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Technical note

Subunit analysis of mitochondrial cytochrome *c* oxidase and cytochrome bc_1 by reversed-phase high-performance liquid chromatography

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ABSTRACT

A rapid separation of the ten nuclearly-encoded subunits of mitochondrial cytochrome *c* oxidase, and ten out of the eleven subunits of cytochrome bc_1, was achieved using a short, 50 mm C_{18}-reversed-phase column. The short column decreased the elution time 4–7 fold while maintaining the same resolution quality. Elution was similar to a previously published protocol, i.e., a water/acetonitrile elution gradient containing trifluoroacetic acid. Isolated subunits were identified by MALDI-TOF. The rapidity of the described method makes it extremely useful for determining the subunit composition of isolated mitochondrial complexes. The method can be used for both analytical and micro-preparative purposes.

Cytochrome *c* oxidase (Complex IV, CcO, E.C. 1.9.3.1) and ubiquinol-cytochrome *c* oxidoreductase (Complex III, Cyt bc_1, E.C. 1.10.2.2.) are electron transfer complexes located within inner mitochondrial membrane. Both, mammalian CcO and Cyt bc_1 are multisubunit enzyme complexes consistent of 13 and 11 non-identical polypeptides, respectively. The three largest, and more hydrophobic subunits of CcO, I, II and III, are encoded by the mitochondrial genome. The remaining smaller subunits are nuclearly-encoded [1]. The Cyt bc_1 complex contains 11 subunits. In addition to the catalytic cytochrome *b* (subunit III), cytochrome c_1 (IV), and Rieske Fe/S (V) subunits, the mammalian enzyme contains eight polypeptides that do not have prosthetic groups [2]. The number of subunits, both hydrophobic and hydrophilic, makes the isolation, purification and analysis of individual polypeptides rather challenging. Reversed-phase HPLC is one of the best analytical approaches. Robinson's group previously applied RP-HPLC to analyse the subunits composition of three mitochondrial electron transfer complexes [3–7]. However, all analysis were performed using 250 mm long columns, either C_{18} or C_4, and, therefore, the chromatographic procedures were time consuming. An alternate approach is to use a short column for HPLC as it was suggested previously [8,9]. In the present work using a 50 mm C_{18} column and a modified gradient we have achieved almost the same quality of subunit separation for both complexes with nearly baseline resolution between most of the eluted peaks, and with a significantly shorter separation time. All isolated subunits were positively identified by MALDI-TOF analysis. Cytochrome *c* oxidase was isolated from bovine heart mitochondria according to modified protocol of Soulimane and Buse [10,11]. The enzyme isolated using anion-exchange chromatography was solubilized in 0.1 M NaH_2PO_4 buffer (pH 7.4) containing 0.1% TX-100, frozen in liquid nitrogen and stored at −80 °C. The concentration of CcO was determined using the molar extinction coefficient $\varepsilon_{422} = 154\ mM^{-1}cm^{-1}$ [12]. The enzyme was a gift from Dr. M. Fabian from The Center for Interdisciplinary Biosciences P.J. Safarik University in Kosice, Slovakia. Cytochrome bc_1 was purified from frozen bovine heart as previously described [13]. The cytochrome c_1 content was determined from the ascorbate-reduced minus ferricyanide-oxidized spectrum using a double difference

Abbreviations: CcO, cytochrome *c* oxidase; Cyt bc_1, cytochrome bc_1; TX-100, Triton X-100; DDM, dodecyl maltoside; TFA, trifluoroacetic acid.

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extinction coefficient $\Delta\Delta\varepsilon$ of 17.5 mM^{-1} cm^{-1} for 552 minus 540 nm [14]. The subunit analysis of both, CcO and Cyt bc_1 complexes were performed using an Agilent 1200 Infinity Series HPLC system (Agilent, USA) with a Poroshell 120EC - C_{18} column, (3 × 50 mm, 2.7 μm) with a 120 Å pore size. Isolated CcO was solubilized in 20 mM phosphate buffer pH 7.4, containing 2 mM DDM. TFA was added to a final concentration of 0.1% and the sample was incubated for 5 min at 20 °C. The same concentration of TFA was included in both mobile phases A (deionized water) and B (100% acetonitrile). The column was equilibrated for 10 min at 1.3 mL/min flow rate with mobile phase A. CcO (5 μL samples containing 5–50 μg of protein) was injected onto the HPLC column via Agilent auto-sampler. Elution was at 1.3 mL/min with an acetonitrile gradient for total time of 10 min. The gradient was: 0–2 min from 0 to 30% B; 2–5 min to 50% B; 5–8 min to 65% B, and 8–10 min to 85% B. The eluate was monitored at 214 nm. Cyt bc_1 was solubilized in 20 mM phosphate buffer pH 7.4, containing 2 mM DDM. TFA was added to a final concentration of 0.2% and sample was incubated for 50 min at 20 °C. The same concentration of TFA was included in both mobile phases A (deionized water) and B (100% acetonitrile). The column was equilibrated with the mobile phase A for 10 min at 1.0 mL/min flow rate. Cyt bc_1 samples (5 μL containing 5–50 μg of protein) were injected onto the HPLC column via Agilent auto-sampler. The acetonitrile/water gradient was as follows: 0–10.5 min from 0 to 50% B; 10.5–12.5 min to 51% B; 12.5–16 min to 51.5% B; 16–18 min to 54% B; 18–20 min to 60% B. The eluate was monitored at 214 nm. All HPLC experiments have been performed at room temperature. Prior of injection, CcO and Cyt bc_1 samples were filtrated through a 0.45-mm pore size filter. The subunit determination within each HPLC elution peak was completed by collecting fractions of eluant and analyzing the contents using Microflex MALDI-TOF (Bruker-Daltonics). HPLC fractions were collected in 0.5 mL volume, dried and dissolved in 10 μl of 60% acetonitrile in water. The aliquots (0.9 μl) of prepared samples were mixed with an equal volume of DHB matrix, loaded onto the surface of a steel plate (Bruker-Daltonics) and dried for several minutes at room temperature. The spectra represent the average of 105–150 laser shots. CcO eluted in nine resolved peaks when analyzed by RP-HPLC using the 50 mm C_{18} column and conditions described above (Fig. 1, panel A). The observed chromatographic pattern is comparable to that obtained by Robinson's group with a 250 mm column [6]. For example, the first two peaks elute early and appear to be very similar to the elution of subunits VIb and V as previously reported [6]. Indeed, MALDI-TOF analysis confirmed the presence of these two subunits in the first two peaks. Moreover, by MALDI-TOF analysis of every HPLC peak, all 10 nuclearly-encoded subunits of CcO were positively identified (Table 1, Fig. 1, panels C and D). All of the determined molecular masses were in agreement with published mass spectrometry data [15].

The three largest and very hydrophobic subunits I, II and III were not recovered from the column since they precipitated in the solvent and could not be analyzed [5,6]. The broad peak eluting at retention time of about 8.5 min contained both, subunit IV and residual TX-100 that remained from the isolation procedure. The presence of TX-100 was confirmed by RP-HPLC analysis of detergent alone (data not shown). The HPLC procedure allows analytical (5 μg of protein) as well as micro-preparative (up to 50 μg of protein) separation with the same quality. Reversed-phase HPLC of the cytochrome bc_1 rapidly separates ten out of eleven subunits (Fig. 1, panel B). We have previously stated that using 250 mm C_{18} column the subunits separation can be achieved in less than 3 h [4]. The present protocol allows the separation of Cyt bc_1 polypeptides with similar quality in less than 1 h, including the sample preparation

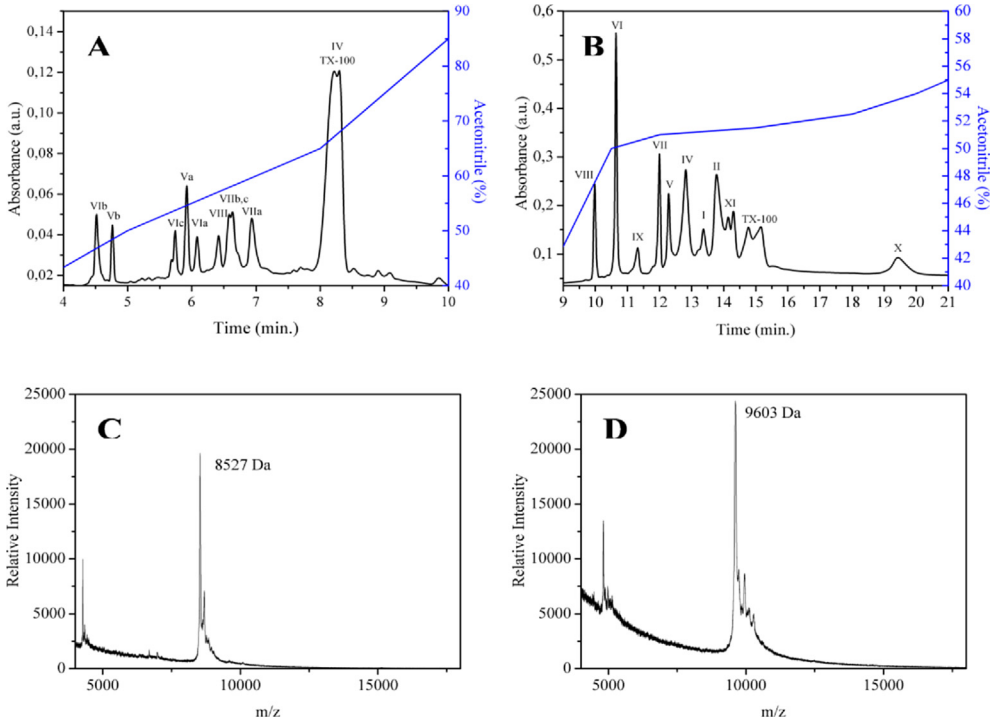

Fig. 1. RP-HPLC separation and MALDI-TOF analysis of cytochrome *c* oxidase and cytochrome bc_1 subunits. Panel A: RP-HPLC separation of cytochrome *c* oxidase subunits (5 μg of protein). Elution of subunits was monitored at 214 nm. The blue line represents % acetonitrile in the gradient as described in Methods. Panel B: RP-HPLC separation of mitochondrial cytochrome bc_1 (50 μg of protein) subunits. Subunits elution was monitored at 214 nm. Blue line represents % acetonitrile in the gradient. Panel C: representative MALDI-TOF mass spectra of CcO subunit VIc. Panel D: representative MALDI-TOF mass spectra of Cyt bc_1 subunit VII. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Molecular weight of cytochrome *c* oxidase and cytochrome bc_1 subunits isolated by RP-HPLC and determined by MALDI-TOF mass spectrometry.

Cytochrome *c* oxidase			Cytochrome bc_1		
Subunit	Reported mass[a]	MALDI/MS[b]	Subunit	Reported mass[c]	MALDI/MS[b]
IV	17150	17142	I	49002	49236
Va	12471	12434	II	46285	46530
Vb	10696	10668	IV	29295	27906
VIa	9529	9531	V	21643	21669/21609
VIb	10105	10063	VI	13427	13389
VIc	8527 (8552)	8521	VII	9603	9587
VIIa	6672	6676	VIII	9184	9172
VIIb	6357	6359	IX	8021	7997
VIIc	5444	5446	X	7341	7298/7326
VIII	4964	4963	XI	6540	6520

[a] Reported mass is the molecular mass of bovine CcO subunits determined previously by MALDI-TOF [15].
[b] Experimentally measured by MALDI/MS in the present work.
[c] Reported mass is the molecular mass of bovine cytochrome bc_1 subunits determined previously by ESI/MS [4]. Subunits I-III of CcO and subunit III of Cyt bc_1 precipitated in acetonitrile and were not analyzed.

time. The chromatography procedure (excluding the sample preparation) was only 20 min long. All eluted subunits were identified by MALDI-TOF and the obtained data were in agreement with published mass spectrometry data (Table 1, Fig. 1 panel D). Subunits I, II and XI were the most difficult to separate. We have tested several gradients with different slopes, all performed with acetonitrile and water, containing TFA. However, residuals of subunit II were always detected within the fraction collected at retention time of ~13.5 min, i.e. subunit II co-eluted with subunit I. The same difficulty was to separate subunit XI from subunit II (residuals of subunit II were found within the peak with the retention time of ~14.25 min). All other subunits were separated with almost baseline resolution. The broad double peak eluted at ~15 min did not contain protein, but was due to residual TX-100.

In conclusion, the methods to separate mitochondrial electron transfer complexes into their subunits performed in the past all share one disadvantage, that the separations were time consuming. The procedure described in this report is rapid, reproducible and requires less than a few micrograms of protein. The method can be used for both analytical and micro-preparative purposes, and isolated subunits are suitable for further characterization. Our preliminary results also suggest that the method is useful for detecting chemical modification of subunits.

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