian and *C. elegans* mitochondria. Around 84% of human CI subunits are also found in *C. elegans* [5].

Several studies have shown that blue native polyacrylamide gel electrophoresis (BN-PAGE) can be used to study OXPHOS complexes in *C. elegans* [3,5–7]. BN-PAGE is a widely used technique for isolating native protein complexes from biological membranes

and cell homogenates. A crucial step of BN-PAGE is the efficient release and isolation of mitochondrial proteins. In *C. elegans*, this part is complicated by the extremely resilient worm exoskeleton. For that purpose, several techniques were used so far in varying combinations, including bead-beater and/or Potter homogenization, Polytron rupture, and proteinase subtilisin A treatment of worm samples [3,5–7]. However, with these approaches, large worm quantities and high amounts of mitochondrial protein were required to obtain clear results (150–300 µg mitochondrial protein; 2–3 g worm pellet) [3,5–7]. Most probably, release of mitochondria is still not optimal under these conditions. Moreover, isolation of OXPHOS complexes with ACBT buffer has not been done in BN-PAGE protocols for *C. elegans*.

To obtain large worm quantities, liquid culture was used in previous studies. This type of culture allows the rapid generation of large animal quantities. However, although a matter of debate, growth in axenic medium (CeMM) may be disadvantageous for studies of mitochondrial function. It has been shown that culture of *C. elegans* in CeMM decelerates development, decreases brood size, and increases life span compared to animals grown on agar plates [8]. It is unclear whether worm metabolism might also adapt to anaerobic pathways under these conditions.

Against this background, culture on agar plates appears favorable for OXPHOS studies. However, with this type of culture, worm amounts needed for current BN-PAGE protocols are difficult to obtain. Moreover, OXPHOS-deficient *C. elegans* often show slow growth and larval arrest, which makes it even more difficult to culture sufficient quantities [4].

Therefore, the aim of this study was to develop an efficient protocol for the application of BN-PAGE to study CI activity and assembly in *C. elegans*, grown on solid culture plates. After testing

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¹ Abbreviations used: BN-PAGE, blue native polyacrylamide gel electrophoresis; CeMM, axenic medium; CI, complex I; IGA, in-gel activity; OXPHOS, oxidative phosphorylation system; PMSF, phenylmethylsulfonyl fluoride.

of various isolation techniques and protein concentrations (data not shown), we here present an optimized approach.

Maintenance of *C. elegans* was carried out as described previously [9]. Bristol N2 was used as wild-type strain. For BN-PAGE analysis, 500 mg worms were collected in 3 ml of Milli-Q water from agar plates (8.5 cm diameter) when most of the bacterial lawn was consumed by the worms. Samples were centrifuged for 5 min at 2000 rpm and resuspended in 10 ml MSME/1 mM PMSF (MSME, 220 mM mannitol + 70 mM sucrose + 5 mM Mops + 2 mM EDTA, pH 7.4; PMSF, phenylmethylsulfonyl fluoride). After two further steps of centrifugation (5 min at 2000 rpm), dry worm pellets were stored at -80°C for later analysis.

In a first step, release of mitochondria was performed by Polytron rupture (PT 1200 C Kinematica), collagenase treatment, and subsequent homogenization by a Potter homogenizer. These are crucial steps to break the worm cuticle, which is composed of a collagenous matrix. Here, the use of collagenase appears to be specifically effective, which is not part of previous BN-PAGE protocols. In detail, worm pellets were suspended in 6 ml MSME/1 mM PMSF and transferred to a 10 ml glass vial. Polytron treatment was performed at stage 6 for 30 s on ice. Then, 300 μl of a collagenase stock solution (33 mg/ml in 5 mM CaCl₂/PBS; Sigma-Aldrich) was added to the sample and incubated for 15 min at room temperature on a tumbler mixer. Finally, worm suspension was transferred to a Potter homogenizer and treated for 3 min on ice.

In a second step, mitochondria-enriched fractions were obtained. To this end, 1 ml of the suspension was transferred to a 2 ml Eppendorf tube and 1 ml of MSME/0.4% BSA was added per sample (see also Kayser et al. [7]). After centrifugation at 2000 rpm for 10 min at 4°C , the supernatant was again transferred to a new tube and centrifuged at 9800 rpm for 10 min at 4°C . The supernatant was now stored, containing the cytoplasmic fraction. The pellet was resuspended in 1 ml MSME/PMSF and centrifuged two further times at 9800 rpm, 10 min, 4°C , yielding the crude mitochondria-enriched fraction.

In a third step, mitoplast isolation was performed (see also Suthammarak et al. [3]). For this approach, mitochondria-enriched pellets were gently resuspended in 100 μl PBS. Then, 100 μl digitonin (Sigma-Aldrich; 12 μl digitonin [200 mg/ml] + 88 μl PBS) was added and the samples were incubated for 10 min at room temperature. After the addition of 0.5 ml PBS, samples were centrifuged at 13,000 rpm for 20 min at 4°C . The pellet constitutes the mitoplast.

In a fourth step, isolation of OXPHOS complexes was performed (see Calvaruso et al. [10]). This technique has not been used in protocols for *C. elegans* so far. To this end, mitoplast pellets were resuspended in 100 μl ACBT (1.5 M aminocaproic acid + 75 mM Bis-Tris). Then, 10 μl of 20% laurylmaltoside (*n*-dodecyl β -D-maltoside; Sigma-Aldrich) was added and samples were incubated for 10 min on ice. Subsequently, suspensions were centrifuged for 30 min at 13,000 rpm and 4°C . The supernatant was collected, containing the native OXPHOS complexes. Protein determination was carried out using a Bio-Rad protein assay.

Electrophoresis was performed using a minigel system (Xcell SureLock Mini Cell, Invitrogen). This size is sufficient for a good separation of OXPHOS complexes and has the advantage that it requires less reagents and worm material. In addition, we used commercial ready-to-use blue native gels (Native-PAGE Novex Bis-Tris Gel system, 3–12% gel; Invitrogen). Electrophoresis was performed as described by Calvaruso et al. [10]. In brief, electrophoresis was started for approximately 30 min at 30 V and then voltage was raised to 100 V. When the front reached the middle of the separating gel, cathode buffer A was replaced by cathode buffer B. Total running time was approximately 4 h.

For detection of dehydrogenase activity characteristic of CI, in-gel activity (IGA) assays were performed by incubating gels for 1 h at room temperature with 5 mM Tris-HCl, pH 7.4, 0.1 mg/

ml NADH, and 2.5 mg/ml NTB (nitrotetrazolium blue, Sigma). After overnight staining at 4°C , gels were washed and immediately scanned. Experiments revealed that optimal IGA was already achieved with about 30–35 μg of mitochondrial protein (about 500 mg worm pellet). Fig. 1A shows the clear IGA band, obtained with this approach. In addition, Coomassie staining of the gel is depicted in Fig. 1B. We used a native marker to identify the height of the individual bands (NativeMark, Invitrogen). This indicates that the IGA band is at 950 kDa, which corresponds very well with the height of IGA bands published by Suthammarak et al. [3]. The low IGA band that we observed in our experiments is probably another protein, which has dehydrogenase activity. Similar findings have been shown in human cells where this band was caused by a protein dihydrolipoamide dehydrogenase [11]. Alternatively it might be a partially assembled/or degraded complex I part.

Western blotting of blue native gels was essentially performed according to standard procedures using PVDF blotting membranes (0.45 μm). For detection of CI, a monoclonal antibody against human NDUFS3 (homologue of *C. elegans* NUO-2) was used (MS112, mouse, MitoSciences) at a dilution of 1:1000. Results are shown in Fig. 1C. Interestingly, Western blotting of *C. elegans* CI revealed two bands. However, only the higher band apparently had an in-gel activity. Possibly, the lower band represents a partially assembled/inactive CI.

Subsequent denaturing electrophoresis was carried out according to Calvaruso et al. [10]. This way the content and distribution of individual subunits can be displayed, allowing the detection of possible subassemblies. In brief, a lane was cut out of the first-dimension gel and incubated with a dissociating solution (1% SDS

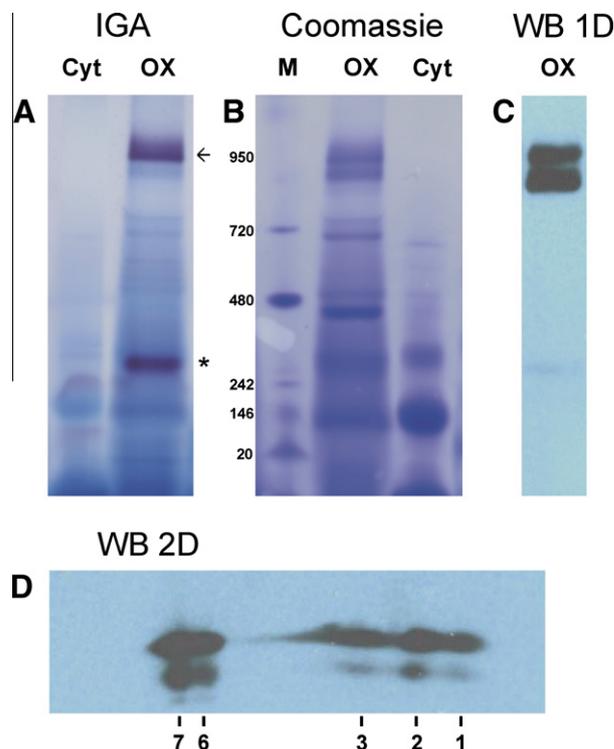


Fig. 1. (A) Mitochondrial complex I in-gel activity assay (IGA). Arrow indicates IGA band of interest; asterisk denotes an unspecific activity band. (B) Coomassie staining of the blue native gel. Cytoplasmic (Cyt) and OXPHOS (OX) protein fractions are indicated. A native marker (M) was used to identify the height of the individual bands. (C) First-dimension Western blotting (WB) of blue native gels, using anti-NUO-2 antibody. (D) Second-dimension BN/SDS-PAGE analysis. Putative subcomplexes (1–3, 6) and holo-CI (7) are indicated.

and 1% β -mercaptoethanol) for 1 h at room temperature. The gel strip was then placed at the top of the glass plate and the gel sandwich was further assembled. For exact composition of the gel sandwich and the running procedure see Calvaruso et al. [10]. Electrophoresis was started at 30 V for 30 min and then continued at 80 V (2–4 h) or at 20 V (overnight run 10–12 h). Results are shown in Fig. 1D. The second dimension shows a double band, indicating the holo-CI. In addition, several assembly intermediates become apparent. The low assembly intermediates resemble very much the subcomplexes 1–3 as found in human cells using this antibody, whereas the high molecular weight one close to CI might resemble subcomplex 6 (see Vogel et al. [12]).

In conclusion, we developed a more efficient protocol for BN-PAGE of mitochondrial CI in *C. elegans*, cultured on solid agar plates. In general, BN-PAGE in *C. elegans* has been rarely used so far and current protocols have the disadvantage that high amounts of mitochondrial protein are required. Key steps of the presented method are an improved approach for cuticle rupture (see first step of the protocol) and the final isolation of OXPHOS complexes (see fourth step). Based on these steps, clear results can be achieved with 30–35 μ g of mitochondrial protein. The strong reduction of required material makes worm culture easier and allows the use of agar plates. Moreover, with smaller loading amounts, electrophoresis can be simplified by the application of commercial ready-to-use blue native gels. In addition, second-dimension BN/SDS-PAGE analysis of CI assembly profiles has not been performed so far. This extension of BN-PAGE may deliver important insights into CI biogenesis and composition in *C. elegans*.

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