Purification of Reiske Iron-Sulfur Protein from Beef Mitochondrial bc_1 complex

Elise Huang
Department of Molecular Cell Biology, University of California, Berkeley

Instructor: Dr. Edward Berry, Dr. Li-shar Huang.
Department of Physical bioscience, LBNL.

Sponsor: Prof. Robert Glaeser
Department of Molecular Cell Biology, University of California, Berkeley

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Introduction

The iron-sulfur proteins (ISP) are a very diverse class of Redox proteins which have an iron-sulfur cluster as the cofactor. Examples are known with one, two, three and four irons in the cluster. The redox potential varies from strong enough to reduce NAD+ to NADH (-350 mv) to quite weak reducing agents like the Rieske ISP (RISP) which reversibly reduces cytochrome c1 (+250 mv).

The Rieske [2Fe-2S] protein is part of the membrane bound bc_1 complex. Figure 1. illustrate the position of RISP in bc_1 complex. bc_1 complex (complex III in Figure 2) participates in aerobic mitochondrial and bacterial respiratory chains, and also in photoredox chains (called b_6f complex). bc_1 complex is also involved in denitrification. bc_1 complex couples hydroquinone oxidation to proton-motive force across membrane, giving rise to membrane potential for cellular metabolism. The function of RISP is to catalyze the one-electron oxidation of hydroquinone to semiquinone anion via cytochrome b, after which the electron is transferred to cytochrome c_1 then the soluble cytochrome c.

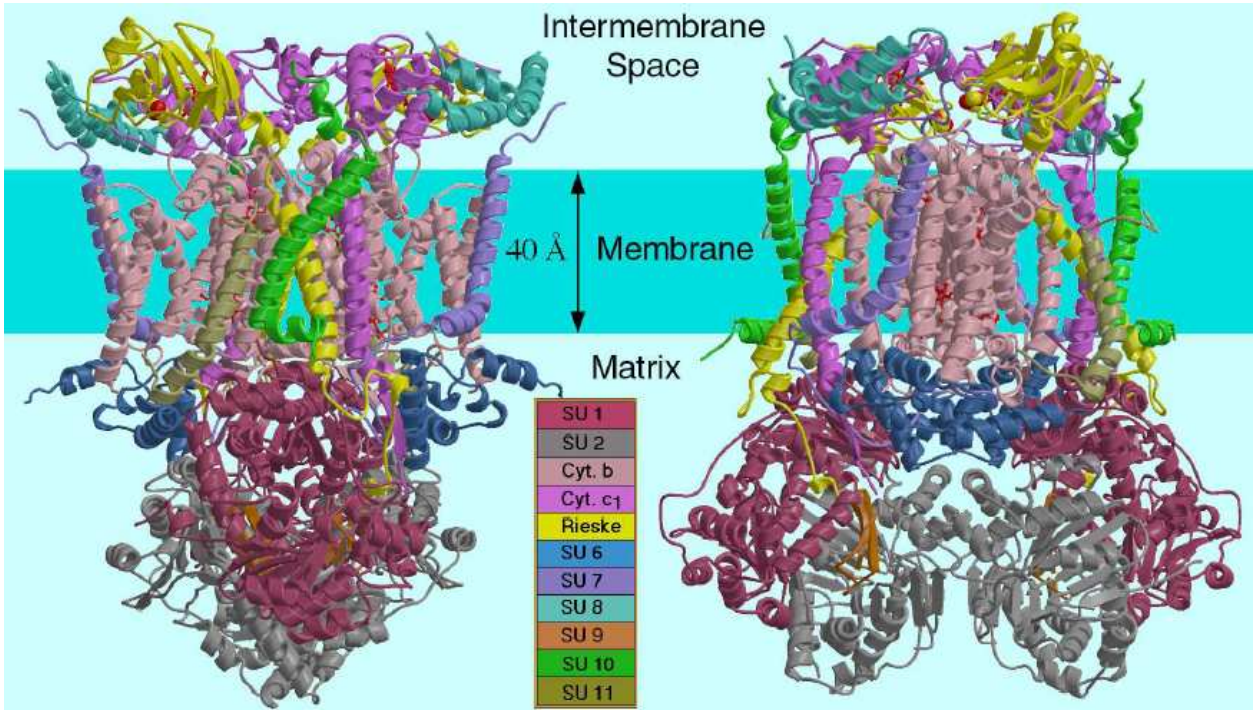

Figure 1. Mitochondrial bc_1 complex. Rieske Iron-sulfur proteins are the yellow subunits.

To understand how Rieske ISP tunes the redox potential to fit the application at hand, it is necessary to understand the electronic structure of the cluster. This can be studied by a number of spectroscopic techniques such as XAFS, Mossbauer, and EPR (iron sulfur clusters generally have an unpaired electron in at least one of their redox states). The reduced form of Reiske ISP (Fe3/Fe2) is paramagnetic and the oxidized (Fe3/Fe3) is not. The oxidized form is electrically neutral, with the 2 Fe+3 charges balanced by one negative charge on each of two cysteinate ligands and two negative charges on each of two inorgainc sulfide ions in the cluster.

In addition to information about the electronic structure of the cluster, spectroscopy can provide information about the orientation of the cluster, and hence the protein bearing it, in oriented samples. EPR spectroscopy of oriented films or single crystals can determine the

orientation of the g-tensor relative to the plane of the membrane or the crystallographic axes. In order to translate this into orientation about the cluster or the protein bearing it, it is necessary to know the orientation of the tensor relative to the interatomic vectors of the cluster. This was explored in a recent paper using single crystals of the bovine bc_1 complex. Although a reasonable conclusion was reached, the results were not very satisfactory. In order to fit the data it was necessary to assume that the answer was significantly different for the two monomers in the asymmetric unit. Only one crystal was used, so the reproducibility is unknown.

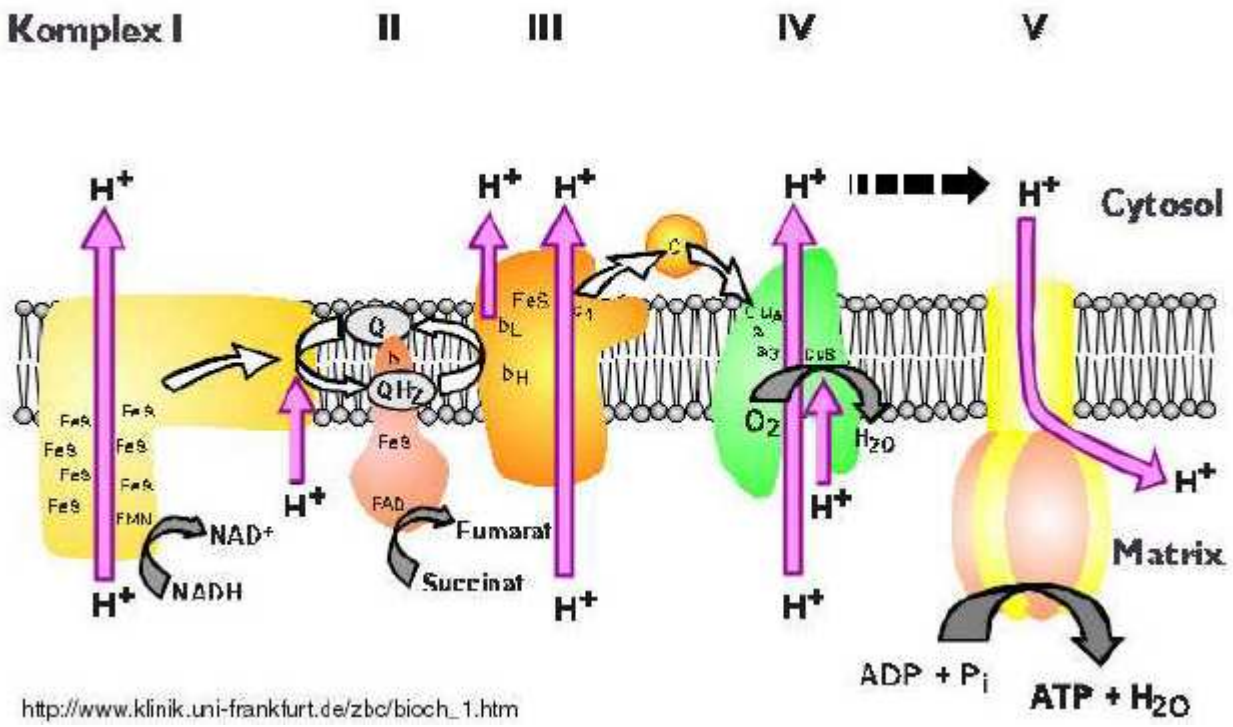

Figure 2. Mitochondrial electron transfer chain membrane protein complexes.

High-resolution X-ray crystallography can also help elucidate the electronic structure of the complex, by obtaining accurate bond lengths and angles (double bonds are shorter than single bonds and so on) and by determining the protonation state of ionizable groups. In addition obtaining crystals of the ISP by itself and determining the crystal structure would allow testing the g-tensor result with a much higher signal to noise ratio- the RISP is about 25 kDa vs. 250 kDa for the bc1 complex, so a crystal the same size would contain roughly ten times as many ISP clusters.

To this end we are aiming to purify and crystallize soluble fragments of several RISP proteins from bc1 complexes. The soluble fragment of RISP contains the catalytic site for redox reaction, which can be obtained by cleavage between the soluble module and the transmembrane hydrophobic helix (available to trypsin, chymotrypsin, and thermolysin). In the case of the bovine bc1 complex and the chloroplast b_6f complex, this has been achieved by other groups, resulting in structures with protein data bank entries 1RIE (bovine) and 1RFS (spinach). For a starter, we are adapting the methods used for the bovine protein to work with our material. After this is done we will extend it to the RISP of Rhodobacter capsulatus, a photosynthetic bacterium, for which there is no high resolution structure available.

Materials and Methods

bc_1 complex purification

bc_1 complex was purified from beef heart mitochondrial membrane. Homogenized beef heart in 50mM K_3PO_4 (pH 7.5), 0.05% Triton, 260mM NaCl, and 0.1 g/L DM was centrifuged at 11.5 krpm for 1 hour. The supernatant was kept for later use. The pellet was resuspended with the same buffer and incubated for 20 minutes. Resolublized pellet was concentrifuged under the same condition. The supernatant, combined with that from the previous centrifugation, was fed into Phenyl Sepharose CL-6B DEAE column. The column was washed with the same buffer, and rewashed with the flow-through. Gradient made of the same buffer and 500mM K_3PO_4 (pH 7.5), 0.05% Triton, 100mM NaCl, and 0.1 g/L DM was used to elute bc_1 complex, which was concentrated using YM-100 membrane. The same chromatography procedure was repeated again with the eluted bc_1 complex to increase the purity.

RISP purification

Eluted bc_1 complex solution was spun down to resuspend the pellet in equilibration/wash buffer of 200mM NaCl, 20% glycerol, 25mM Tris-HCl (pH 8.0), 1mM DTT, 0.25% sodium deoxycholate, and 1mM EDTA. Phenyl Sepharose CL-4B hydrophobic column (0.5 x 32 cm) was equilibrated and washed with the buffer. RISP and core proteins were eluted separately with salt gradient made of washing buffer and buffer containing 25mM Tris-HCL (pH 8.0), 1mM DTT, 1% sodium deoxycholate, and 20% glycerol. The CL-4B column is regenerated with 10mM NaCl and 0.5% Triton.

RISP proteolysis

Eluted RISP solution was buffer-exchanged with 20mM MOPS (pH 7.5), 100mM NaCl, and 0.5mM EDTA via membrane centrifugation. Time-course proteolysis of RISP was performed with thermolysin and trypsin separately at room temperature. In the use of thermolysin, additional calcium ions were added to balance off present EDTA, which was later used as reaction stopping reagent. In trypsin proteolysis, PMSF was used as reaction inhibitor.

Purification of soluble RISP fragment

AcA44 gel filtration column (0.5 x 48cm) was connected to HPLC instrument and equilibrated with 20mM MOPS (pH 7.5), 100mM NaCl, and 0.5mM EDTA. Proteolyzed RISP was injected onto AcA44 column. Fractions containing RISP fragment were concentrated for crystallization experiment.

Results and Discussion

Proteolysis-choice of starting material and protease

We attempted to directly digest bc_1 complex with chymotrypsin or thermolysin. The reaction ratio was chymotrypsin: bc_1 complex = 1.5: 50 (w/w), or thermolysin: bc_1 complex = 1:50 (w/w). Results on SDS-PAGE gel indicated incomplete digestion after 3 hours of incubation. Higher protease concentration was needed to efficiency attack RISP, which constituted only 10% of bc_1 complex. Comparison of gel results showed chymotrypsin as a better candidate for proteolysis due to its faster action. See Figure 3 & 4 for details. From literature, cut RISP fragment could come in sizes of 18 kDa (V_o), 20 kDa (V''), and 23 kDa (V'). However, we could not determine which band below RISP band was our proteolyzed product; the bands could be multiple-cut fragments from core proteins or cytochromes. Antibody specific to RISP or protein sequencing was needed to reveal the band identity.

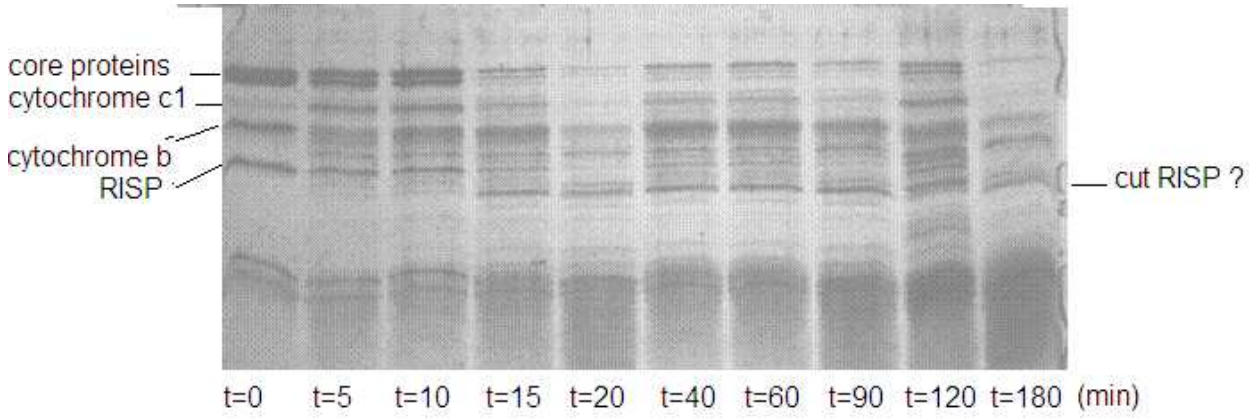

Figure 3. Time course proteolysis of bc_1 complex with chymotrypsin.

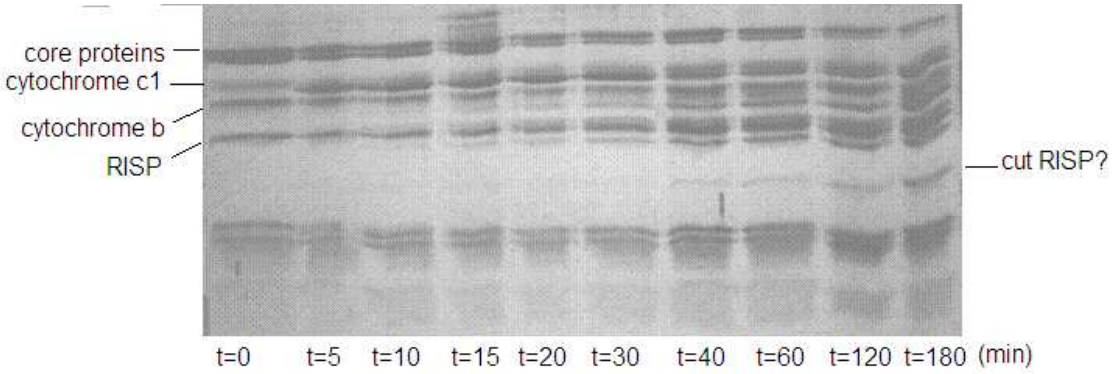

Figure 4. Time course proteolysis of bc_1 complex with thermolysin.

Proteolyzed bc_1 complex was run through 2cm long CL-6B DEAE column to separate RISP fragment. Figure 5. illustrates the co-elution of RISP with core proteins. Uncut RISP was washed out due to its hydrophobic tail that did not adhere to charged resins. A step gradient from 260mM NaCl to 500mM NaCl was used in elution.

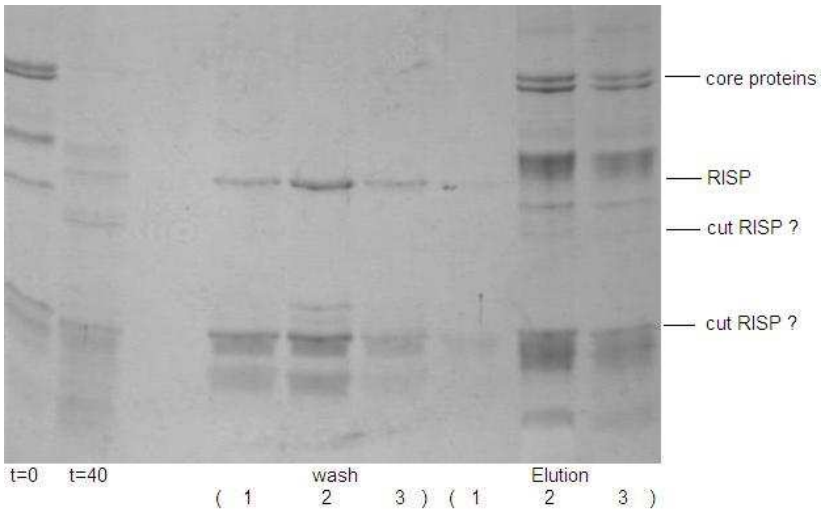

Figure 5. Column profile of proteolyzed bc_1 complex through CL-6B DEAE column.

Density gradient was proposed to extract RISP from the proteolysis solution. The density gradient was made of 20mM Tris-HCl (pH 7.5), 100mM NaCl, 0.5mM EDTA, 0.5 g/L DM, and with 20% and 40% glycerol (w/v). The reaction mixture was spun through the density gradient at 50 krpm for 18 hours. Fractions were pulled from bottom to top of the density gradient. Those near the top of density gradient contained protein fragments of expected RISP size (Figure 6.) UV-visible spectrums of the RISP-presence fractions showed dominating signals from cytochrome c, which likely had masked the RISP.

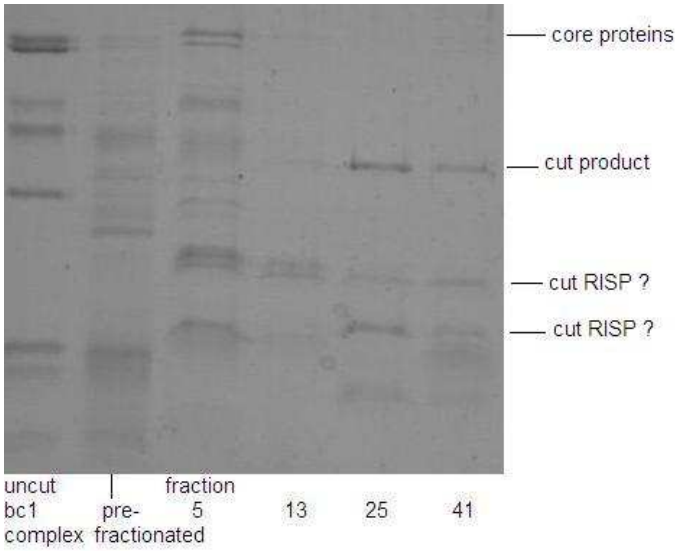

Figure 6. Fractions from density gradient separation of proteolyzed bc_1 complex with chymotrypsin.

Main difficulty in using bc_1 complex as starting material was that proteolysis produced fragments unrecognizable for RISP without protein sequence information. Presence of cytochrome c made UV-visible spectroscopy unavailable for RISP determination. Uncertainty overrode the urge for further purification; there is a need to acquire RISP as starting material for crystallography study.

Purification of RISP

bc_1 complex was run through the CL-4B hydrophobic column, which was prepared with 4 column volume of equilibration/wash buffer. Step gradient of low salt elution buffer co-eluted core proteins and RISP (Figure 7). Longer wash was needed to delipidize bc_1 complex so that RISP could come out of the complex and attach to the resin via its hydrophobic tail. The long wash could also enhance the binding of RISP while other subunits were washed away. UV-visible spectrum indicated presence of cytochrome c masking RISP signal. This is determined by increased absorbance after additional of oxidizing agent Ferricyanide (Figure 8). RISP alone would only yield an increased absorbance peak at 420nm rather than 420nm, 520nm, and 550nm, later of which were characteristics of cytochrome c and b_1. To remove cytochrome c and b_1, the RISP fractions were passed through CL-6B DEAE. Elution still contained residual cytochromes after testing on US-visible spectrometer.

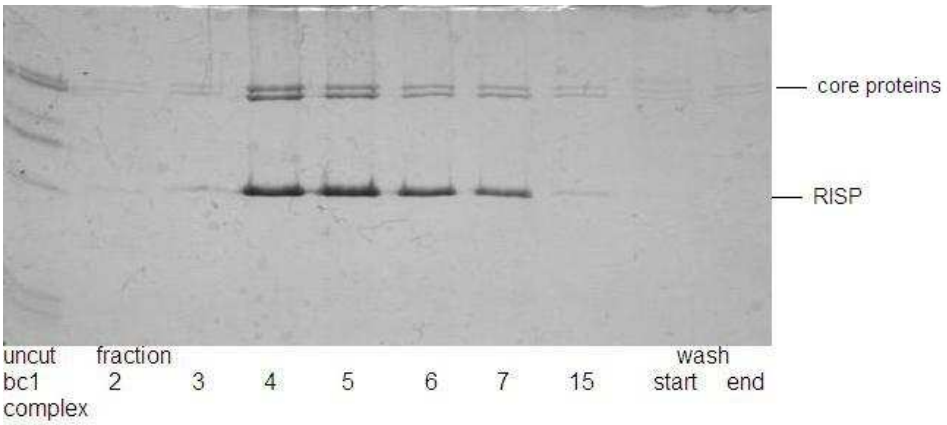

Figure 7. Elution of CL-4B by step gradient.

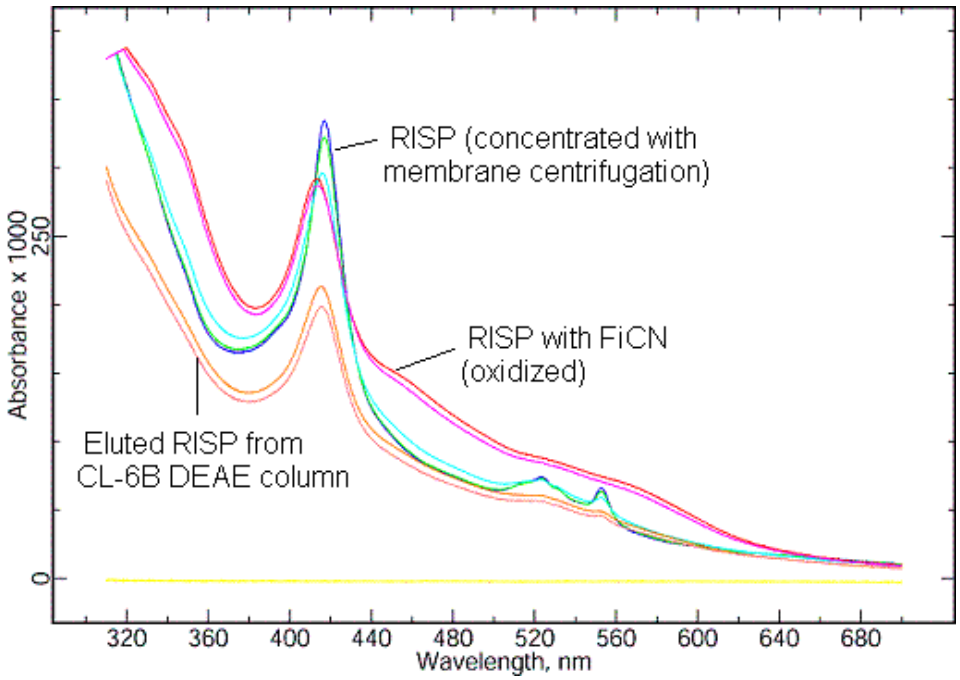

Figure 8. Spectrum of step-gradient eluted fraction.

To extract RISP, 20-40% glycerol density gradient separation was applied to recollected and concentrated fractions. SDS-PAGE showed longer operation time was needed to increase separation (Figure 9) of RISP from core proteins. Proceeding with pooled fractions containing RISP, proteolysis using trypsin was performed. The choice of protease was made due to its common use in literature. SDS-PAGE results showed uncut RISP bands. Perhaps trypsin-acting sites were not accessible. Another trypsinization on the same sample was done, tweaking the limit of proteolysis. Turbid white cloud emerged during proteolysis, resulting in high viscosity that obstructed time course sampling. Examination of the solution under microscope showed needle and brown cube crystals (Figure 10). Artifact of PMSF was eliminated by comparison of PMSF crystals. Ethanol presented in PMSF stopping reagent could have served as a catalyst of crystallization, forcing proteins to aggregate in an orderly manner. RISP of high purity was brownish yellow, and there grain-like crystals were of the same color. We suspected that there were RISP and core protein crystals. The crystals were stored for future crystallography analysis in available beam time.

The turbid reaction mixture was spun down, analysis of which showed RISP presented in pellet. Aggregation of deoxycholate could be a possible reason of this phenomenon due to its observed solidification at cold temperature. The amyloid could have trapped RISP via hydrophobic interaction with RISP tail. Buffer-exchange would be needed before proteolysis if this was the case. Fresh working material, RISP, was needed.

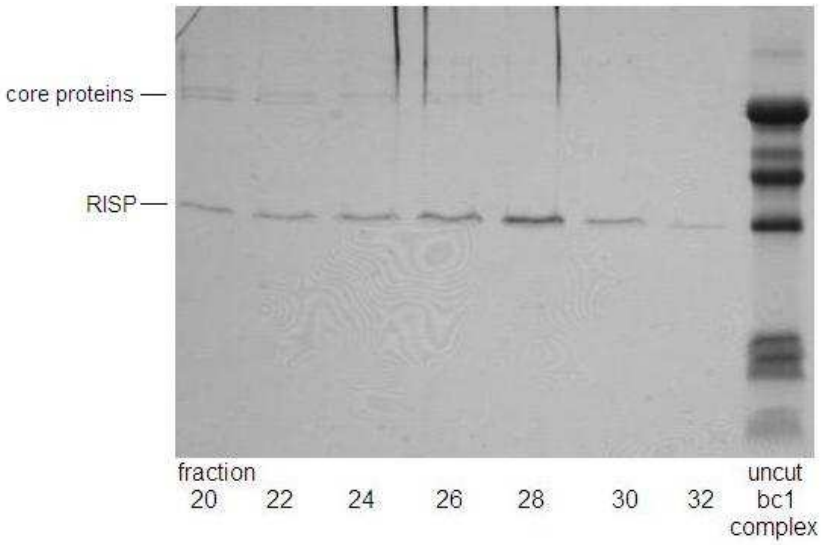

Figure 9. Glycerol density separation of CL-4B elute.

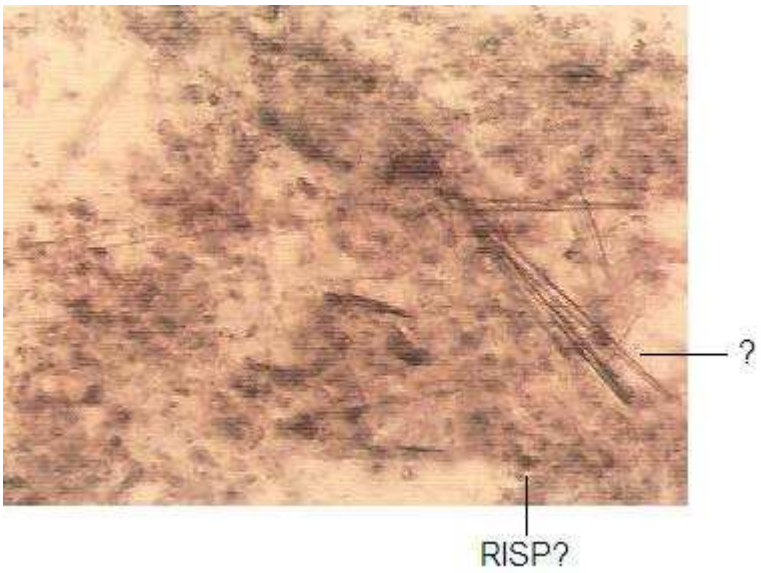

Figure 10. Needle and cube crystals from turbid proteolysis solution.

Salt gradient was proposed to elute RISP apart from core proteins from CL-4B column. SDS-PAGE of pooled fractions in early and late elution phases revealed good separation of RISP from core proteins (Figure 11). The core protein, seemly of high purity, was dialyzed and applied in crystallization. RISP fractions were concentrated for later proteolysis experiments.

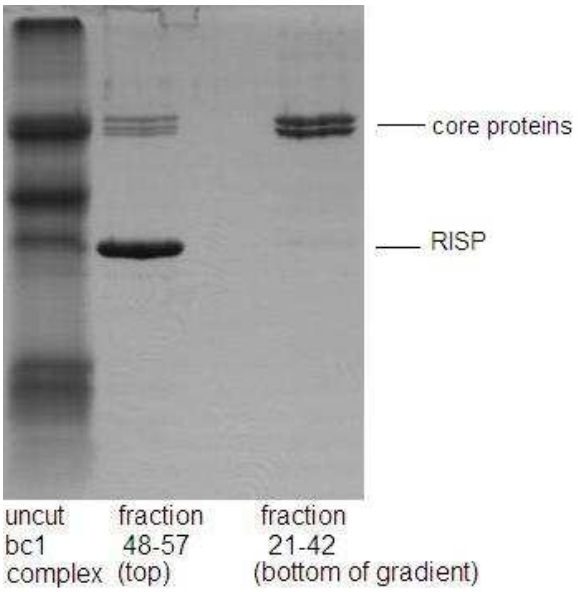

Figure 11. Pooled fractions from density gradient separation of RISP and core proteins.

RISP solution was exchanged with buffer (see *Methods*) and proteolyzed with thermolysin (5 g/L). Turbidity of high viscosity emerged again, which was directly analyzed on SDS-PAGE gel (Figure 12). Smears of continuous protein bands may be caused by amyloids which perhaps were made of RISP hydrophobic tails instead of deoxycholate. To test the presence of amyloid, the sample was run through AcA44 gel filtration column, which yielded only one peak in addition to known references. The peak's relative position in the column profile indicated its

composition RISP fragment. Another test, using Triton X-100 was done; the column profile revealed a semi-gausian distribution of various micelle sizes. If amyloid hypothesis was true, various sizes of micelle should have been detected. Detailed investigation would be needed to determine the smear.

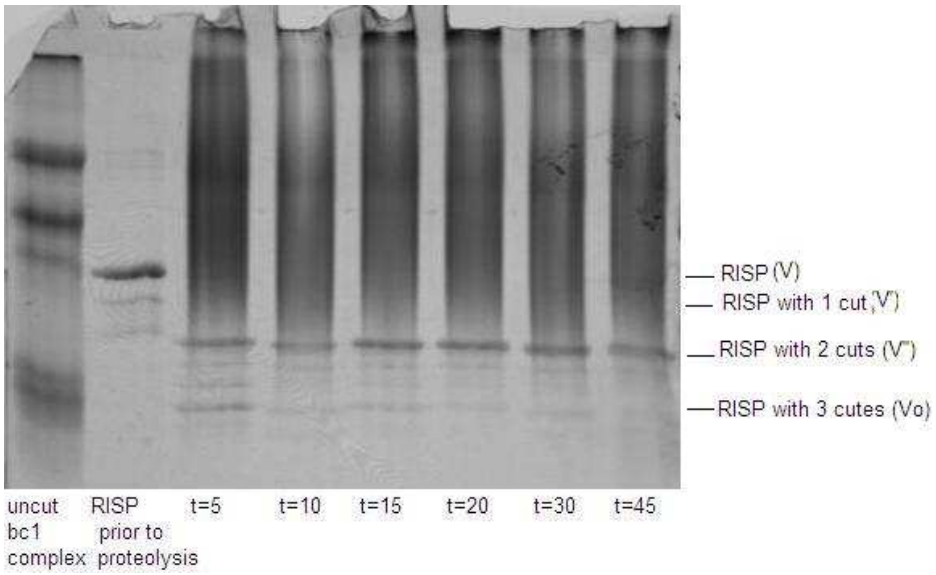

Figure 12. Results of RISP proteolysis.

In the attempt to avoid formation of possible amyloids of RISP tails by providing purer sample for proteolysis, we passed the RISP, obtained from CL-4B column, through AcA44 gel filtration column. SDS-PAGE gel of the fractions showed separation of cytochromes from RISP (Figure 13). The UV-visible spectrum indicated high purity of RISP from this preparation (Figure 14). Proteolysis of pooled RISP fractions can yield high purity of soluble RISP fragments, the purification of which will be achieved with AcA44 gel filtration column. Crystallization of soluble RISP fragment is close.

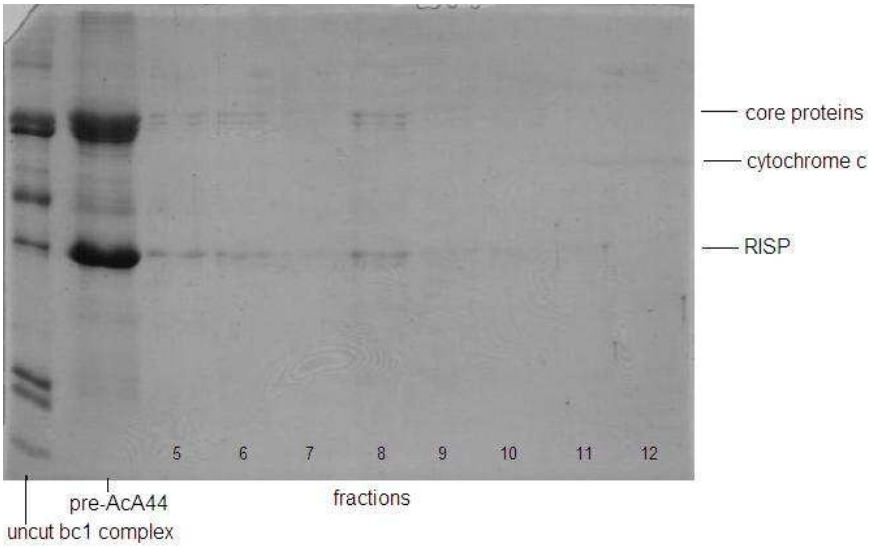

Figure 13. Fractions collected from AcA44 column.

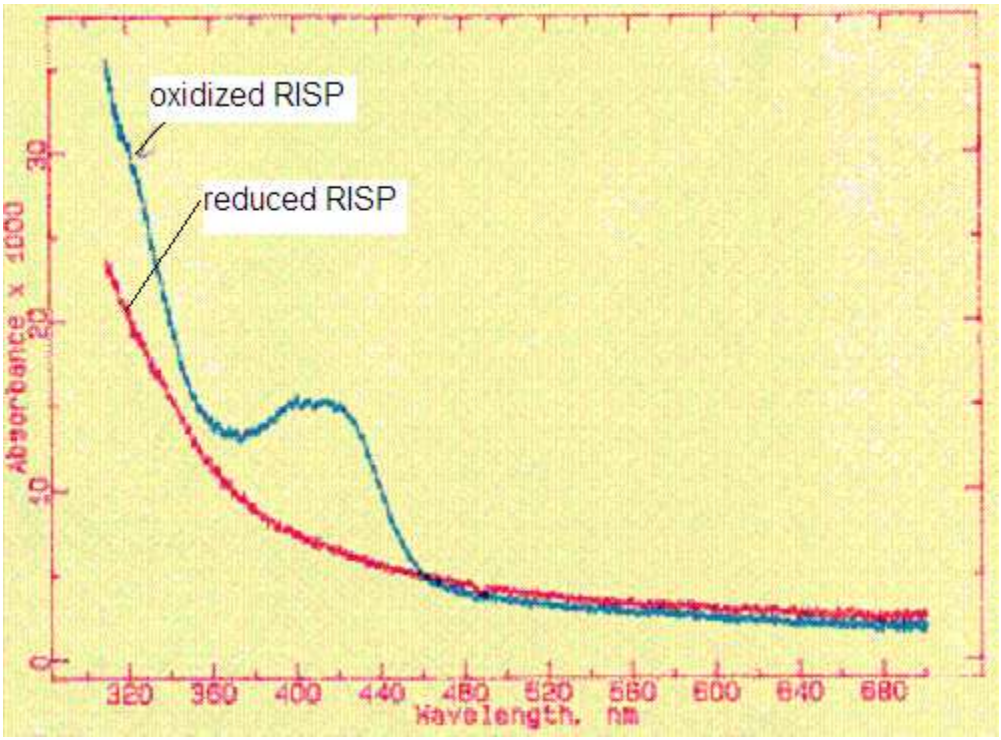

Figure 14. UV-vis spectrum of RISP from AcA44 column.

For future experiments, purity of RISP prior to proteolysis is important to obtain high-grade soluble RISP fragment for crystallization. Long equilibration with CL-4B buffer is essential to achieve ideal separation of RISP from core proteins. If AcA44 gel filtration column is to be bypassed before proteolysis, information regarding cytochrome cut sites is needed to assess the separation extent of AcA44 column afterwards. Detailed structures of core proteins are not yet available, and the high purity core proteins, as a by-product from RISP purification, can be a potential crystallography project.

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