Effective Quality Improvement of Protein Crystals under Microgravity

---Recent Progress of Technical Development---

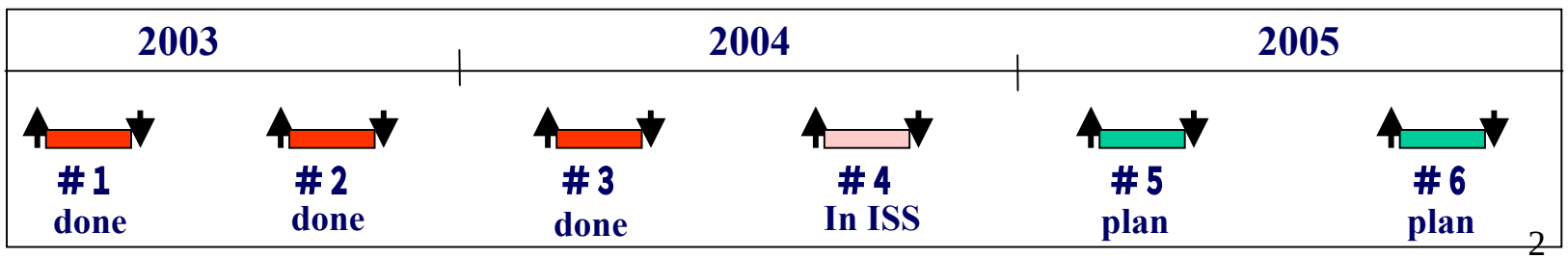
微小重力環境での蛋白質結晶の高品質化と最近の技術開発成果

2004.8.

JAXA

Outline of JAXA(NASDA)-GCF Project

Mission	JAXA(NASDA)-GCF-1~6
Launch from	Baikonur (Kazakhstan)
Vehicle	Progress
Landing at	Kazakhstan
Spacecraft	Soyuz
Experimental opportunities	2003~2005 (twice a year, 6 opportunities)
Duration	2~4 months
Flight Facility	Granada Crystallization Facility (GCF) After 2004 (4th flight), a new crystallization device developed by JAXA will be used.



Granada Crystallization Box (GCB)

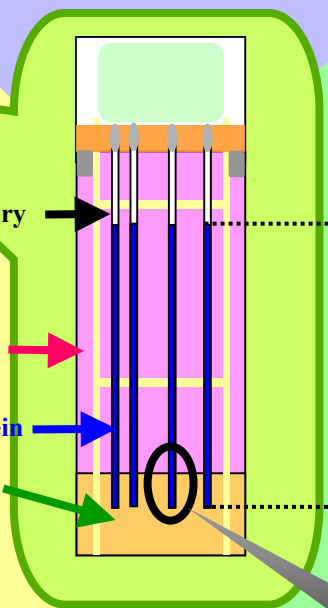
Outlook of the crystallization device

GCF contains 23 GCB.

GCF

(Granada Crystallization Facility)

After liquid tightness test conducted, GCF is mounted in Progress.

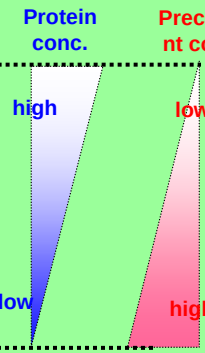


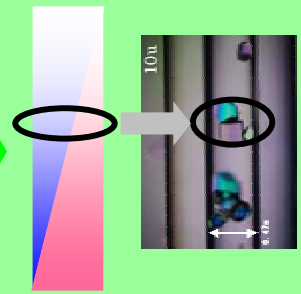
GCB

(Granada Crystallization Box)

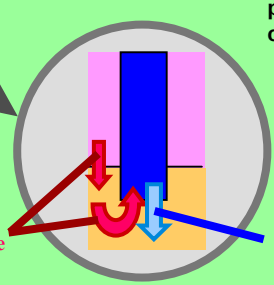
Principle of the counter-diffusion method

Concentration gradient in the capillary





Crystallization occurs where the concentrations of the protein and the precipitant are optimal.



Diffusion of the precipitant molecule

Diffusion of the protein molecule

Space Experiments

Mission	Odissea	NASDA-GCF#1	NASDA-GCF#2	JAXA-GCF#3	JAXA-GCF#4
Launch	25/09/2002	02/02/2003	29/08/2003	29/01/2004	11/08/2004
at	Baikonur (Kazakhstan)				
Vehicle	Progress				
Landing	07/12/2002	03/05/2003	28/10/2003	29/04/2004	19/10/2004
at	USA	Kazakhstan			
Spacecraft	Space Shuttle	Soyuz			
Duration	10 weeks	13 weeks	9 weeks	13 weeks	9 weeks
Flight Facility	Granada Crystallization Facility (GCF)				GCF and JCF (JAXA Crystallization Facility)
Number of GCBs (No. of protein samples)	2 GCBs (2 proteins)	46 GCBs (36 proteins)	69 GCBs (53 proteins)	50 GCBs (41 proteins)	28GCB (GTmethod) Total 37GCBs (38 proteins)
Protein for JAXA technical verification	2 proteins	1 protein	1 protein	2 proteins	6 proteins
Installation Location		Russian Service Module	CGBA (US module) in ISS	CGBA	TBU/Cryogem-3M

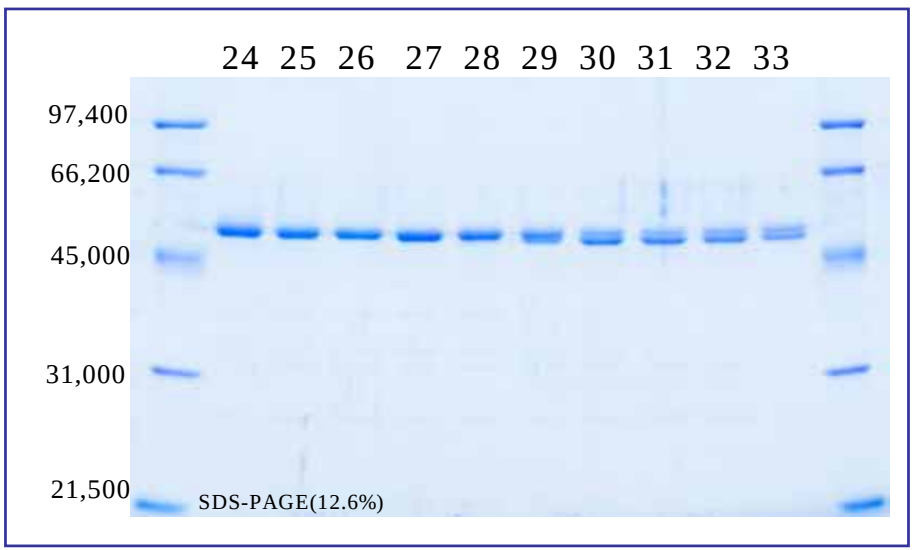
Crystallization of JAXA's Technical Verification Protein **Alpha-Amylase**

JAXA's R&D Example: Alpha-Amylase (E.C.3.2.1.1)

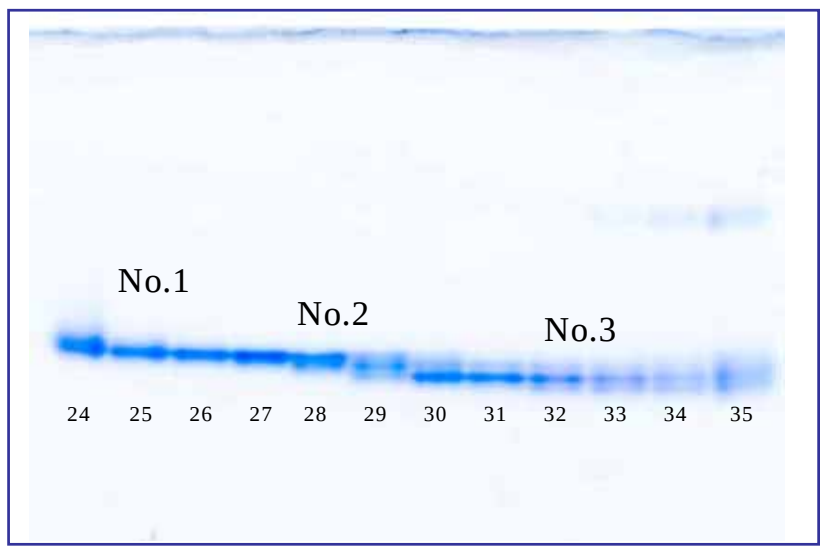
- Derived from *Aspergillus oryzae*
- 476 amino acids
- M.W. 50,000
- Acidic protein
- Catalyze the hydrolysis of the alpha-1,4 glycosidic linkage in starch
- Protein structure 2.1Å (6TAA)
  - *Acta Cryst*(1991)B47, 535-544

Sample Preparation

- Anion-exchange and hydrophobic chromatography were applied for further purification of the commercially available sample.

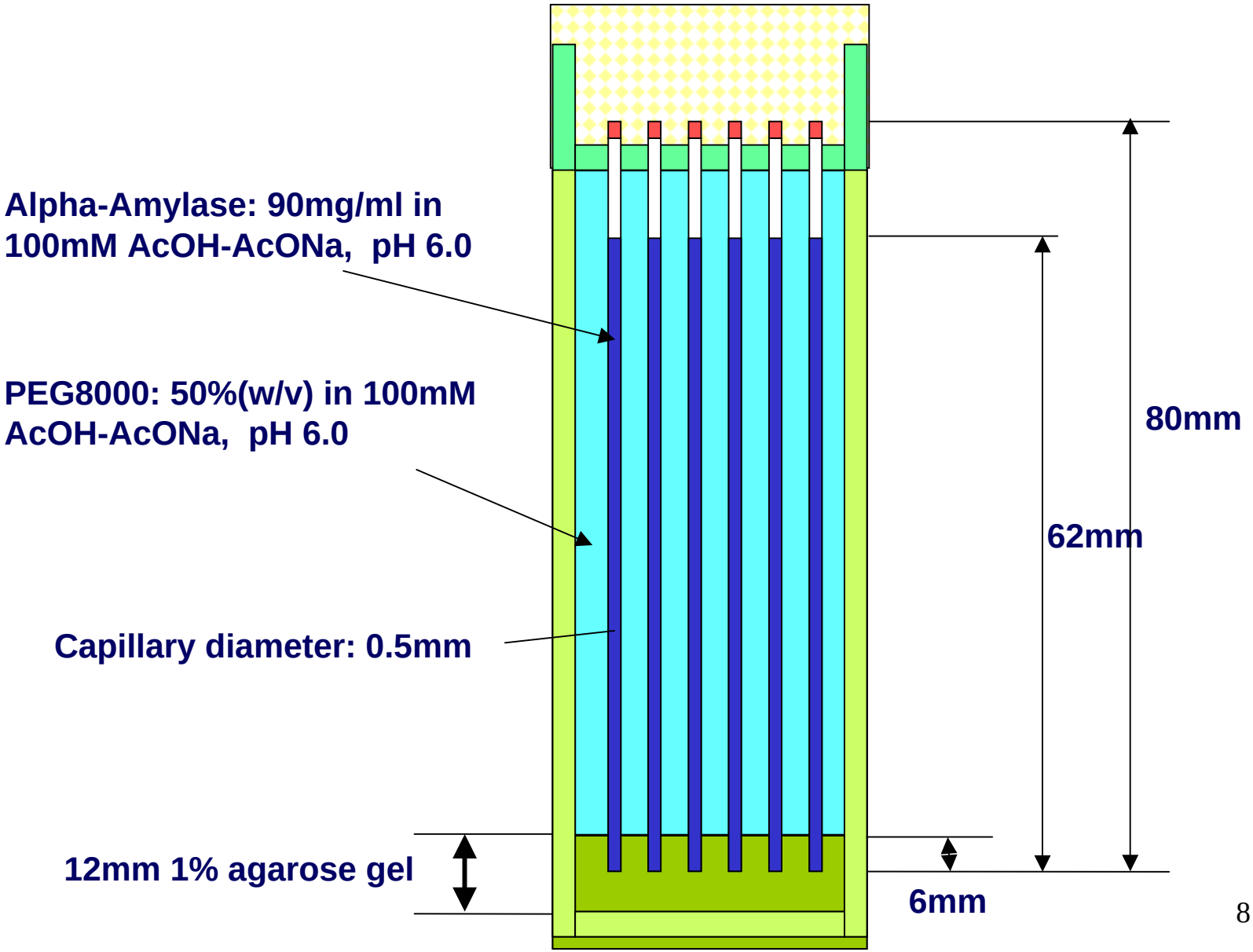


SDS-PAGE



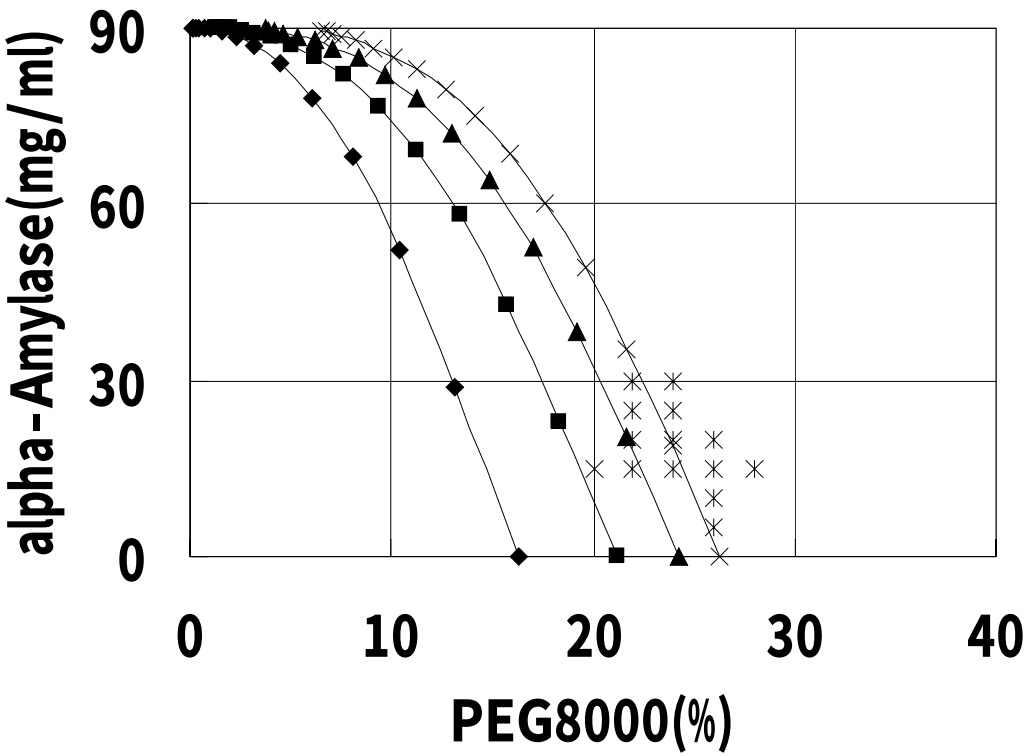
Native-PAGE

Configuration of GCB



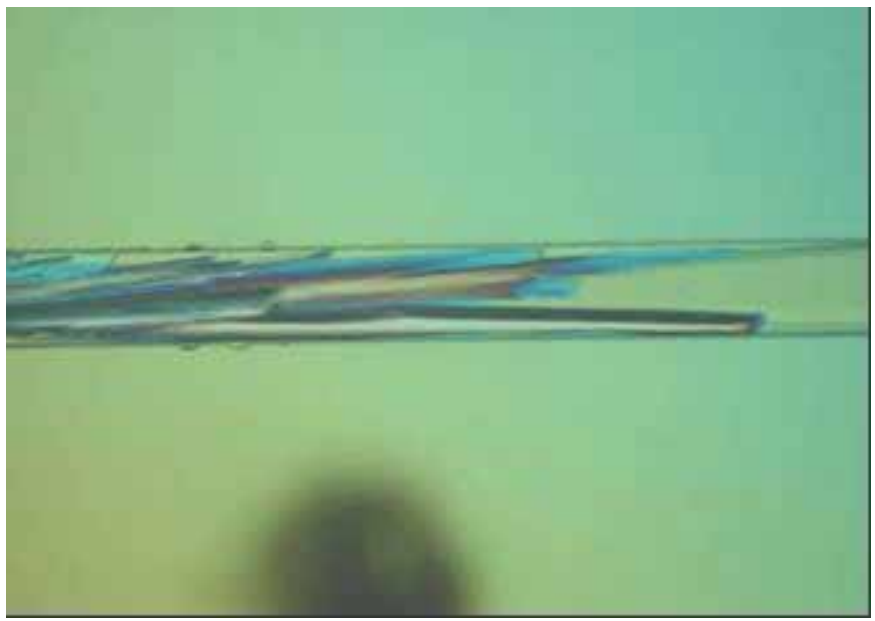
Crystallization Condition

We performed 1-D simulation to optimize the crystallization condition.



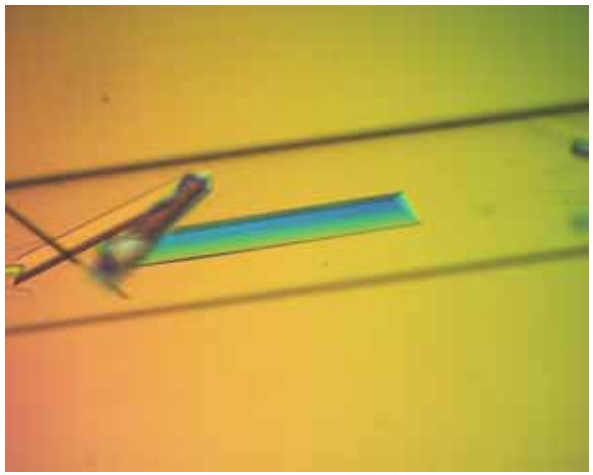
Crystallization Alpha-Amylase on the Ground using PEG8000 as a Precipitant

- Highly cluster-like morphology
- Maximum resolution: 1.12Å (by seeding)

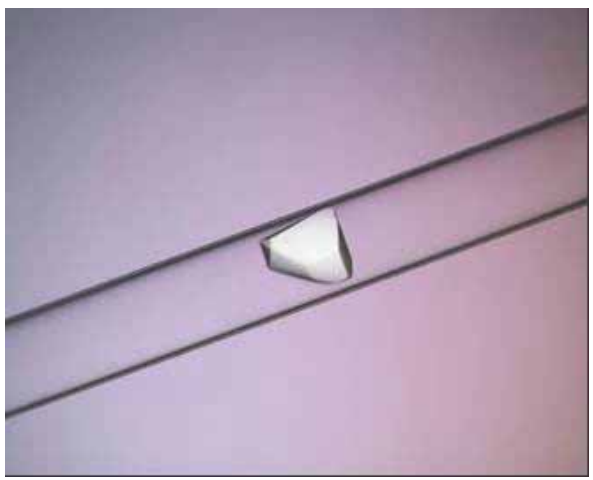


Grown on the ground

Crystallization of Alpha-Amylase in Space using PEG8000 as a precipitant



JAXA-GCF#1

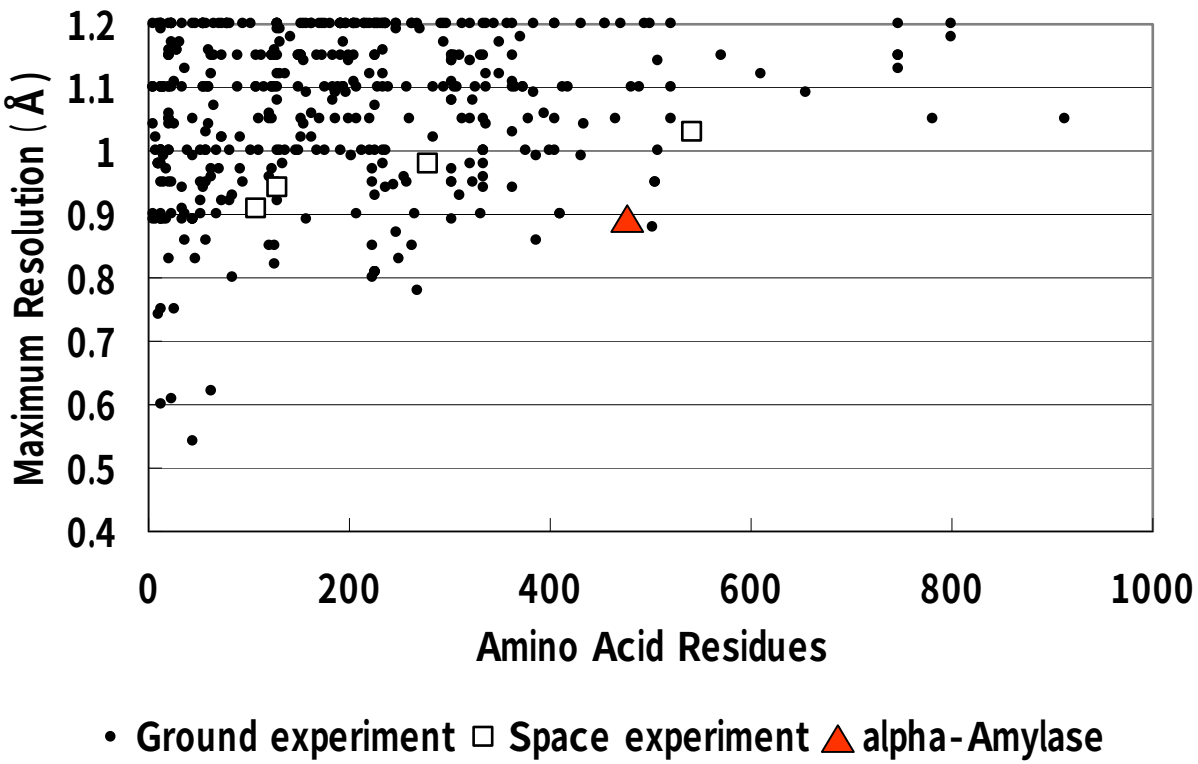


Odissea mission

- No cluster-like morphology
- Single crystal with same morphology as ground-grown crystal
- Maximum resolution: beyond 0.89Å (tentative)
  - May be the champion data among this M.W. proteins

- Single crystal with different morphology

Data extracted from PDB



Before X-ray Diffraction Data Collection…

- Harvest crystals from a capillary
  - Break the capillary
  - Shell the capillary like husking eggshell
  - Immerse a crystal in the appropriate harvest solution
- Prepare the appropriate harvest solution
  - 1-D Simulation Program and preliminary check
- Prepare the cryoprotectant solution
  - 20~30% glycerol is recommended
    - » See the poster No. P213
  - Check if no ice formation is observed beforehand when it is flash-frozen

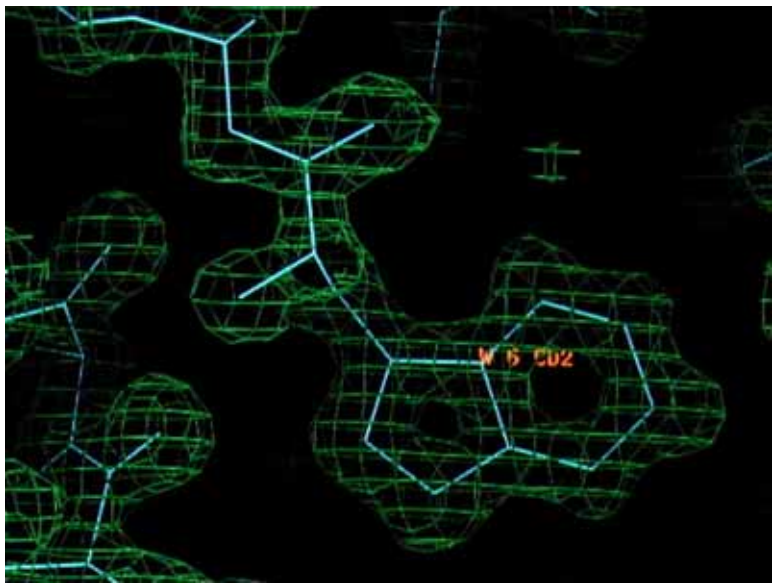
Crystal Data and X-ray Data Processing Statistics

Crystals / Diffraction data	6TAA (Swift et al.*)	Ground experiment (Batch / Seeding)	Space experiment (GCB)**	Space experiment (GCB)
X-ray source	Conventional	BL12B2	BL12B2	
Wavelength(Å)		1	0.7	
Space Group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	
Cell constant (Å)	a=51.0, b=67.2, c=133.6 α=β=γ=90°	a=50.8, b=67.7, c=130.1 α=β=γ=90°	a=50.4, b=67.4, c=130.4 α=β=γ=90°	
Volume of the cell	457,960	447,187	442,807	
Maximum resolution(Å)	2.1	1.12	0.89	
Mosaicity		0.312(~1.4Å)	0.241(~0.9Å)	
Avarage of I/σ(I)		22.2	39.2	26.6
Rmerge(overall)		0.063(30-1.40 Å)	0.036(30-1.40 Å)	0.062(15-1.0 Å)
Rmerge(outer shell)		0.188(1.45-1.40 Å)	0.039(1.45-1.40 Å)	0.262(1.04-1.00 Å)
Completeness(overall)(%)		94.5(30-1.40 Å)	99.3(30-1.40 Å)	96.5(15-1.0 Å)
Completeness (outer shell)(%)		89.9(1.45-1.40 Å)	99.3(1.45-1.40 Å)	95.7 (1.04-1.00 Å)

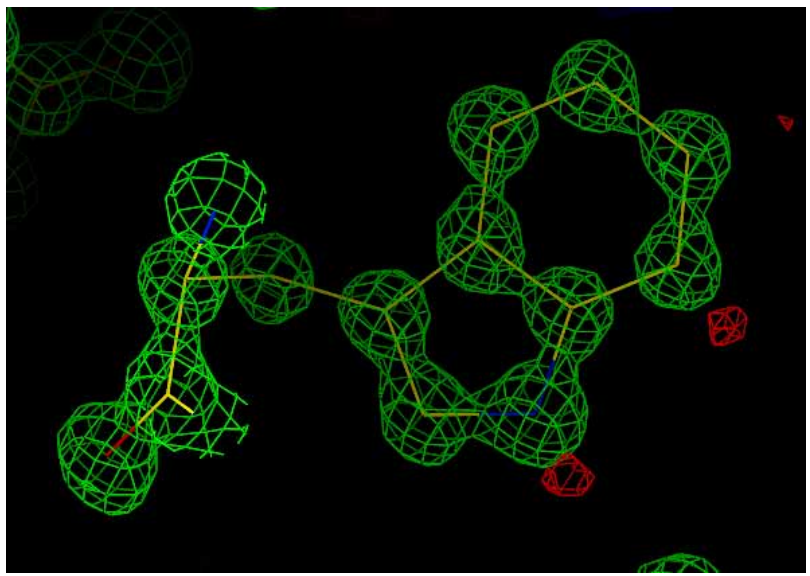
*Swift, H.J. et al.:Acta Cryst., B47, 535-544 (1991)

**Calculated for the comparison with the data of the ground experiment.

Electron Density Map of Alpha-Amylase (tentative)



Crystal grown on the ground



Crystal grown in space

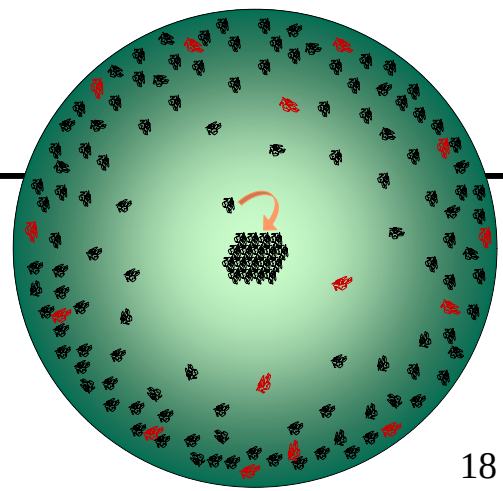
Alpha-Amylase crystals obtained in space

- The cluster-like formation was suppressed.
- The maximum resolution was improved and fine electron density map was obtained.
- The crystal with unfamiliar morphology was sometimes obtained.

Numerical Analysis of the Microgravity Effects

Expected Microgravity Effects

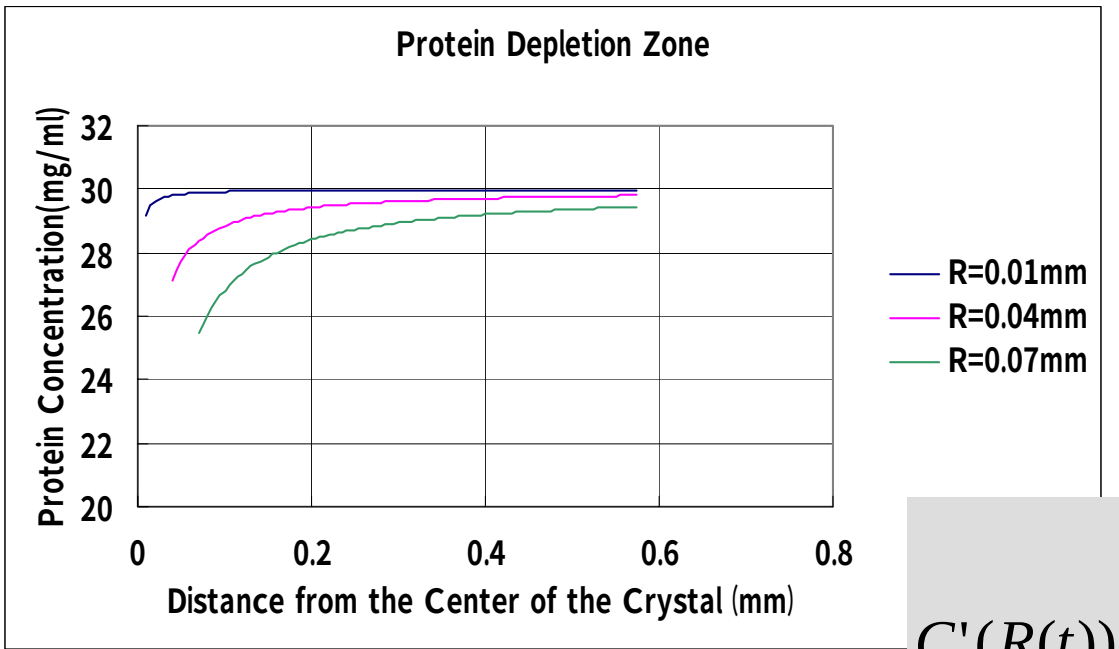
Mechanism	Effects on the crystal
Protein depletion zone (PDZ) formation	Suppression of the cluster formation
Impurity depletion zone (IDZ) formation	Suppression of the disorder
Suppression of the step bunching	
Suppression of the microcrystal capture	



Estimate PDZ and IDZ effects numerically in the case of alpha-Amylase

Diffusive field around a growing crystal

In case of 30mg/ml alpha-Amylase in 20% PEG8000



Ce=10mg/ml

$D=3.0\times10^{-12}m^2s^{-1}$

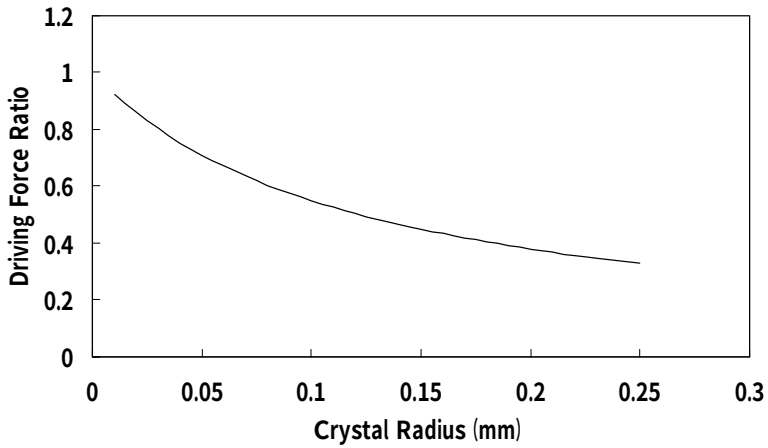
$\beta=0.09mmhr^{-1}$

$$C'(R(t)) = \frac{\frac{R(t)\cdot\beta\cdot C'e}{D} + C'(\infty)}{1+\frac{R(t)\cdot\beta}{D}}$$

- **Concentration of the protein around the crystal surface becomes lower if the viscosity of the solution is high.**

Depletion zone around the growing crystal (alpha-Amylase/PEG8000)

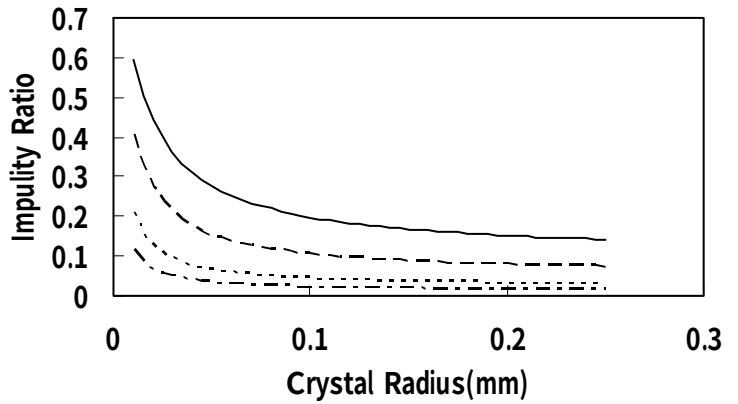
Protein depletion zone



Ce=10mg/ml
D=3.0x10^{-12}m^{2}s^{-1}
ß=0.09mmhr^{-1}

$$DFR = \frac{DF_{0G}}{DF_{1G}} = \frac{C(R)-Ce}{C(\infty)-Ce} = \frac{1}{1+\frac{R\cdot\beta}{D}}$$

Impurity depletion zone



A=10 A=20 A=50 A=100

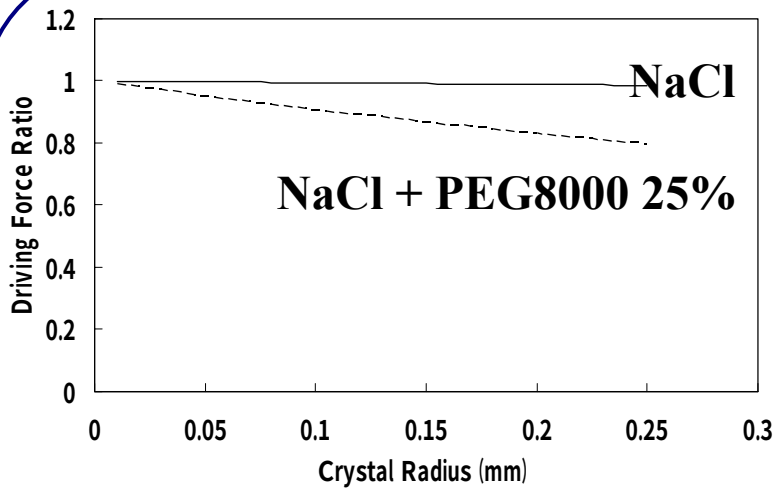
$$IR = \frac{IUR_{0G}}{IUR_{1G}} = \frac{1+\frac{R\cdot\beta}{D}}{1+\frac{R\cdot\beta i}{Di}} = \frac{1+\frac{R\cdot\beta}{D}}{1+A\cdot\frac{R\cdot\beta}{D}}$$

$$A = \frac{\beta i\cdot D}{\beta\cdot Di}$$

- **The effects on DFR and IR are expected in case of alpa-Amylase using PEG8000 as a precipitant.**

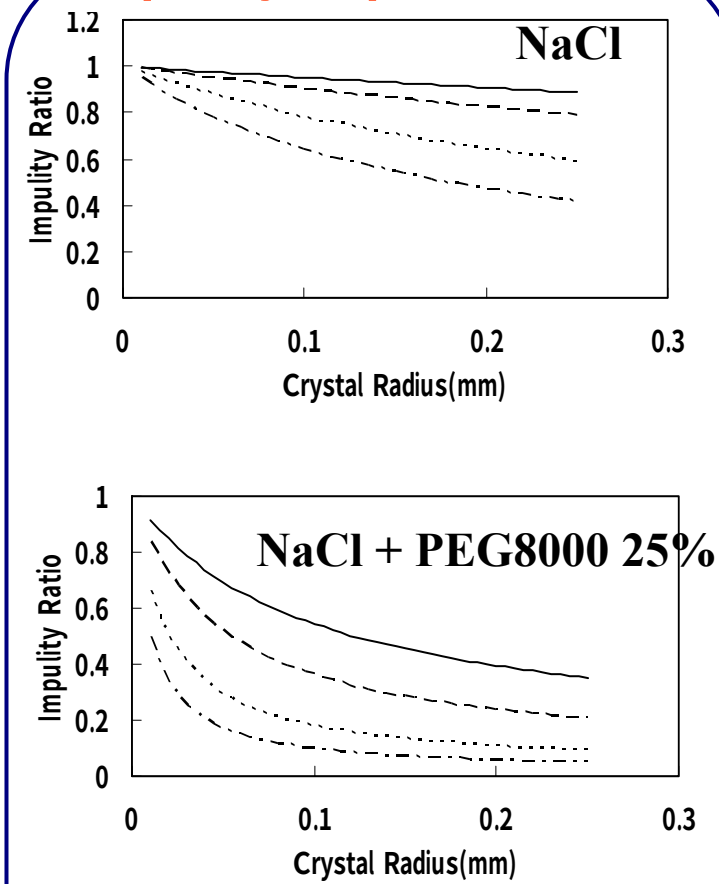
Depletion zone around the growing crystal (Lysozyme/NaCl)

Protein depletion zone



Ce=25mg/ml
$D=1.04\times10^{-10}m^2s^{-1}$ (NaCl)
$D=5.74\times10^{-12}m^2s^{-1}$ (NaCl+25%PEG8000)
$ß=0.04mmhr^{-1}$

Impurity depletion zone



- If the viscosity of the solution is higher, the effects on DFR and IR can be expected.

Results of the Numerical Analysis

- **Suppression of the convection**
  - **The protein and the impurity depletion zones are formed effectively.**
  - **Improvement of the quality of the crystals can be expected.**
  - **Suppression of the cluster-like morphology can be expected.**
- **The depletion zones can be expected in case of**
  - **○ Alpha-Amylase/PEG**
  - **× Lysozyme/NaCl**
  - **○ Lysozyme/NaCl+PEG8000**
- **The depletion zone effects are enhanced in high viscous solution**
- **To obtain high quality crystals in microgravity experiment**
  - **Use PEG as a precipitant**
  - **Add PEG to increase viscosity**

» **See the poster No. P203**

Crystallization of JAXA's Technical Verification Protein **Lysozyme**

Crystallization of Lysozyme

- **Method: Counter Diffusion Method**
- **Precipitant Solutions**

  I. 3% NaCl in 100mM Acetate buffer (pH 4.5)

  II. 10% NaCl in 100mM Acetate buffer (pH 4.5)

  III. 20% PEG8000 in 100mM Acetate buffer (pH 4.5)

  IV. 3% NaCl and 20% PEG8000 in 100mM Acetate buffer (pH 4.5)

  V. 12% NaCl and 20% PEG8000 in 100mM Acetate buffer (pH 4.5)

Experimental results for lysozyme

- **The crystals grew in the experiments using the precipitants I, II, IV and V, but did not grow using III.**

→ In case of lysozyme, PEG8000 was not sufficient to grow crystals, but did not inhibit the crystallization.

↓

In space, we performed crystallization experiment of lysozyme using

<u>12% NaCl and 20% PEG8000 in 100mM Acetate buffer (pH 4.5) as a precipitant</u>

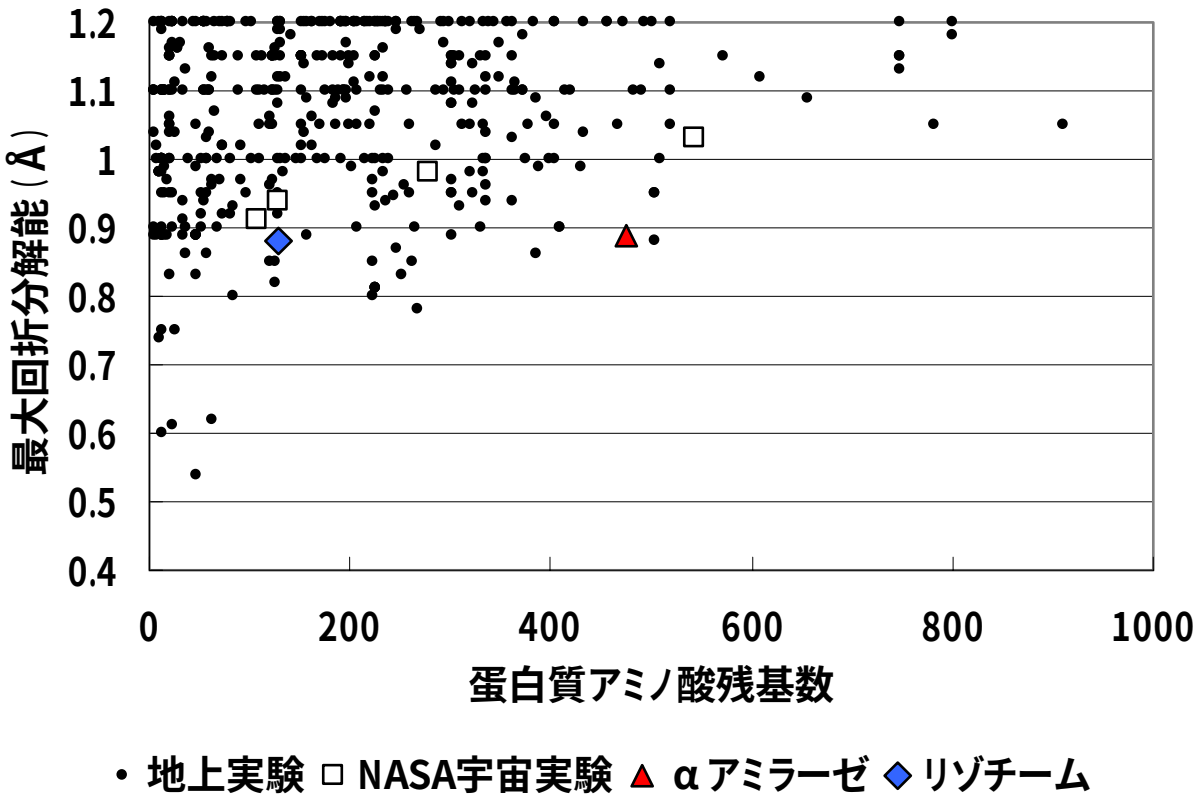
by GCB gel acupuncture method (JAXA-GCF#3, 2004, Jan-Apr).

Crystal Data and X-ray Data Processing Statistics (Tentative)

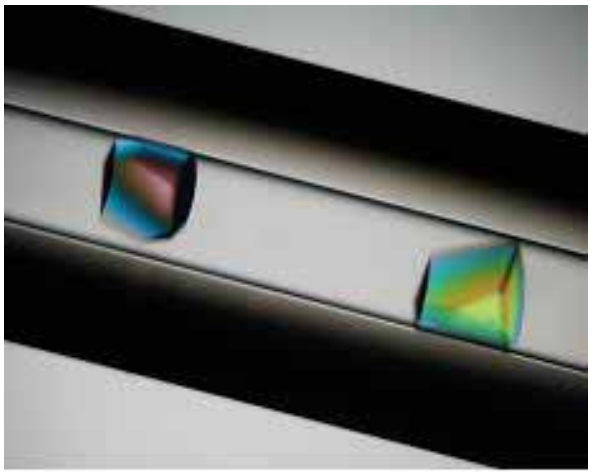
	1iee[*1] (Space crystal) (APCF)	Ground crystal (GCB)	Space crystal (GCB)
X-ray source	X11/BW7B	BL12B2	BL12B2
Space Group	$P4_32_12$	$P4_32_12$	$P4_32_12$
Cell Constants (Å)	a=b=77.06 c=37.22	a=b=77.3 c=37.9	a=b=78.0 c=37.7
Max Resolution(Å)	0.94-0.95	1.08	0.88
Mosicity	0.47	0.584~0.628	0.242~0.303
Rmerge % (total)	5.2(~0.94 Å)		6.9(~0.88 Å)
		3.3(~1.08 Å)	4.0(~1.04 Å)
Rmerge % (outer)	32.9(0.94-0.96 Å)		25.8(0.91-0.88 Å)
		16.8(1.12-1.08 Å)	6.6(1.11-1.04)
Completeness % (total)	98.9	92.3	89.9
Completeness % (outer)	88.6(0.94-0.96 Å)		81.4(0.91-0.88 Å)
		83.6(1.12-1.08 Å)	86.9(1.11-1.04 Å)

*1) Sauter et al., Acta Cryst., (2001) D57, 1119-1126

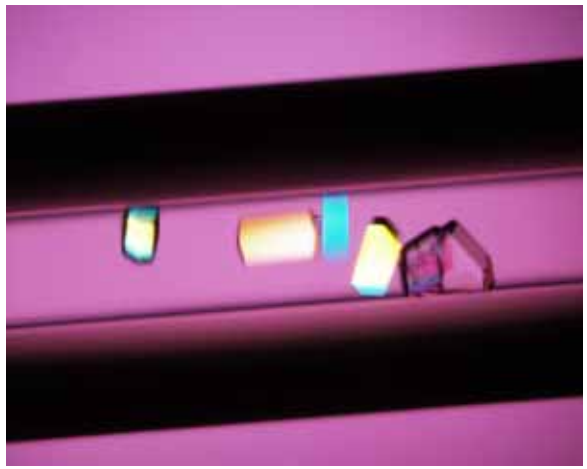
Data extracted from PDB



The effect of the addition of PEGs



II. 10% NaCl



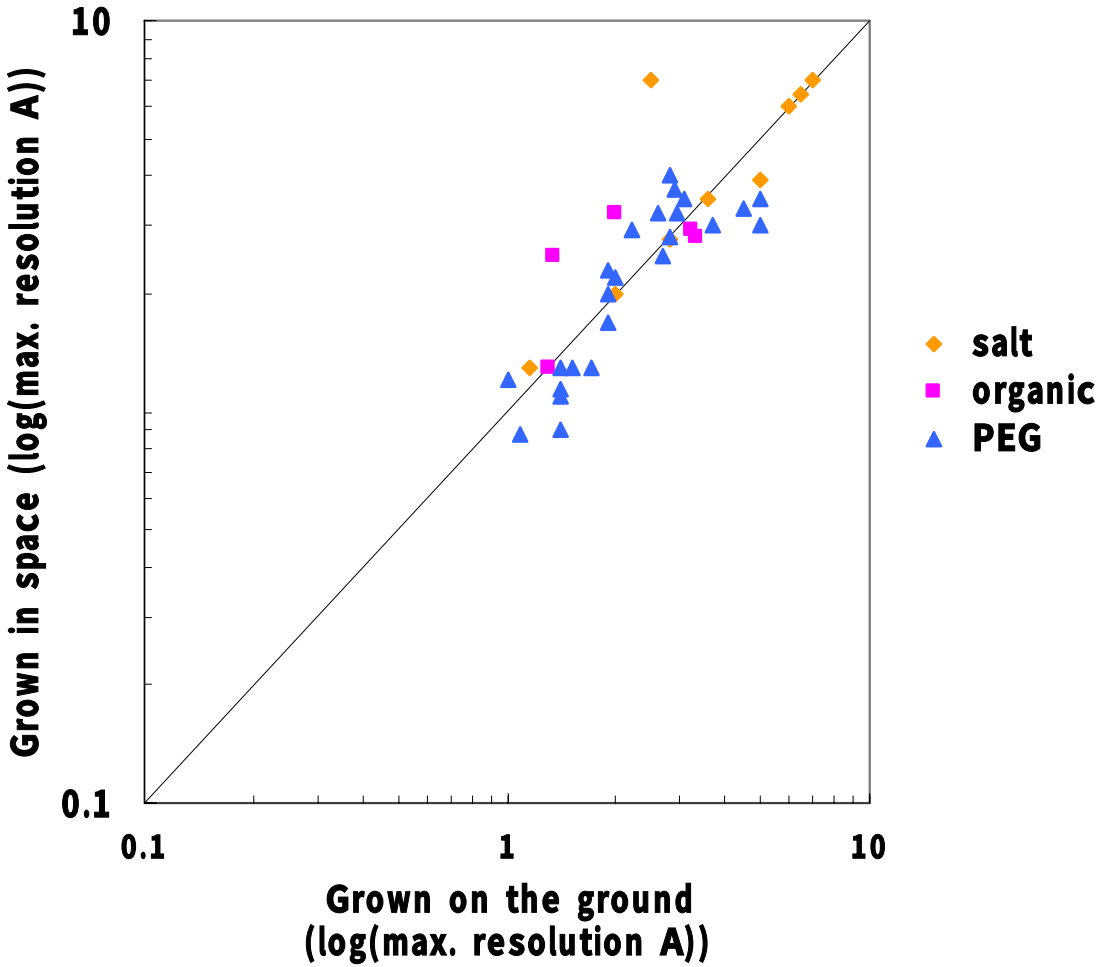
IV. 3% NaCl + 20% PEG8000

The crystals occurred 1 day after the start of experiment in both condition. PEG seemed to have generated the synergy effect, because it takes 1 week till the crystals occur using solution I (3% NaCl without PEG) as a precipitant.

Conclusions

- **The lysozyme crystals grew with PEGs in combination with NaCl as precipitant solutions, though they did not grow with PEGs alone.**
- **We could get the highest resolution ever collected with lysozyme crystals grown in space.**
- **The addition of PEGs to the precipitant solution could brought about a synergistic effect. The utilization of PEGs will help to use lower concentration of salt solutions as a precipitant.**

Comparison between Maximum Resolution of Crystals grown on the ground and in space



Data are collected from crystals grown in JAXA(NASDA)-GCF#1, #2, and #3 space experiments

Reasons for the successful results

- **Microgravity effects**
- **Samples with high purity**
- **Counter-diffusion technique**
- **Polyethylene glycol for a precipitant**
- **Technical improvement of harvesting crystals from a capillary**
- **Proper cryoprotection**

Acknowledgement

- ESA/ Dr. Olivier Minster
- CSIC-University of Granada/ Prof. García-Ruiz and the members of the laboratory
- Belgian government
- Russian Federal Space Agency and RSC Energia
- NASA