

Cheiron School BL Practice of BL38B1 (Protein Crystallography)

Title:

Data Collection and S-SAD Phasing of Insulin Crystals

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Abstract:

Phase problem is a major difficulty in protein crystallography. As a recent advancement, S-SAD phasing method has been proposed. It solves the problem by the anomalous effect of sulfur atoms involved in most protein samples. In this exercise, we plan to the data collection of insulin crystal and the determination of its structure. The alignment of the beamline and a sample mounting robot will also be introduced.

Contents:

1. Brief Introduction

Energy range 6 ~ 17.5 keV

Energy resolution $\Delta E/E = 10^{-4}$

Spot size horizontally 250 μm , vertically 200 μm (@ 12.4 keV ($\lambda = 1.0 \text{ \AA}$)) at detector position



Fig 1. BL38B1. (Left) Inside of experimental hutch. (Right) Outside. Operators desks and computers are located.

1.1. Optical Components

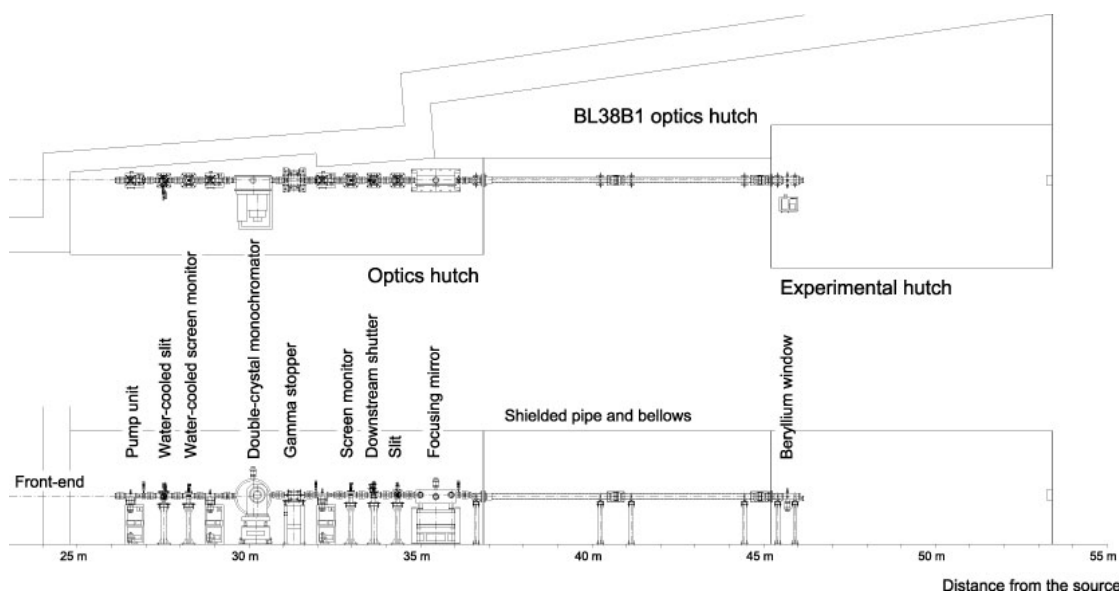


Fig. 2: Schematic View of Beamline

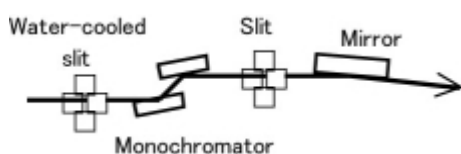


Fig. 3: Outline of Optics

1.2. Experimental Station

Rapid data collection system for protein crystallography is installed at BL38B1. Two detectors, CCD (Rigaku/MSJ Jupiter 210) and imaging plate (RIGAKU RAXIS-V), are equipped on the experimental table and users can easily change these detectors depending on their experimental purpose. Combination of the coaxial-microscope to observe crystal along x-ray beam path and the goniometer having motor driven x, y and z stage make centering of crystal very easy. Data collection, including measurement of XAFS spectrum and automatic MAD (Multi-wavelength Anomalous Diffraction) data collection, is performed through controlling software BSS (Beamline Scheduling Software). Since BSS can control all of the instruments required for data collection, switch of detector and change of camera length, wavelength are automatically performed at the beginning of measurement. Therefore, what beamline users have to do after crystal mounting and centering is to input measurement conditions to BSS.

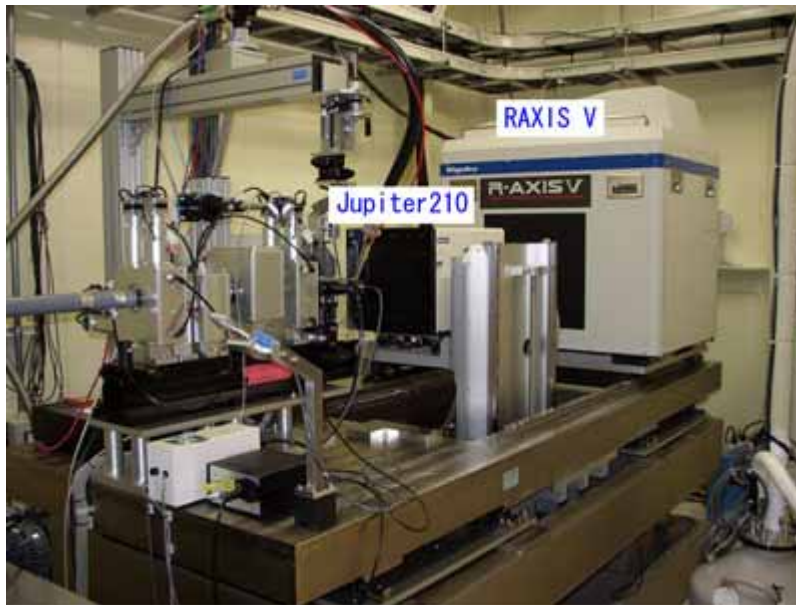


Fig. 4. Diffractometer for protein crystallography
(a : Rigaku/MSK Jupiter210, b : RIGAKU RAXIS-V)

When RAXIS-V is used, the CCD stage goes down into the experimental table and the IP stage can move toward the goniometer without any physical interference

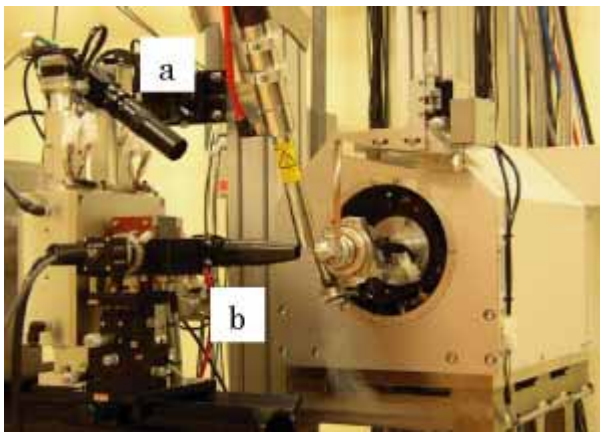


Fig.5. Magnified view around the goniometer
Two microscopes are used for crystal centering.
(a) low magnification microscope
(b) high magnification coaxial-microscope

1.3. Specification of beamline equipments

* CCD detector (Jupiter 210 (Rigaku / MSC Corporation))

Detector area : $210 \times 210 \text{ mm}^2$

Pixel size : $51.27 \times 51.27 \text{ }\mu\text{m}^2$

No. of pixels : 4096×4096

Camera length : 150 ~ 330 mm

* Imaging plate detector (RIGAKU RAXIS-V)

Detector area : $400 \times 400 \text{ mm}^2$

Pixel size : $100 \times 100 \text{ }\mu\text{m}^2$

No. of pixels : 4000×4000

Camera length : 170 ~ 1000 mm

* Horizontal axis goniometer having motor-driven x, y, z stage

* Cryostat : temperature control range 80 ~ 350 K

* Gas-flow type ionization chamber

* Si PIN photodiode detector for fluorescence measurement

* Multi-channel analyzer of fluorescence measurement

* Control Software BSS

* Sample Automated Changer



Fig. 6: Sample Auto Change SPACE

2. Optical Alignment and Adjustment

2.1. Control Software BSS

The software of BSS (Beamline scheduling software) can deal with all of the beamline operation as follows: 1) Control the optical and experimental components as monochromator, goniometer, and detector. 2) Data collection of crystal diffraction, X-ray fluorescence etc.

The GUIs of the software is as follows:

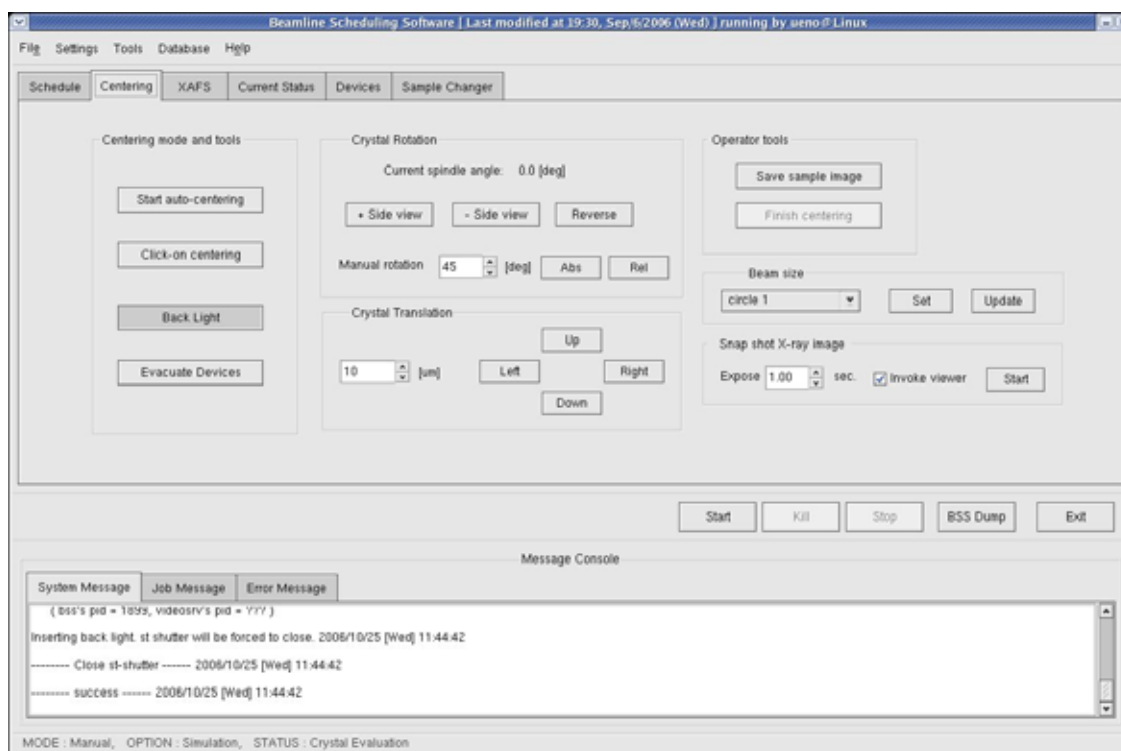


Fig. 7: BSS Crystal Centering Tab

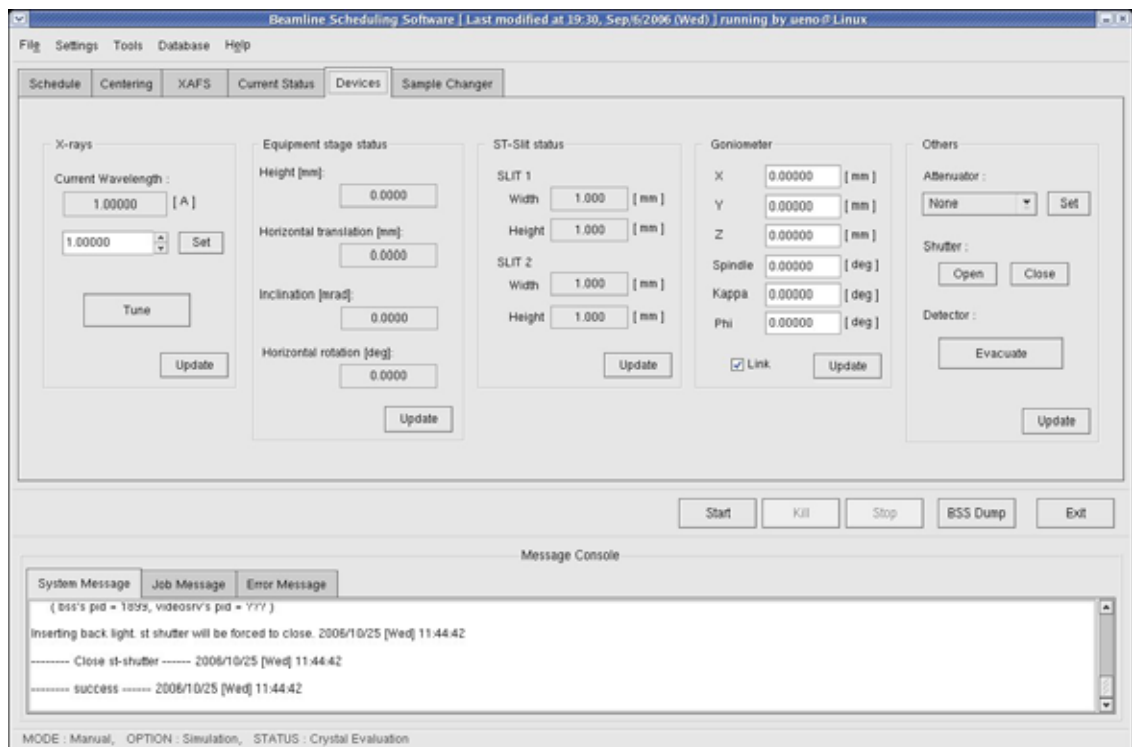


Fig. 8: BSS Device Control Tab

2.2. Operations of Beam Alignment

- 2.2.1. To confirm and define the rotational center of the goniometer head at the window of the sample monitor software using a sample mount Lithographic loop. If the position is not located at the center of the display, the position should be aligned by the micrometer of the sample monitor CCD camera. The cross of the center position can also be aligned by the software definition of the video capture software.
- 2.2.2. BSS Run Option is set to "Admin" mode.
- 2.2.3. Set the spindle axis to 0 degree.
- 2.2.4. Put the fluorescent plate on the goniometer head. The detector cover should be placed.
- 2.2.5. Focalize the sample monitor CCD camera with the stl_gonio_1_x axis of Axis Tools.
- 2.2.6. Turn off the sample illuminator.
- 2.2.7. Open X-ray shutter and the wavelength should be adjusted to 1 A.
- 2.2.8. Record the intensity value of the X-ray at 1A.
- 2.2.9. Display the window of monochromator on BL-Work Station. Record the position of $\Delta\theta_1$ axis.
- 2.2.10. The size of two slits (ST1/ST2) is set to 3 mm by Axis Tools.
- 2.2.11. Scanning of $\Delta\theta_1$ with Energy tab. Record the value of I_0 , the position of the beam, and the shift of $\Delta\theta_1$.

- 2.2.12. Beam position should be aligned to the rotational center of the goniometer head by the movement of the experimental stage with opened slits. In Z axis direction (height), beam position should be placed on the spindle axis. In Y axis direction (horizontal), the position should be the central cross of the video monitor.
- 2.2.13. Return the slit width. The width of ST1 and ST2 are 0.5/0.3mm and 0.4/0.4mm, respectively. Record the value of I_0 .
- 2.2.14. Do the $\Delta\theta_1$ scanning.
- 2.2.15. BSS Run Option is set to "None" mode.

3. Data Collection and Phasing of Insulin Crystals

3.1. Theoretical background of SAD phasing

3.1.1. Anomalous effect

Thomson scattering occurs the following scheme: 1) X-ray photon strikes a free electron, 2) the electromagnetic field of the incident oscillates the electron. 3) the oscillated electron scatters X-ray with the same wavelength as the incident X-ray.

However, the electrons of atoms are bound tightly and its oscillation has a certain resonance frequency. Therefore the scattering factor is depends on the incident wavelength. It can be expressed as the following formula.

$$f = f_o + f' + if''$$

In the case of sulfur atom, the wavelength dependent f' and f'' terms are illustrated as following graphs.

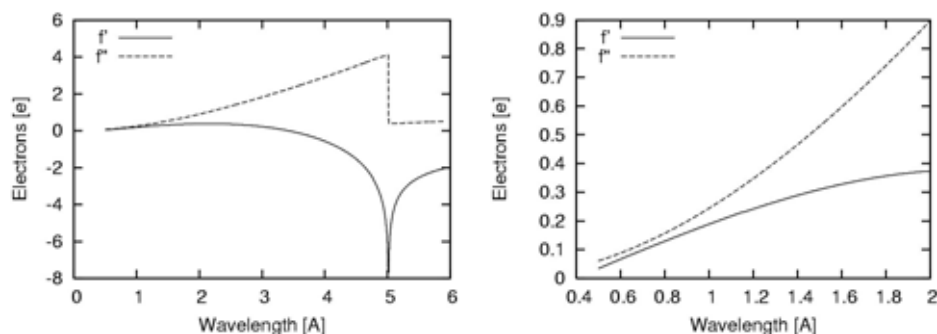


Fig. 9. Anomalous scattering terms of sulfur atom. Calculated by CCP4/Crossec.

3.1.2. Harker diagram and phase

Crystallographic $F(\mathbf{h})$ values (structure factors) are given by Fourier transformation of electron density $\rho(\mathbf{x})$. $F(\mathbf{h})$ value is a complex and composed of absolute $|F(\mathbf{h})|$ and

phase $a(\mathbf{h})$. Inverse Fourier transformation of $F(\mathbf{h})$ gives electron density of crystal, however $|F(\mathbf{h})|$ is only obtained from diffraction experiments. The phase $a(\mathbf{h})$ can be recovered from several experimental methods. Here we describe the scheme of SAD method.

Harker diagram (phase diagram) is a picture for understanding phase determination.

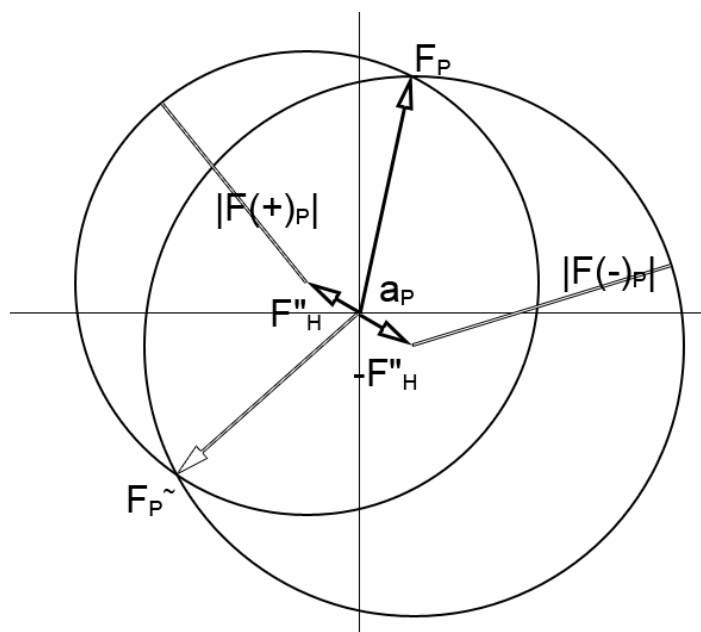


Fig 10: Harker diagram of SAD method.

Anomalous effect breaks down Friedel's Law where a reflection $F(\mathbf{h})$ and its centrosymmetric reflection $F(-\mathbf{h})$ have same value of $|F|$. ΔF , the difference of $F(\mathbf{h})$ and $F(-\mathbf{h})$ is a good approximation of the structure factor amplitude of anomalous scatterers. Therefore, Patterson method or direct phasing can extract the position of the anomalous scatterers.

Once the positions of anomalous scatterers are determined, their structure factor (\mathbf{F}''_H) is calculated. The vector of \mathbf{F}''_H and its negative can be drawn in the diagram. Next, we can draw two circles: one with radius of $|F(+)_P|$ centered on the end of \mathbf{F}''_H . another with radius $|F(-)_P|$ centered on the end of $-\mathbf{F}''_H$. We can see the two intersections F_P and $F_{P\sim}$ of the two circles. One of the two points is correct answer. Although the two possibilities cannot be distinguished without any information, we can obtain good phases by the treatment of electron density shape (density modification; solvent flattening etc.) in some cases.

3.2. Data Collection

3.2.1. Sample mounting on goniometer

1) Pick up a desired crystal by a cryoloop device: Firstly, a desired crystal is scooped with a cryoloop under a microscope. Protein crystal is mostly small and its typical dimension of 0.1~0.2 mm. Protein crystal is obtained from water solution, therefore it is easy to dry up under atmospheric condition and also very fragile. Scooping process should be conducted rapidly.

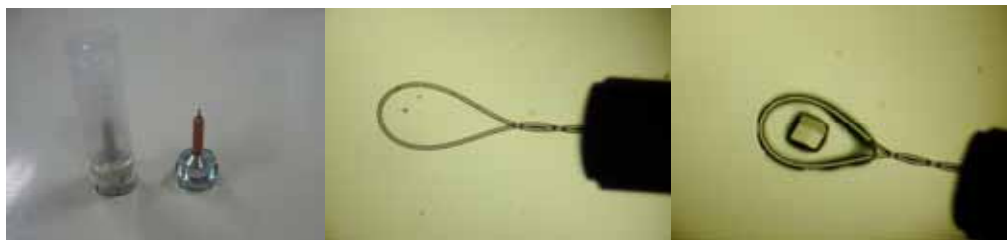


Fig. 10. Cryoloop. (Left) Cryoloop, (Middle) Nylon loop. The tip of the cryoloop. (Right) Cryoloop with a crystal.

2) Soaking the crystal into cryoprotectant: The scooped crystal is transferred into the solution containing cryoprotectant (typically it contains 30% glycerol) which prevents ice formation during sample cooling.

3) Picking the crystal again: To mount the crystal onto a goniometer head, the crystal is picked again. Since glycerol containing solutions are viscous, the excess of the solvent is sometime scooped onto the cryoloop. The excess causes unfavorable scattering which degrades the statistics of diffraction data. Since the anomalous signal for S-SAD phasing is weak, the solvent should be removed possibly.

4) Mounting and flush cooling the crystal: Immediately, the picked crystal should be put on the goniometer head with magnetic support. To cool the sample during X-ray irradiation and prevent the radiation damage of the sample, cold nitrogen gas stream is blowing at the crystal position. Before mounting the crystal, the blow is blocked out by the shutter system. And after mounting, the shutter should be immediately opened. This is 'flush cooling technique' for preventing ice formation in solvent and crystal.

3.2.2. Collection of diffraction images

1) Set up the data collection software: To take diffraction images, the program BSS is used at this beamline. Following two figures are the windows of data collection GUI.

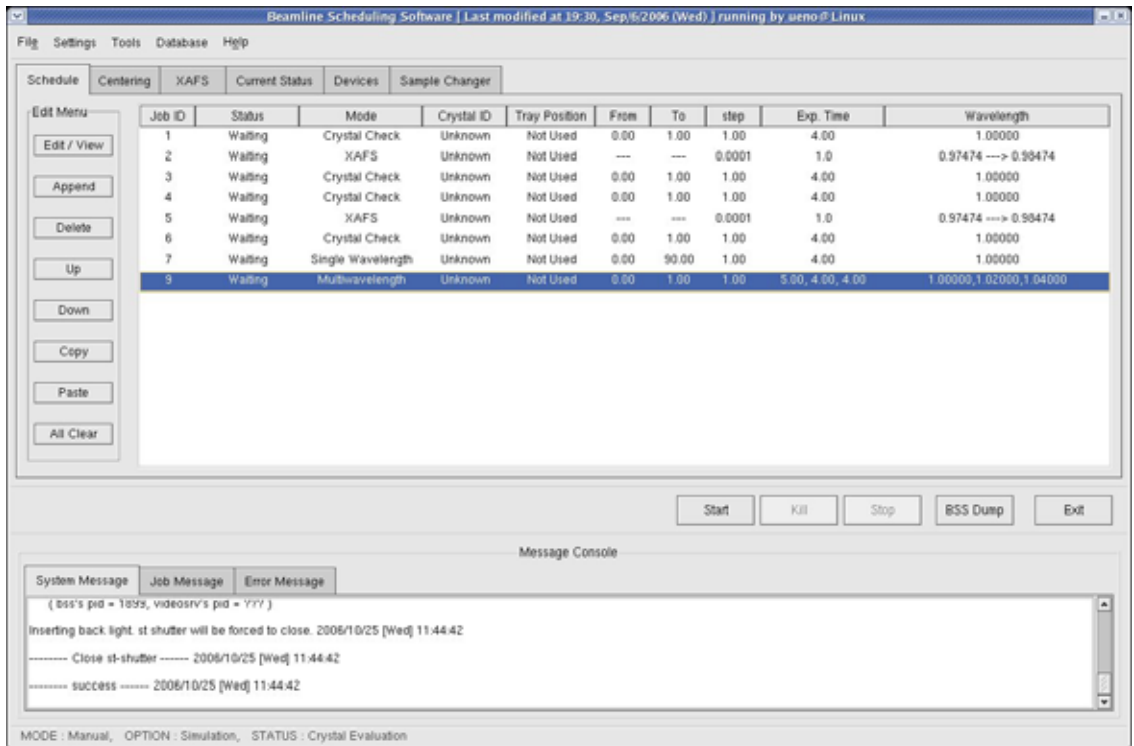


Fig. 11: Main window of BSS

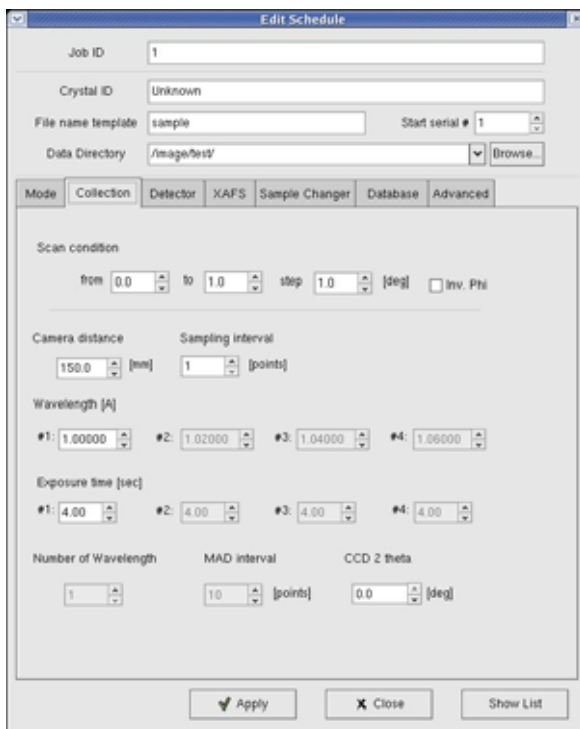


Fig. 12: The window for editing data collection condition.

2) Centering the sample to beam position: Diffraction images are taken by oscillation

method. In the method, crystal rotates and oscillates with the spindle axis of goniometer. To keep the crystal position during the rotation against X-ray beam, the crystal is aligned to the center of the spindle axis, because X-ray beam is already aligned to the spindle axis. This operation can be achieved in the program BSS using video camera and monitor.

3) Closing experimental hutch and opening down stream shutter of the beamline.

4) Take a few images and check the diffraction: To estimate the quality of the mounted crystal, one or two images should be taken before taking data set.

5) Take a full data set: When the crystal passes through the quality check, the consecutive series of diffraction images are taken. In most cases, 180 images will be taken if you do not have any information of the crystal as space group, cell constant etc. In the case of SAD, highly redundant data collection is required, because it makes the statistics better.

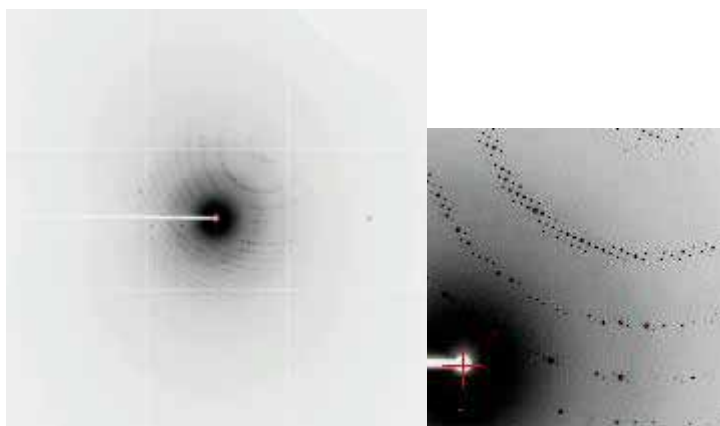


Fig. 13: A diffraction pattern of a protein crystal.

3.3. Data processing

Diffraction images are processed by a special computer program. Against the raw data of each image, the following process is performed: 1) the prediction of reflections (diffraction spots) through the determination of lattice constants and crystal orientation and the parameter-refinement of the camera-distance etc [indexing and prediction]. 2) the determination of reflection index and the estimation of its intensity [integration]

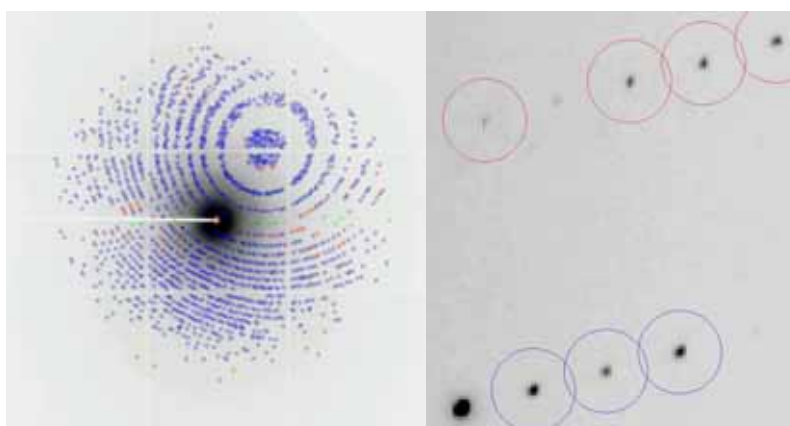


Fig: 14: Spot prediction

After obtaining the index and intensity of each reflection in all images, the intensity should be merged into reflections which are equivalent in crystallographic symmetry [scaling]. Finally we obtain F-data (containing hkl : Mirror index and $F(hkl)$: structure factor amplitude). In the case of SAD, Friedel mates $F(+)$ and $F(-)$ should be recorded independently.

3.4. Phase Calculation by SAD

There are several program packages for this purpose. In this practice, HKL2MAP will be used. This program is a GUI software for SHELX crystallographic analysis package. This analysis is performed as following procedure: 1) SHELXC: the preparation of ΔF data from experimental F data. 2) SHELXD: finding the positions of sulfur atoms as anomalous scatterers. 3) SHELXE: phase calculation and phase improvement by density modification. Finally, the calculated phases are checked by electron density maps using the program XtalView/Xfit. Its details will be introduced at the BL practice. Only several pictures are shown.

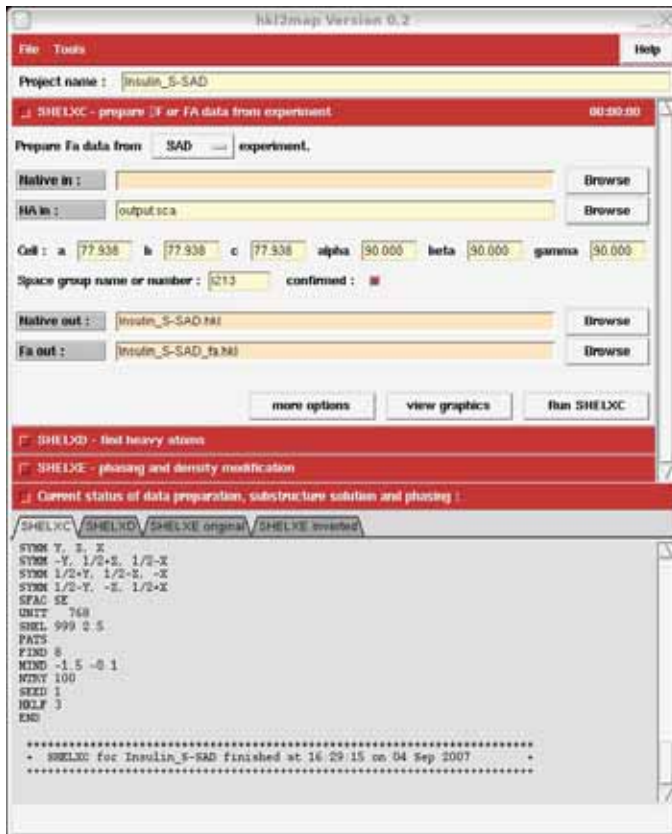


Fig. 15: hkl2map (Shelxc)

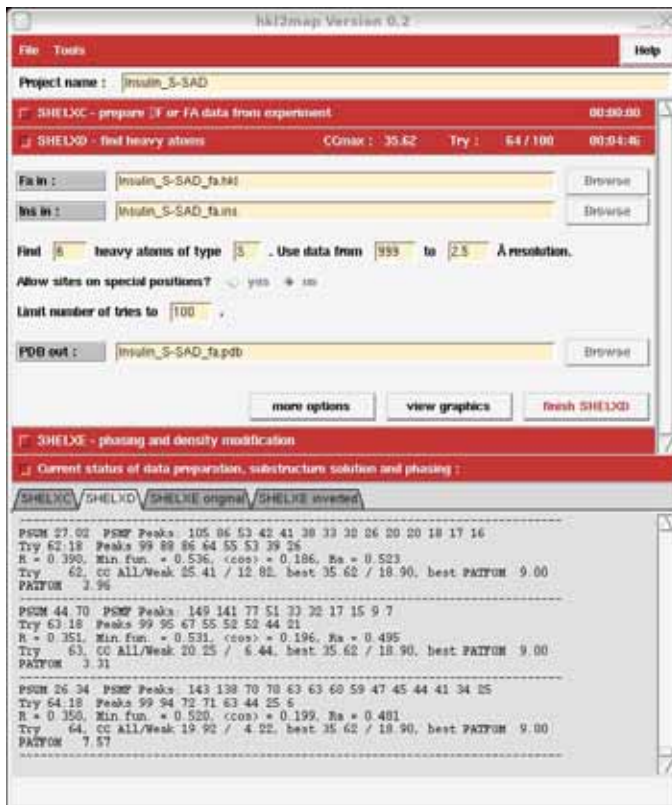


Fig. 16: hkl2map (Shelxd)

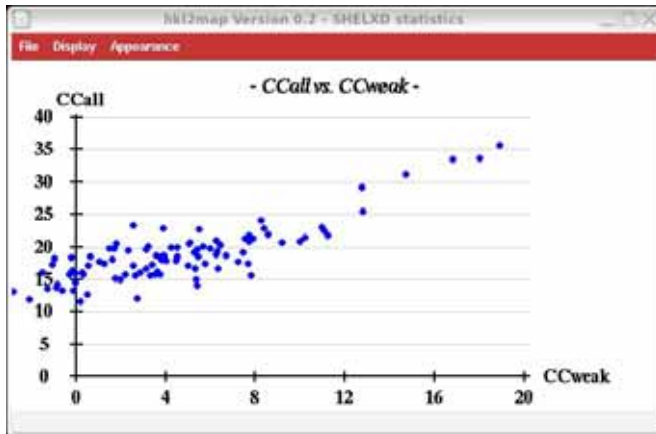


Fig. 17: CCall vs. CCweak

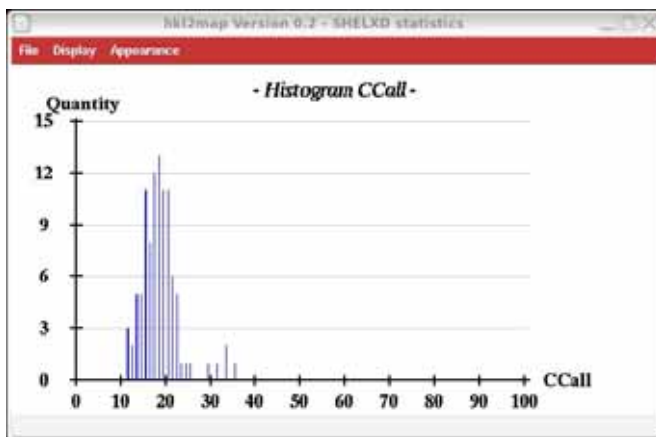


Fig. 18: Histogram CCall

Estimate solvent content

Number of residues in asymmetric unit : 51

Estimated fractional solvent content : 0.638

close window copy to SHELXE

Fig. 19: Estimate Solvent Content. Insulin is composed of 51 amino acids

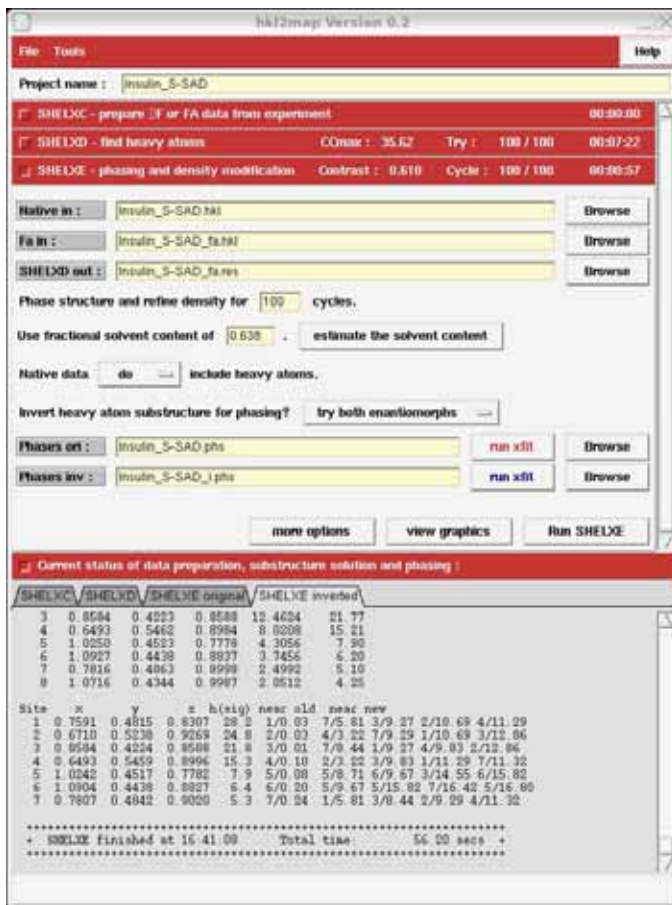


Fig. 20: Shelxe

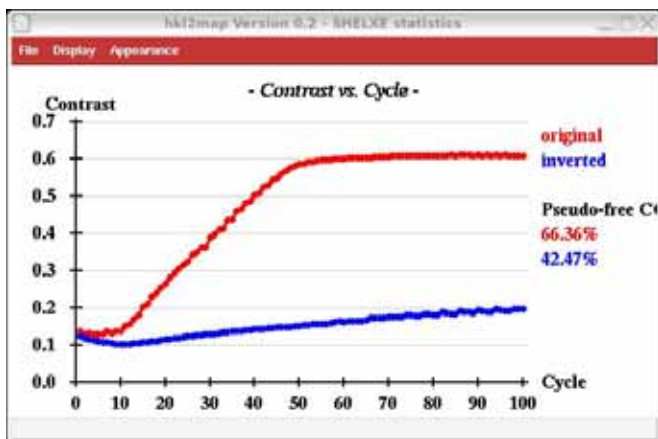


Fig 21: Contrast graph.

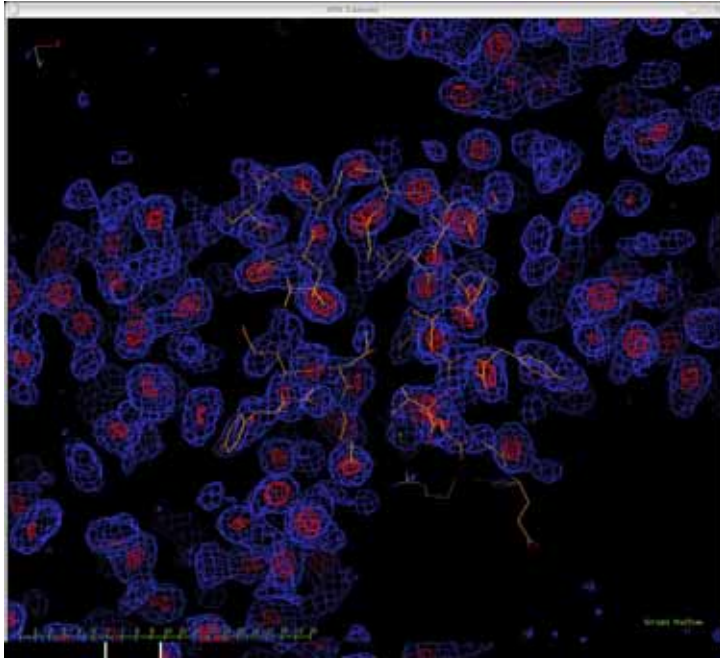


Fig. 22: Nicely determined phases. High contrast electron density map was obtained.