Preliminary X-ray analysis of the binding domain of the soybean vacuolar sorting receptor complexed with a sorting determinant of a seed storage protein

Nobuyuki Maruyama,^{a,*} Tomohiro Goshi,^a Shigeru Sugiyama,^b Mayumi Niiyama,^c Hiroaki Adachi,^d Kazufumi Takano,^{d,e} Satoshi Murakami,^{d,f} Tsuyoshi Inoue,^{c,d} Yusuke Mori,^{c,d} Hiroyoshi Matsumura^{c,d} and Bunzo Mikami^g

Received 29 September 2014

Accepted 17 December 2014

Keywords: soybean; seed storage protein; β -conglycinin; vacuolar sorting receptor.

^aLaboratory of Food Quality Design and Development, Graduate School of Agriculture, Kyoto University, Gokasho, Uji 611-0011, Japan, ^bGraduate School of Science, Osaka University, Machikaneyama 1-3, Toyonaka, Osaka 560-8531, Japan, ^cGraduate School of Engineering, Osaka University, Yamadaoka 2-1, Suita, Osaka 565-0871, Japan, ^dSOSHO Inc., 313 Photonics Center Building, Yamadaoka 2-1, Suita, Osaka 565-0871, Japan, ^eGraduate School of Life and Environmental Sciences, Kyoto Prefectural University, 1-5 Hangi-cho, Shimogamo, Sakyo-ku, Kyoto 606-8522, Japan, ^fGraduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, Nagatsuka 4259, Midori-ku, Yokohama, Kanagawa 226-8501, Japan, and ^gLaboratory of Applied Structural Biology, Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Gokasho, Uji, Kyoto 611-0011, Japan. *Correspondence e-mail: marunobu@kais.kyoto-u.ac.jp

β -Conglycinin is a major seed storage protein in soybeans, which are an important source of protein. The major subunits (α , α' and β) of β -conglycinin are sorted to protein-storage vacuoles in seed cells. Vacuolar sorting receptor (VSR) is an integral membrane protein that recognizes the sorting determinant of vacuolar proteins, including β -conglycinin, and regulates their sorting process. Vacuolar sorting determinants of the α' and β subunits of β -conglycinin exist in their C-terminal peptides. Here, the preliminary X-ray diffraction analysis of the binding domain of soybean VSR crystallized with the peptide responsible for the sorting determinant in β -conglycinin is reported. X-ray diffraction data were collected to a resolution of 3.5 Å. The crystals belonged to space group $P3_121$, with unit-cell parameters $a = b = 116.4$, $c = 86.1$ Å.

1. Introduction

Soybeans are used as a source of protein in many types of food products as well as in fodder for farm animals. Soybean seeds contain large quantities of storage proteins, mainly β -conglycinin and glycinin. β -Conglycinin has a trimeric structure with a molecular mass of 150–180 kDa (Maruyama *et al.*, 1998). Three subunits (α , α' and β) have been identified in β -conglycinin, of which the α and α' subunits contain an extension domain at the N-terminus in addition to the core region common to all of the subunits, whereas the β subunit consists of the core domain alone (Maruyama *et al.*, 1998). The α and α' subunits are synthesized as the pre-pro form and the β subunit is synthesized as the pre-form. All subunits are N-glycosylated with high-mannose glycans (Yamauchi & Yamagishi, 1979; Picariello *et al.*, 2013). They assemble as trimers in the endoplasmic reticulum (ER) and are then transported to protein-storage vacuoles (Mori *et al.*, 2004).

The vacuolar sorting receptor (VSR) recognizes the sorting determinants of vacuolar proteins, including seed storage proteins (Xiang *et al.*, 2013; Shimada *et al.*, 2003). VSR is a type I integral membrane-spanning protein with a luminal ligand-binding domain and a cytosolic tail (Kirsch *et al.*, 1994; Ahmed *et al.*, 1997). The C-terminus of the luminal ligand-binding domain of the VSR contains three epidermal growth factor (EGF)-like motifs (Cao *et al.*, 2000), the second and the

© 2015 International Union of Crystallography

clone and a BaculoDirect linear DNA using LR Clonase II Enzyme Mix (Life Technologies, USA). Sf9 cells were then transfected with the recombinant baculovirus DNA using Cellfectin (Life Technologies, USA). 4–5 d after transfection, we harvested the cell-culture medium and used it as a viral stock. The optimal expression time for GmVSR mBD was determined by monitoring the cellular extract and medium by SDS–PAGE analysis and an immunoblotting assay with an anti-His-tag antibody. The medium collected by centrifugation was applied onto Ni Sepharose 4 FF (GE Healthcare, UK) and eluted with a gradient of 40–500 mM imidazole in buffer A (20 mM HEPES–NaOH pH 7.0, 150 mM NaCl, 0.4% CHAPS, 1 mM CaCl₂). The fractions containing GmVSR mBD were concentrated using Vivaspin 30 (Sartorius, Germany) and loaded onto a Superdex 200 pg column (GE Healthcare, UK) equilibrated with buffer A. N-terminal amino-acid sequences were determined using a Procise 492 protein sequencer (Life Technologies, USA).

2.2. Crystallization

GmVSR mBD was concentrated to 5–10 mg ml⁻¹ in buffer A. Initial screening for crystallization conditions was performed at 293 K using the sitting-drop vapour-diffusion method with screening kits including Crystal Screen, Crystal Screen 2, PEG/Ion, PEG/Ion 2, SaltRx1, SaltRx2, PEGRx1 and PEGRx2 (Hampton Research, USA). Small crystals appeared in several conditions, and their diffraction patterns were checked using the beamlines at the SPring-8 facility, Hyogo, Japan. When the crystals were prepared at neutral pH using PEG as a precipitant, diffraction from the crystals was observed. However, owing to the low resolution, we could not collect adequate data. Previously, we have shown using the photoaffinity casting method that the peptide for the C-terminal sorting determinant of the α' subunit of β -conglycinin binds to GmVSR mBD (Morimoto *et al.*, 2013). Therefore, to improve the quality of the crystals, GmVSR mBD was co-crystallized with the peptide for the sorting determinant of the α' subunit of β -conglycinin. The peptide for the sorting determinant of the α' subunit of β -conglycinin (the C-terminal six residues; ILRAFY) was synthesized, purified to >98% purity by GL Biochem Ltd (Shanghai, People's Republic of China) and dissolved in distilled water. The protein was incubated with the peptide (at a molar ratio of 2–5:1 peptide:protein) for more than 1 h before

For expression of the GmVSR minimal binding domain (GmVSR mBD; Fig. 1) in insect cells, the BaculoDirect Baculovirus Expression system (Life Technologies, USA) was used according to the manufacturer's instructions. In order to obtain entry clones, we amplified GmVSR mBD cDNAs by using the primers 5'-AAGCTTCGGAGATCTTCGCTGTGCGTCTTTC-3' and 5'-AATCCGCTCGAGTCAGTTGGTGGTGGTGGTGAGCTGCACAGGTTGTATATCCGTCTCCTTT-3' (where italicized and underlined letters indicate the regions corresponding to the His tag and XhoI site, respectively). The amplified fragments digested by XhoI were then ligated with the NcoI (filled in)-XhoI-digested entry vector pENTR4 (Invitrogen, San Diego, California, USA) to produce the entry clone. To construct the recombinant baculovirus DNA, a recombination was performed between the entry

crystallization. The protein–peptide mixture was loaded into 5 or 10 μl ring-cap glass capillaries (Hirschmann Laborgeräte) which were capped with silicone tubes filled with agarose gel for the capillary-diffusion method.

2.3. Diffraction data collection and processing

Crystals obtained by the capillary-diffusion method were transferred into reservoir solution containing 30% glycerol, mounted in cryoloops and immediately cooled in a stream of cold nitrogen gas at 100 K. Initially, diffraction images were checked using an in-house Bruker HI-STAR detector coupled to a Mac Science M18XHF rotating-anode generator. After checking the images, the crystals were stored in liquid nitrogen for X-ray diffraction data collection on the BL38B1, BL41XU or BL44XU beamlines at the SPring-8 facility, Hyogo, Japan. The best crystals were those grown in 0.1 M HEPES–NaOH, 10% (v/v) PEG 1000, 0.1 M Tris–HCl pH 8.5 at 293 K, which

Figure 2
SDS–PAGE analysis of purified GmVSR mBD. Lane 1, molecular-weight marker (labelled in kDa); lane 2, purified GmVSR mBD.

Figure 3
Crystal of GmVSR mBD in complex with the C-terminal peptide of β -conglycinin.

Table 1

Crystallographic measurements for GmVSR mBD in complex with the C-terminal peptide of β -conglycinin.

Values in parentheses are for the outermost resolution shell.

Wavelength ($\text{\AA}$)	0.9
Resolution range ($\text{\AA}$)	48.22–3.50 (3.56–3.50)
Space group	$P3_121$
Unit-cell parameters ($\text{\AA}$)	$a = b = 116.4$, $c = 86.1$
Total reflections	70885 (3511)
Unique reflections	8775 (418)
Multiplicity	8.1 (8.4)
Completeness (%)	99.7 (99.9)
Mean $I/\sigma(I)$	12.5 (3.64)
R_{merge}	0.083 (0.675)
$R_{\text{p.i.m.}}$	0.031 (0.249)
$CC_{1/2}$ in highest resolution shell	0.813
Solvent content (%)	55%
No. of molecules in asymmetric unit	2

diffracted to 3.5 $\text{\AA}$ resolution. Data were obtained at a wavelength of 0.9 $\text{\AA}$ on the BL44XU beamline using a MAR225HE CCD detector at a crystal-to-detector distance of 286.0 mm. Diffraction patterns were indexed, integrated and scaled using *HKL-2000* (Otwinowski & Minor, 1997) followed by programs from the *CCP4* suite (Winn *et al.*, 2011).

3. Results and discussion

Purified GmVSR mBD (calculated molecular weight 49.1 kDa) migrated as a single band on SDS–PAGE, with a purity greater than 95% (Fig. 2). The N-terminal sequence was KFFVE, indicating that the signal peptides had been cleaved. The best crystals had dimensions of approximately $0.05 \times 0.05 \times 0.05$ mm (Fig. 3).

A summary of the data-collection statistics is given in Table 1. The crystal belonged to space group $P3_121$, with unit-cell parameters $a = b = 116.4$, $c = 86.1$ $\text{\AA}$, $\gamma = 120^\circ$. Two monomers were estimated to be present in an asymmetric unit assuming $V_{\text{sol}} = 55\%$ and a molecular weight of 49 146. The oscillation range was 1° per frame and the exposure time was 1 s per frame. A total of 136 frames were collected and the collected images were processed with *HKL-2000* and *SCALEPACK*. A total of 70 885 reflections were collected containing 8775 unique diffractions, with 99.7% completeness and an R_{merge} of 0.083 to 3.5 $\text{\AA}$ resolution.

In order to determine the structure of GmVSR mBD in complex with the sorting determinant of the α' subunit of β -conglycinin, data collection from heavy-atom and selenomethionine derivatives in addition to further optimization of the crystallization is currently being carried out. We anticipate that our structural study of GmVSR in complex with the C-terminal sorting determinant will contribute to elucidation of the sorting mechanism of storage proteins into plant vacuoles in seeds.

Acknowledgements

The synchrotron-radiation experiments were performed on BL38B1, BL41XU and BL44XU at SPring-8 with the approval of the Japan Synchrotron Radiation Research Institute (Proposal Nos. 2009B6826, 2010B6538, 2011A1085,

2011B1076 and 2011B1419). This work was supported in part by grants to NM from the Ministry of Education, Culture, Sports, Science and Technology (Nos. 21780093 and 24658286). We thank E. Okuda (Kyoto University) for technical assistance. The study is related to the subject of the Development and Assessment of Sustainable Humanosphere (DASH) system of Kyoto University (NM).

References

- Ahmed, S. U., Bar-Peled, M. & Raikhel, N. V. (1997). *Plant Physiol.* **114**, 325–336.
- Cao, X., Rogers, S. W., Butler, J. M., Beevers, L. & Rogers, J. C. (2000). *Plant Cell*, **12**, 493–506.
- Kirsch, T., Paris, N., Butler, J. M., Beevers, L. & Rogers, J. C. (1994). *Proc. Natl Acad. Sci. USA*, **91**, 3403–3407.
- Maruyama, N., Katsube, T., Wada, Y., Oh, M. H., Barba De La Rosa, A. P., Okuda, E., Nakagawa, S. & Utsumi, S. (1998). *Eur. J. Biochem.* **258**, 854–862.
- Mori, T., Maruyama, N., Nishizawa, K., Higasa, T., Yagasaki, K., Ishimoto, M. & Utsumi, S. (2004). *Plant J.* **40**, 238–249.
- Morimoto, S., Tomohiro, T., Maruyama, N. & Hatanaka, Y. (2013). *Chem. Commun.* **49**, 1811–1813.
- Nishizawa, K., Maruyama, N., Satoh, R., Fuchikami, Y., Higasa, T. & Utsumi, S. (2003). *Plant J.* **34**, 647–659.
- Nishizawa, K., Maruyama, N., Satoh, R., Higasa, T. & Utsumi, S. (2004). *Plant Sci.* **167**, 937–947.
- Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
- Picariello, G., Amigo-Benavent, M., Del Castillo, M. D. & Ferranti, P. (2013). *J. Chromatogr. A*, **1313**, 96–102.
- Shimada, T., Fuji, K., Tamura, K., Kondo, M., Nishimura, M. & Hara-Nishimura, I. (2003). *Proc. Natl Acad. Sci. USA*, **100**, 16095–16100.
- Watanabe, E., Shimada, T., Kuroyanagi, M., Nishimura, M. & Hara-Nishimura, I. (2002). *J. Biol. Chem.* **277**, 8708–8715.
- Winn, M. D. *et al.* (2011). *Acta Cryst. D* **67**, 235–242.
- Xiang, L., Etxeberria, E. & Van den Ende, W. (2013). *FEBS J.* **280**, 979–993.
- Yamauchi, F. & Yamagishi, T. (1979). *Agric. Biol. Chem.* **43**, 505–510.