

PURIFICATION OF RAVE COMPLEX FROM *Saccharomyces cerevisiae* USING FLAG TAG-AFFINITY PURIFICATION METHOD

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Received: 25 December 2012; Accepted for publication: 7 November 2013

ABSTRACT

RAVE (Regulator of the H⁺-ATPase of the Vacuolar and Endosomal membranes) is an essential factor of assembly and reversible disassembly of V-ATPase. RAVE complex has three subunits, which are Rav1p, Rav2p and Skp1p. There are few studies on RAVE so it is very important to study structure of RAVE complex to understand more about the regulation of the assembly and reassembly at V-ATPase. In this study the RAVE complex was purified by affinity purification by fusing FLAG tag to subunit Rav1p or Rav2p. Experimental process: yeast cells were incubated in 8 L YEPD medium at 30 °C, 200 rpm (OD_{600nm} around of 3). Furthermore harvested cells were broken by a French pressure cell disruptor at 25,000 p.s.i in TBSE (50 mM Tris/Cl, 150 mM NaCl, 1 mM EDTA, pH 7.4) with 1 mM PMSF. The cell lysate was centrifuged at 20,000 xg for 20 minutes at 4 °C. Then, the supernatant, was achieved by centrifugation, and loaded onto a small column contained 1 ml of anti-FLAG M2 gel. After washing anti-FLAG column with TBSE, RAVE complex was eluted with TBSE containing 100 µg/ml FLAG peptides. The results showed RAVE complex purification from strain with FLAG tag fused in C-terminus of Rav2 is better than RAVE complex purification from the yeast strain *S. cerevisiae* with FLAG tag fused in N-terminus of Rav1 or C-terminus of Rav1.

Keywords: RAVE complex, Rav1, Rav2, Skp1, V-ATPase, Yeast, protein purification.

1. INTRODUCTION

In eukaryotic cells, Vacuolar proton-translocating ATPases (V-ATPases) are present in organelles, such as endosomes, lysosomes, Golgi-derived vesicles, secretory vesicles and the plasma membrane of some cells. V-ATPase plays a central role in acidification of these organelles [1, 2, 3, 4]. And it plays a key role in many normal and disease processes such as regulation of the vacuolar fission–fusion equilibrium [5], neurotransmitter release [6], endocytic and secretory trafficking [7], ovulation and embryogenesis [8], virus infection [9], cancer [10, 11]

and Alzheimer disease [12]. Therefore the studies about the structure, mechanism and regulation of V-ATPase are very important with several studies on structure, function and regulation being done with *S. cerevisiae* due to the ease of incubation and gene manipulation.

The V-ATPase is a large, multi-subunit enzyme and composes of two domains V_1 and V_0 . The V_1 domain is a peripheral complex of 650 kDa, consists of eight different subunits named A, B, C, D, E, G and H with molecular weight 13 – 70 kDa. It hydrolyzes ATP to ADP, Pi and releases energy. The V_0 domain is an integral complex of 260 kDa includes six different subunits as a, d, e, c, c' and c'' of molecular mass 9 – 100 kDa. The energy released in ATP hydrolysis is transferred to the V_0 domain to drive protons translocation from cytoplasm to the lumen or extracellular space [1, 2, 13, 14, 15, 16].

Many studies in yeast V-ATPase were carried out, but until now the regulation of V-ATPase is not completely understood [2, 14, 17, 18, 19]. A few years ago, a novel factor was found which played an important role in both assembly of V-ATPase and reassembly of disassembled V_1 and V_0 domains, was named the RAVE complex (Regulator of the H^+ -ATPase of the Vacuolar and Endosomal membranes), includes Rav1p, Rav2p and Skp1p [17, 18, 19]. Skp1p subunit has molecular weight about 22 kDa and is also a subunit of SCF ubiquitin ligases. The molecular weight of Rav2p is 40 kDa and Rav1p is 155kDa [17, 20, 21, 22]. In RAVE complex, Rav1p binds to Rav2p and Skp1p, but Rav2p do not bind to Skp1p. When RAVE complex regulates for assembly and reassembly of this enzyme, RAVE complex binds to V_1 sector at subunits E and G [17]. Further, RAVE can affect the efficient assembly of C subunit with the V-ATPase. In absence of RAVE complex, in the regulation of V-ATPase, the subunit C is not stable with the V-ATPase [19]. The topology and V-ATPase's regulation of the RAVE complex is shown in Fig. 1. It is very important to study the structure of RAVE complex to understand the regulation of V-ATPase. Therefore, in this study, we want to purify RAVE complex for further study.

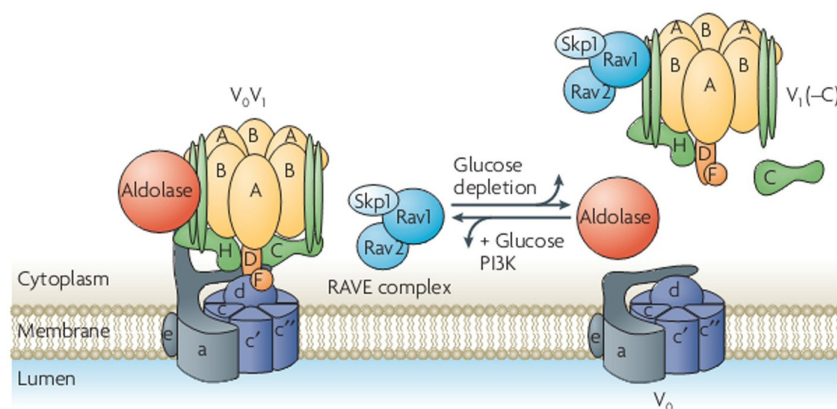


Figure 1. Models for RAVE activity in assembly and reassembly of disassembly of the V-ATPase [2].

2. MATERIALS AND METHODS

2.1 Reagents and growth media

Oligonucleotides (table 1), herring sperm ssDNA and DNA marker S plus were synthesized by Sangon Biotech (Shanghai) Co. Ltd. *Taq* DNA polymerase, *pfu* DNA polymerase, dNTP, agarose, Tris base, EDTA, phenylmethylsulfonyl fluoride, yeast extract, peptone and G418 were purchased from Bio Basic Inc. Lyticase, Anti-FLAG M2 Affinity gel and FLAG peptide were

purchased from Sigma-Aldrich Shanghai Trading Co Ltd. Anti-FLAG Tag Monoclonal Antibody and HRP AffiniPure Goat Anti-Mouse IgG were purchased from EarthOX. 0.45 µm PVDF membrane was purchased from Millipore. GeneRuler 1kb DNA Ladder and PageRuler Prestained Protein Ladder were purchased from Fermentas. All other chemicals and reagents, were not noted, were from Sinopharm Group Co.Ltd. Yeast cells were grown in 1 % yeast extract, 2 % peptone and 2 % dextrose (YEPD) medium at 30 °C. For growth of recombinant strains, YEPD was contained 2 % agar and added G418 to the final concentration of it is 200 µg/ml.

2.2 Strains

The two strains were used in this study are *Saccharomyces cerevisiae* BY4742 from State Key Laboratory of Food Science and Technology of Jiangnan University and *S. cerevisiae* SF838–5A RAV1-Myc13 2.3 from Patricia Kane in the Department of Biochemistry and Molecular Biology, State University of New York, Upstate Medical University.

2.3 Strain Construction

Flag tag was fused to the N-terminus of Rav1, C-terminus of Rav1 and C-terminus of Rav2. Using *S. cerevisiae* BY4742 genome for synthesizing the fragments are homogeneous with yeast genome. *S. cerevisiae* genome was used for synthesizing KanMX6 marker. For fragments have length less than 1,000 bp in length, *Taq* DNA polymerase was used to run PCR. For the fragments have length more than 1,000 bp in length, *Taq* and *pfu* DNA polymerase were used to run PCR. Fusion PCR was also used *Taq* and *pfu* DNA polymerase. Primers from YNR1-1 to YNR1-8 were used to construct FLAG tag to N-terminus of Rav1, primers from YCR1-1 to YCR1-4 were used to construct FLAG tag to C-terminus of Rav1 and primers from YCR2-1 to YCR2-6 were used to construct FLAG tag to C-terminus of Rav2 (table 1). Yeast transformation method was used from Gietz R D and Woods RA [23].

Table 1. Oligonucleotides used in this study.

Oligonucleotide name	Oligonucleotide sequence 5' → 3'
YNR1-1	TGCTGGCTGTTTCAGAAGTTTTAG
YNR1-2	CAAGCTAAACAGATCTATATTACCATGGTAGCTAGTTGTAAAGATCAG
YNR1-3	CTGATCTTTACAACACTAGCTACCATGGTAATATAGATCTGTTTAGCTTG
YNR1-4	GTGCGTTTGTAGATATCAAAGAATTAGAAAACTCATCGAGCATC
YNR1-5	GATGCTCGATGAGTTTTTCTAATTCTTTGATATCTACAAACGCAC
YNR1-6	CTTGTCGTCATCGTCTTTGTAGTCCATGAGAACTACCTTTGCGTGTGC
YNR1-7	ATGGACTACAAAGACGATGACGACAAGTCATTGAACCTTTCTTCCAGG
YNR1-8	CTAACTCATTATCAGAAGACCATC
NR1-1	GGACAAACCGAATAAAAGAGC
NR1-2	TCGTACTTCCCAATACTGACTATC
YCR1-1	ATTACCTGGCGGTTTCACTTG

YCR1-2	CTACTTGTCATCGTCGTCCTTGTAATCAGCTACAAAGTCATCTAG-TAAGTTCTTGG
YCR1-3	GCTGATTACAAGGACGACGATGACAAGTAGGCGCCACTTCTAAATAAGCG
YCR1-4	ATCAAAGAATCTGGGACGCTC
CR1-1	ATTGGATCTCTAAATGTTGAAGCA
CR1-2	AACTATGGGTGTCCCGCTT
YCR2-1	GGTGACTAAAAACCTTGG
YCR2-2	TTACTTGTCATCGTCGTCCTTGTAAGTCCTTATATGTACTTAACTGTTTGCTA
YCR2-3	GACTACAAGGACGACGATGACAAGTAAAATTTCTTATGATTTATGATTTTAA
YCR2-4	GTTAATGTTATCGCTAGTAGAGCGGCGTTAGTATCGAATC
YCR2-5	GCTGTGATTCGATACTAACGCCGCTCTACTAGCGATAACATTAACA
YCR2-6	ATTAGATTTGATTGAAGAG
CR2-1	CTTTGTAGGAATGAGCTTTGC
CR2-2	AGTTCCCGAATATGATGTTG

2.4 Genomic DNA extraction

Yeast strain was incubated overnight at OD₆₀₀ approximately 10 in YPD medium, 30 °C, 200 rpm. 3 mL samples were collected by centrifugation at 8,000 xg for 2 minutes. Wash the cells with sterile water and resuspend the cells in 500 µl of sorbitol solution (0.9 M sorbitol, 0.1 M Tris-HCl, 10 mM EDTA, pH 7.5). Add 1 µl β-mercaptoethanol and 20 – 50 µL stock solution of lyticase (1,000 U/ml). Incubate at 37 °C for 3 hours to achieve 80 % spheroplasting, centrifuged at 8,000 xg for 5 minutes then resuspended in 500 µl TE (10 mM Tris, 1 mM EDTA) (pH8.0). Add 50 µl 10 % SDS and 10 µl proteinase K (10 mg/ml). Incubate at 65 °C for 20 minutes. Add 10 µl RNaseA (10 mg/ml) and incubate 37 °C for 1 hour. Add 550 µL chloroform:isoamyl alcohol (24:1), vortex, then centrifuge. Precipitate DNA with isopropanol (volume ratio 1 : 1), place on dry ice for 10 minutes or at -20 °C for 1 hour. Centrifuge at 15,000 xg for 10 minutes at 4 °C. Wash twice with 70% methanol. Dry the pellets and resuspend the DNA in 50 µl of TE or ddH₂O. Test the result with agarose gel electrophoresis.

2.5 RAVE complex purification

RAVE complex was purified from three yeast strains. BY4742 *FLAG-Rav1* strain has FLAG tag which was introduced into the N terminus of Rav1 gene of *S. cerevisiae* BY4742, BY4742 *Rav1-FLAG* strain has FLAG tag which was introduced into the C terminus of Rav1 gene of *S. cerevisiae* BY4742 and BY4742 *Rav2-FLAG* with FLAG tag was introduced into the C terminus of Rav2 gene of *S. cerevisiae* BY4742. Yeast strain was grown in 50 ml YEPD medium overnight to A_{600nm} of around 3, and add 50 ml of this culture was added 1 litre of YEPD media in a 3 liter Fernbach flask (total volume for one time purify is 8 liters). The flasks were incubated at 30 °C under vigorous shaking until an A_{600nm} of around 3 (early log phase). Yeast was harvested by centrifugation, washing and resuspended TBSE (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 7.4) and the protease inhibitor (at 1 mM phenylmethylsulfonyl fluoride) were added shortly before cells were lysed by two passages through a French pressure cell at 25,000 p.s.i. The cell lysate was centrifuged at 20,000 xg for 20 minutes at 4 °C. The supernatant,

was achieved after centrifugation, is loaded onto a small column contained 1 ml of anti-FLAG M2 gel. The anti-FLAG column was washed with 20 column volumes of TBSE, and RAVE complex was eluted with TBSE containing 100 µg/ml FLAG peptides.

2.5 Western blot

After separation by SDS-PAGE, proteins were transferred onto a 0.45 µm PVDF membrane, using electron transport. Then, the membrane was washed three times (5 minutes each time) with TBS (10 mM Tris/Cl, 150 mM NaCl, pH 7.4). The membrane was blocked with 3 % bovine serum albumin in TBST (10 mM Tris/Cl, 150 mM NaCl, pH 7.4, 0.01 % Tween 20) for 2 hours at room temperature. Then it was incubated with anti-FLAG tag monoclonal antibody (1 : 2,000 in TBST) overnight at 4 °C. The membrane was washed three times (10 minutes each time) with TBST and incubated with HRP AffiniPure Goat Anti-Mouse IgG (1:1000 in TBST) for 1h at 37 °C. Finally, the membrane was washed three times with TBST and once with TSB. The result was show with diaminobenzidine (DAB).

3. RESULTS

3.1. Constructed recombinant strains

We constructed three strains, strain 1 which contained FLAG tag at N-terminus of Rav1, strain 2 with FLAG tag at C-terminus of Rav1 and strain 3 with FLAG tag at C-terminus of Rav2. The method from Lorenz et al [24] was partially modified and used in this study. For constructing FLAG tag to N-terminus of Rav1, first, four fragments were synthesized by PCR (Fig. 2a). Fragment 1 (using primers YNR1-1 and YNR1-2), fragment 3 (using primers YNR1-5 and YNR1-6) and fragment 4 (using primers YNR1-7 and YNR1-8) are homogeneous with *S. cerevisiae* genome, and fragment 4 is the 5'-end of Rav1 with FLAG tag DNA sequence inserted in after the start codon of Rav1. Fragment 2 (using primers YNR1-3 and YNR1-4) contains KanMX6 marker. Primers YNR1-1 and YNR1-8 were used to fuse these 4 fragments (Fig. 2b). After that, fusion PCR products were used to transform to *S. cerevisiae* BY4742. Recombinant strains were screened on YEPD medium containing 200 µg/ml G418. Furthermore, PCR was performed by using primers NR1-1 and NR1-2 to test for integration of constructing FLAG tag to the 5'-end of Rav1 and the PCR product was sequenced for confirmation (Fig. 2c). The resulted-recombinant strain was named *S. cerevisiae* BY4742 *FLAG-RAV1*.

For constructing FLAG tag to C-terminus of Rav1, two fragments were synthesized by PCR (Fig. 3a). Fragment 1 (using primers YCR1-1 and YCR1-2) is the 3'-end of Rav1 with FLAG tag DNA sequence inserted before the stop codon of Rav1. Fragment 2 (using primers YCR1-3 and YCR1-4) contains KanMX6 marker and homogenes of *S. cerevisiae* genome. The results of fusion PCR product using these fragments are shown in Fig. 3b. And test for integration of constructing FLAG tag to the 3'-end of Rav1 by PCR using primers CR1-1 and CR1-2 are shown in Fig. 3c. Then recombinant strain was confirmed by DNA sequencing. Finally, *S. cerevisiae* BY4742 *RAV1-FLAG* was achieved.

For constructing FLAG tag to C-terminus of Rav2, three fragments were synthesized by PCR (Fig. 4a). Fragment 1 (using primers YCR2-1 and YCR2-2) and fragment 3 (using primers YCR2-5 and YCR2-6) are homogeneous with *S. cerevisiae* genome, and fragment 1 is the 3'-end of Rav2 with FLAG tag DNA sequence inserted before the stop codon of Rav2. Fragment 2 (using primers YCR2-3 and YCR2-4) contains KanMX6 marker. The results of fusion PCR

product using these fragments were shown in Fig. 4b. Furthermore, PCR was performed by using primers CR2-1 and CR2-2 to test for integration of constructing FLAG tag to the 3'-end of Rav2 and the PCR product was sequenced for confirmation (Fig. 4c). The achieved strain was named *S. cerevisiae* BY4742 RAV2-FLAG.

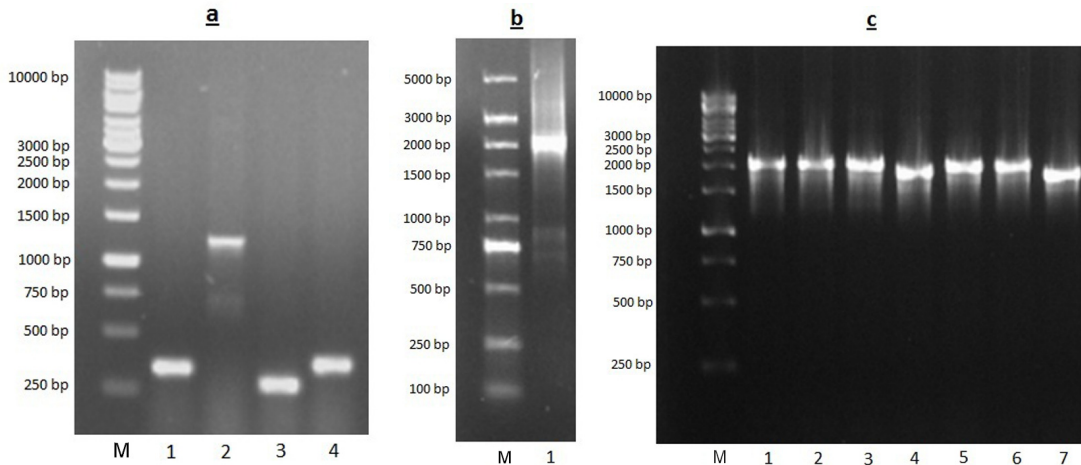


Figure 2. The *S. cerevisiae* BY4742 FLAG-RAV1 constructed

- (a) Land 1: Fragment 1 using primers YNR1-1 and YNR1-2 for PCR, land 2: Fragment 2 using primer YNR1-3 and YNR1-4 for PCR, Land 3: Fragment 3 using primers YNR1-5 and YNR1-6 for PCR, Land 4: Fragment 4 using primers YNR1-7 and YNR1-8 for PCR, land M: GeneRuler 1kb DNA Ladder.
 (b) Land 1: Fusion PCR products of 4 fragments using primers YNR1-1 and YNR1-8.
 (c) Lands 1 to 7: Test for integration of constructing FLAG tag to the N-terminal of Rav1 using primers NR1-1 and NR1-2 of 7 strains.

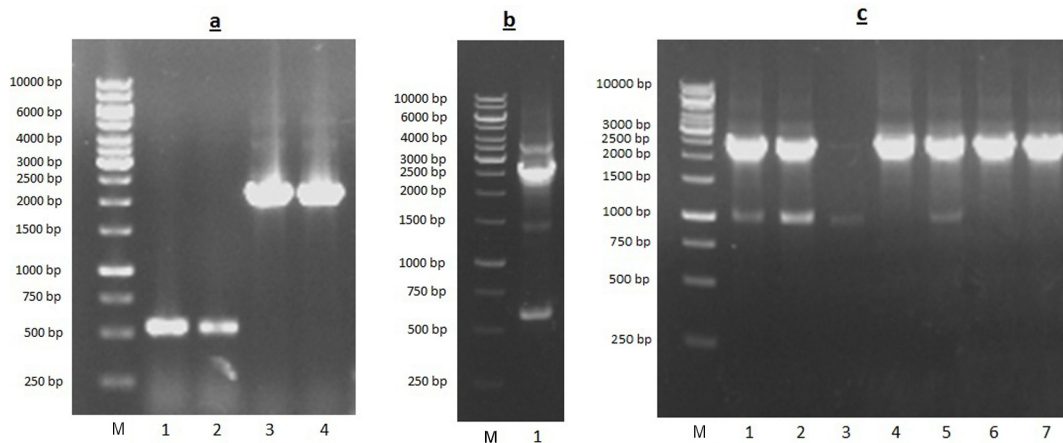


Figure 3. The *S. cerevisiae* BY4742 RAV1-FLAG constructed.

- (a) Land 1 and 2: Fragment 1 using primers YCR1-1 and YCR1-2 for PCR, land 3 and 4: Fragment 2 using primer YCR1-3 and YCR1-4 for PCR, land M: GeneRuler 1kb DNA Ladder. (b) Land 1: Fusion PCR products of 2 fragments using primers YCR1-1 and YCR1-4. (c) Lands 1 to 7: Test for integration of constructing FLAG tag to the C-terminal of Rav1 using primers CR1-1 and CR1-2 of 7 strains.

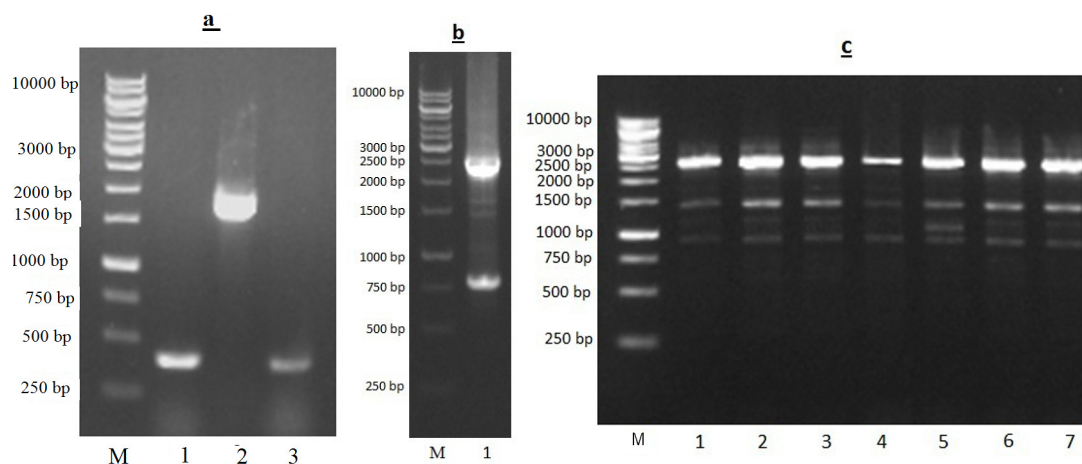


Figure 4. The *S. cerevisiae* BY4742 RAV2-FLAG constructed.

(a) Land 1: Fragment 1 using primers YCR2-1 and YCR2-2 for PCR, land 2: Fragment 2 using primer YCR2-3 and YCR2-4 for PCR, Land 3: Fragment 3 using primers YCR2-5 and YCR2-6 for PCR, land M: GeneRuler 1kb DNA Ladder. (b) Land 1: Fusion PCR products of 3 fragments using primers YCR2-1 and YCR2-6. (c) Lands 1 to 7: Test for integration of constructing FLAG tag to the C-terminal of Rav2 using primers CR2-1 and CR2-2 of 7 strains.

3.2 Purification of rave complex

For purifying RAVE complex, we chose FLAG tag, due to it is two necessary characteristics: protein purification with high purity and little opportunity to impair tagged protein structure (FLAG tag has 8 amino acids, so the size of FLAG tag is small), additionally, FLAG tag can be removed by enterokinase. With another tag, if they can bring high purity to protein purification like *Staphylococcus* protein A (58 amino acids) or Streptavidin-binding peptide (38 amino acids), they may impair the tagged protein structure because, normally, they have big size. If they has small size and a little impair to structure of tagged protein, they may provide poor purity to protein purification [25].

In the Fig. 5, the band of the protein was not big which may attribute the low protein concentration. The low concentration of RAVE in yeast might be due to the reason that RAVE complex was a regulation factor which necessarily may not remain in large amounts in the cell. Comparatively seen in SDS-PAGE Fig. 5a (RAVE complex was purified from BY4742 *FLAG-RAV1*), 5b (RAVE complex was purified from BY4742 *RAV1-FLAG*) and 5c (RAVE complex was purified from BY4742 *RAV2-FLAG*), the efficiency of purification using strains BY4742 *FLAG-RAV1* and BY4742 *RAV1-FLAG* were almost the same, but RAVE complex purification using strain BY4742 *RAV2-FLAG* was better. Beside three bands are Rav1p, Rav2p and Skp1p, there were other bands of 95 kDa, 72 kDa and 55 kDa.

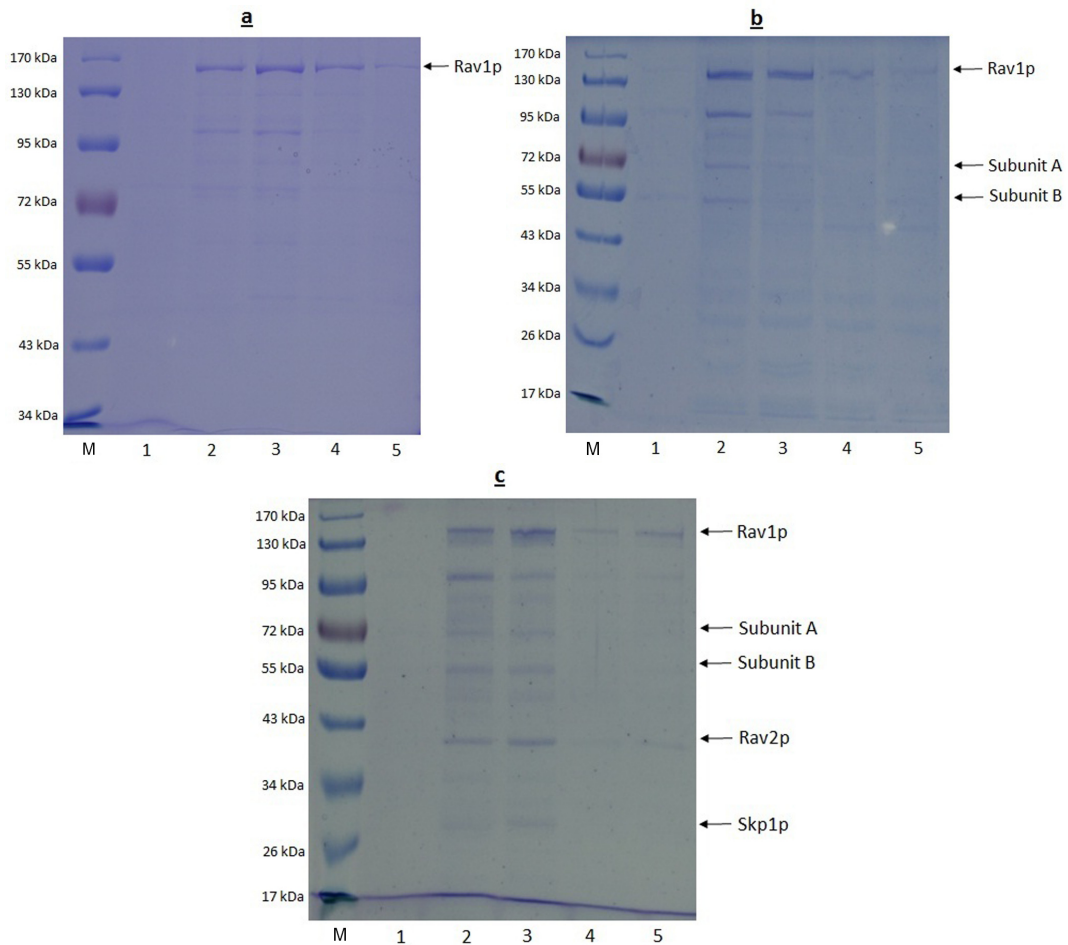


Figure 5. SDS-PAGE of RAVE complex purification.

- RAVE complex purification from BY4742 *FLAG-RAVI*. From land 1 to 5: RAVE complex eluted from anti-FLAG affinity column at fraction from 1 to 5 respectively, land M: PageRuler Prestained Protein Ladder
- RAVE complex purification from BY4742 *RAVI-FLAG*. From land 1 to 5: RAVE complex eluted from anti-FLAG affinity column at fraction from 1 to 5 respectively.
- RAVE complex purification from BY4742 *RAV2-FLAG*. From land 1 to 5: RAVE complex eluted from anti-FLAG affinity column at fraction from 1 to 5 respectively.

The subunit A of V_1 domain may present at 72 kDa site, and the subunit B of V_1 domain may present at 55 kDa site [17]. From the results of Western blot, shown in Fig. 6a and 6b, Rav1p is not stable, can be degraded to 6 fragments, respectively with 6 bands in Fig. 6b. So the band at 95 kDa site can be a fragment of Rav1p or be a other protein. To confirming this sequencing of protein band is necessary. In Fig. 6c, it is showed Rav2p is stable and easy digested, this is sensible. Because the expression of Rav1p is toxic to yeast [26], so degradation of Rav1p may occur due to it is protease enzyme in cytoplasm of yeast.

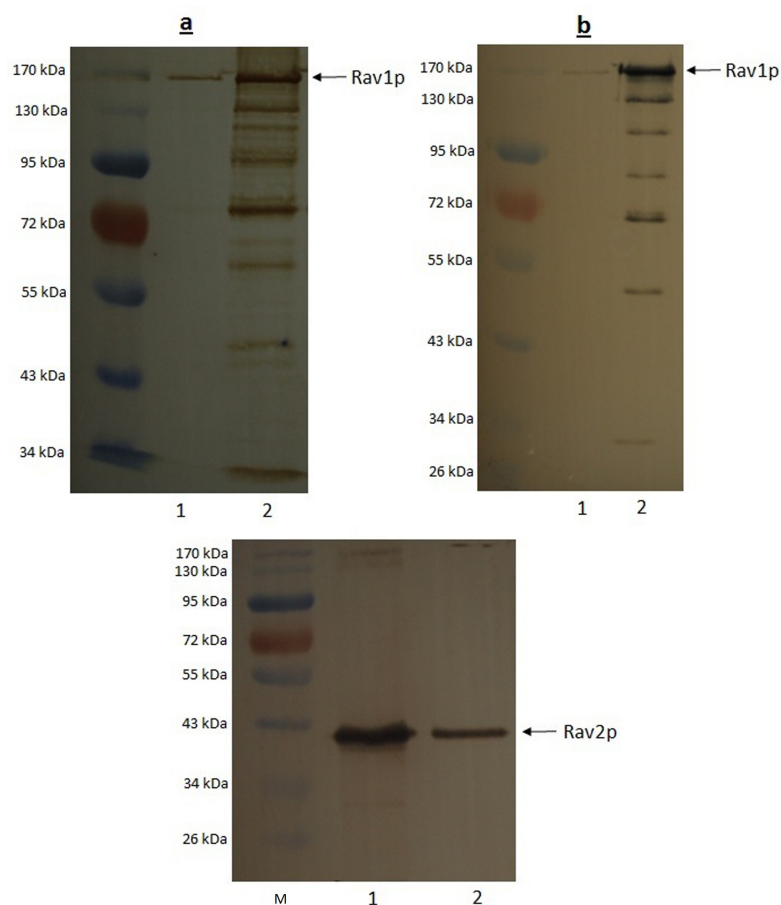


Figure 6. Western blots of extracted proteins.

(a) Western blot of RAVE complex extracted from BY4742 *FLAG-RAV1* with FLAG tag fused to N-terminus of Rav1. Land 1: Fraction 1 of RAVE complex elution. Land 2: Fraction 2 of RAVE complex elution. Land M: PageRuler Prestained Protein Ladder. (b) Western blot of RAVE complex extracted from BY4742 *RAV1-FLAG* with FLAG tag fused to C-terminus of Rav1. Land 1: Fraction 1 of RAVE complex elution. Land 2: Fraction 2 of RAVE complex elution. (c) Western blot of RAVE complex extracted from BY4742 *RAV2-FLAG* with FLAG tag fused to C-terminus of Rav2. Land 1: Fraction 1 of RAVE complex elution. Land 2: Fraction 2 of RAVE complex elution.

4. CONCLUSION

The purification of RAVE complex is not only supports 3-D structure determination but also supports for *in vitro* or *in vivo* studies of V-ATPase regulation. So it necessary removes all other proteins out of RAVE complex. To perform this, we should find some condition to release them to RAVE complex. Thus that investigation can helps us understand more about the interaction of RAVE complex and subunits of V-ATPase.

Acknowledgements. The fund was supplied by the National Natural Science Foundation (30800018); the National Natural Science Foundation (30970058); Doctoral Program of Higher Specialized Research Fund

(200802951036); the Industrial Biotechnology, Ministry of Education Key Laboratory Director Fund (KLIB-ZR200801).

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TÓM TẮT

TINH SẠCH PHỨC HỢP RAVE TỪ *SACCHAROMYCES CEREVISIAE* BẰNG VIỆC SỬ DỤNG FLAG TAG

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RAVE (Regulator of the H⁺-ATPase of the Vacuolar and Endosomal membranes) là một nhân tố cần thiết cho quá trình lắp ráp và tái lắp ráp của V-ATPase. Phức hợp RAVE có ba tiểu đơn vị đó là: Rav1p, Rav2p và Skp1p. Hiện nay có rất ít những nghiên cứu về phức hợp RAVE.

Vì vậy việc nghiên cứu cấu trúc của phức hợp RAVE là rất quan trọng để hiểu rõ về quá trình điều hòa sự lắp ráp cũng như là tái lắp ráp của V-ATPase. Trong nghiên cứu này, phức hợp RAVE được tinh sạch bằng ái lực, dựa vào việc gắn FLAG tag vào tiểu đơn vị Rav1p hoặc là Rav2p. Quá trình thí nghiệm: Tế bào nấm men được nuôi trong 8 lít môi trường lỏng YEPD ở 30 °C, lắc ở 200 rpm, cho đến khi OD_{600nm} khoảng 3. Sau đó tế bào sẽ được phá vỡ bằng thiết bị French pressure cell disruptor ở 25000 p.s.i trong đệm TBSE (50 mM Tris/Cl, 150 mM NaCl, 1 mM EDTA, pH 7,4) với 1 mM PMSF. Dịch tế bào sau khi được phá vỡ sẽ được ly tâm ở 20000 xg trong 20 phút ở 4 °C. Tiếp theo, toàn bộ dịch nổi được chạy qua cột sắc ký có chứa 1 ml anti-FLAG M2 gel. Sau khi rửa cột anti-FLAG với đệm TBSE, phức hợp RAVE sẽ được rửa giải với đệm TBSE có chứa 100 µg/ml FLAG peptides. Kết quả thu được chỉ ra rằng, việc tinh sạch phức hợp RAVE bằng cách gắn FLAG tag vào đầu C của dưới đơn vị Rav2 từ nấm men *S. cerevisiae* tốt hơn là khi tinh sạch phức hợp RAVE bằng cách gắn FLAG tag vào đầu N hoặc đầu C của dưới đơn vị Rav1.

Từ khóa: phức hợp RAVE, Rav1, Rav2, Skp1, V-ATPase, nấm men, tinh sạch protein.