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Crystallization and preliminary X-ray diffraction studies of the GhKCH2 motor domain: alteration of pH significantly improved the quality of the crystals

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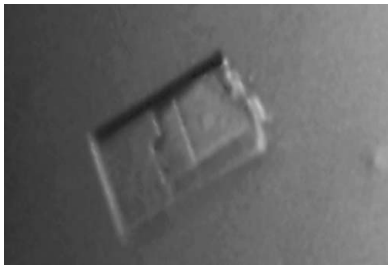
GhKCH2, a member of the kinesin superfamily, is a plant-specific microtubule-dependent motor protein from cotton with the ability to bind to both microtubules and microfilaments. Here, the motor domain of GhKCH2 (GhKCH2MD; amino acids 371–748) was overexpressed in *Escherichia coli*, purified and crystallized using the sitting-drop vapour-diffusion method. The pH of the crystallization buffer was shown to have a significant effect on the crystal morphology and diffraction quality. The crystals belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 60.7$, $b = 78.6$, $c = 162.8$ Å, $\alpha = \beta = \gamma = 90°$. The Matthews coefficient and solvent content were calculated as 2.27 Å^3 Da^{-1} and 45.87%, respectively. X-ray diffraction data for GhKCH2MD were collected on beamline BL17U1 at Shanghai Synchrotron Radiation Facility and processed to 2.8 Å resolution.

1. Introduction

Kinesins are microtubule-based motor proteins that are widespread in eukaryotic organisms and play important roles in microtubule dynamics, transportation of vesicles and organelles, cell division and signal transduction (Bloom & Endow, 1994). The kinesin superfamily, which has hundreds of members, has been divided into 14 subfamilies according to alignment of motor-domain sequences (Lawrence *et al.*, 2004); distinct structures and functions are exhibited among members of different subfamilies and even within a subfamily (Sack *et al.*, 1999; Marx *et al.*, 2005).

Since the publication of the first kinesin structure (*Homo sapiens* kinesin heavy chain; HsKHC; also called the conventional kinesin; Kull *et al.*, 1996), an increasing number of kinesin structures have been deposited in the Protein Data Bank (Marx *et al.*, 2009). These kinesins are mostly from animals and fungi; only KCBP, a Ca^{2+}-binding kinesin, is from a plant (Vinogradova *et al.*, 2004, 2008, 2009). The highly conserved core of the kinesin motor domain is composed of an eight-stranded mostly parallel β-sheet flanked by three α-helices on each side; the MgADP-binding site lies in an exposed surface cleft (Kull *et al.*, 1996; Sablin *et al.*, 1996; Gulick *et al.*, 1998; Kikkawa *et al.*, 2001; Turner *et al.*, 2001).

GhKCH2 (GenBank accession No. EF432568) was first cloned from cotton (*Gossypium hirsutum*) fibre and identified in our laboratory. It is a 112 kDa protein consisting of 1015 residues and belongs to the kinesin-14 subfamily. The motor domain (amino acids 396–734) of GhKCH2, which shares substantial amino-acid sequence identity with those of HsKHC (38% identity) and *Arabidopsis thaliana* KCBP (43% identity), has microtubule-activated ATPase activity just like the conventional kinesin, but its binding affinity to microtubules is much lower (Xu *et al.*, 2007). Interestingly, the N-terminal calponin homology (CH) domain of GhKCH2 has been shown to be able to bind to microfilaments, and full-length GhKCH2 can bind to and cross-link microtubules and microfilaments *in vitro* and *in vivo* (Xu *et al.*, 2009). It is not currently understood whether the dual ability of GhKCH2 to bind to both microfilaments and



microtubules is related to interaction between the motor domain and the N-terminus.

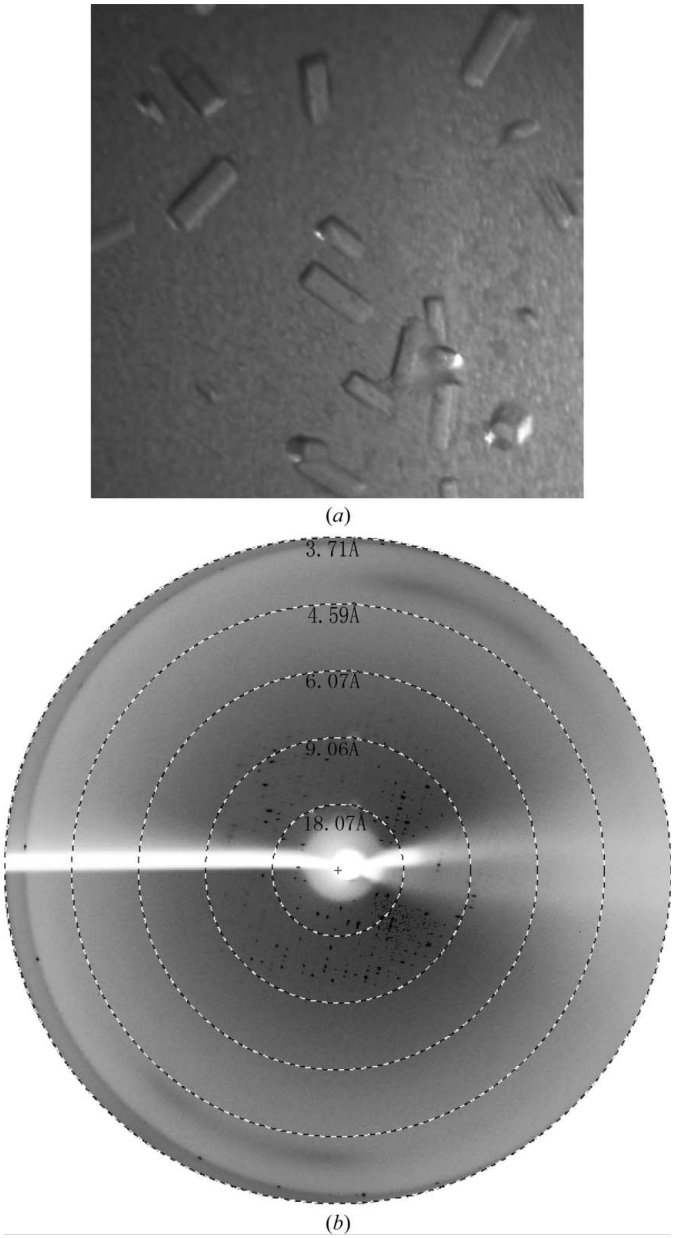
We purified and crystallized the motor domain of GhKCH2 (GhKCH2MD; amino acids 371–748) and identified a critical parameter (the pH of the crystallization buffer) that affects the crystal morphology and diffraction quality. A diffraction data set was collected from a GhKCH2MD crystal and processed to 2.8 Å resolution and preliminary phasing was performed, which suggested that the crystal was promising for structural determination.

2. Materials and methods

2.1. Protein expression and purification

The GhKCH2MD truncation fragment (amino acids 371–748) was cloned and inserted into modified pGEX-4T-2 vector (kindly provided by Dr Zhongzhou Chen, China Agricultural University, Beijing, People's Republic of China), with a TEV cleavage site between GST and the target gene, at the *Bam*HI and *Sal*I sites. The correct certified vector was transformed into *Escherichia coli* strain BL21. The cells were cultured in 10 ml LB medium containing 100 µg ml^{-1} ampicillin at 310 K for 8–12 h and then transferred to 1 l LB medium and grown at 310 K until the OD_{600} reached 0.6–0.8. Expression of GhKCH2MD was induced using 0.1 m*M* isopropyl *β*-D-1-thiogalactopyranoside (IPTG) at 295 K overnight. The cells were collected and lysed by gentle sonication in lysis buffer (0.1 *M* Tris–HCl pH 7.0, 150 m*M* NaCl, 1 m*M* DTT, 1 m*M* PMSF). The supernatant after centrifugation was loaded onto a glutathione Sepharose 4B column (GE Healthcare), incubated for 1 h and washed with ten column volumes of wash buffer (0.1 *M* Tris–HCl pH 7.0, 150 m*M* NaCl). GST-fused GhKCH2MD was cleaved on-column overnight using TEV protease. The flowthrough was further purified using a HiLoad 16/60 Superdex 200 pg gel-filtration column (GE Healthcare). Peak fractions containing GhKCH2MD were pooled, concentrated to 10–15 mg ml^{-1}, flash-cooled in liquid nitrogen and stored at 193 K.

All of the purification procedures described above were conducted at 277 K.



(*a*)

(*b*)

Figure 1
(*a*) Crystals of GhKCH2MD grown using 0.1 *M* Tris–HCl pH 8.5, 15% PEG 20 000. (*b*) Diffraction pattern of a crystal at pH 8.5.

Table 1
Data-collection statistics for a GhKCH2MD crystal.

Values in parentheses are for the last shell.

Wavelength (Å)	0.97923
Space group	$P2_12_12_1$
Unit-cell parameters (Å, °)	a = 60.7, b = 78.6, c = 162.8, $\alpha = \beta = \gamma$ = 90
Resolution (Å)	50–2.8 (2.85–2.8)
Multiplicity	5.2 (5.5)
Unique reflections	18939 (933)
Completeness (%)	94.6 (94.2)
$\langle I/\sigma(I)\rangle$	21.2 (2.5)
$R_{\rm merge}$†	0.104 (0.667)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of reflection i and $\langle I(hkl)\rangle$ is the average of the intensities of all observations of reflection.

2.2. Crystallization of GhKCH2MD

Initial screening for crystallization conditions was carried out in 48-well sitting-drop plates using three commercially available kits, Crystal Screen, Crystal Screen 2 and Index (Hampton Research, USA), at 295 K. Crystals of GhKCH2MD were first obtained after 4 d in Index condition No. 45 [0.1 *M* Tris–HCl pH 8.5, 25%(*w*/*v*) PEG 3350] by mixing 1 µl protein solution [15 mg ml^{-1} in 50 m*M* HEPES pH 7.0, 150 m*M* $MgCl_2$, 1 m*M* EGTA, 10%(*v*/*v*) glycerol] with 1 µl well solution. After optimization of the crystallization conditions, high-quality crystals were obtained in 0.1 *M* Tris–HCl pH 7.0–7.5, 15%(*w*/*v*) PEG 20 000 at 277 K.

2.3. Data collection and processing

Mounted crystals were soaked in a cryoprotectant solution [0.1 *M* Tris–HCl pH 7.5, 15%(*w*/*v*) PEG 20 000, 20%(*v*/*v*) ethylene glycerol] for a few seconds and flash-cooled in liquid nitrogen. Initial data collection was performed on beamline 1W2B at Beijing Synchrotron Radiation Facility (BSRF). The final high-quality diffraction data set was collected at 100 K on BL17U1 at Shanghai Synchrotron Radiation Facility (SSRF) using an ADSC Q315 CCD detector. A total of 360 images with an oscillation angle of 1° each were collected with a crystal-to-detector distance of 300 mm and an exposure time of 1 s per frame. The data were processed to 2.8 Å resolution and were

indexed and scaled using the *HKL*-2000 software package (Otwinowski & Minor, 1997). Data-processing statistics are listed in Table 1. All diffraction images were generated using the program *ADXV* (http://www.scripps.edu/~arvai/adxv.html).

3. Results and discussion

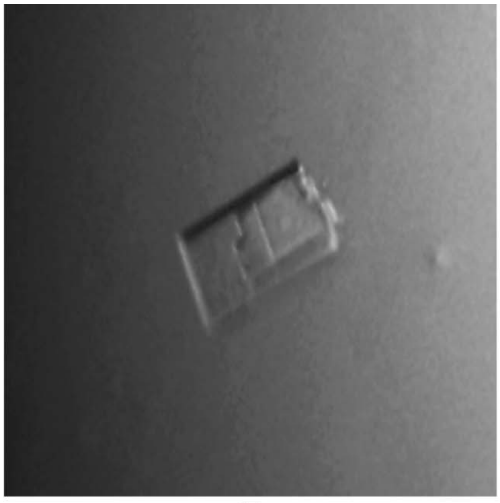
The purified GhKCH2MD was dissolved in a buffer consisting of 20 m*M* HEPES pH 7.0, 150 m*M* NaCl, 1 m*M* EGTA, 5 m*M* $MgCl_2$ at the beginning of the crystallization trials. GhKCH2MD was unstable in this solution and massive precipitation rapidly appeared in most of the drops. Several types of salts were tested according to the method of Jancarik *et al.* (2004), in which $MgCl_2$ was shown to have a strong impact on the solubility of GhKCH2MD. We subsquently found that the buffer concentration also affected protein solubility. Finally, the purified protein was stored in 50 m*M* HEPES pH 7.0, 150 m*M* $MgCl_2$, 1 m*M* EGTA, 10%(*v*/*v*) glycerol. GhKCH2MD could be concentrated to 15 mg ml^{-1} in this solution without any precipitation.

In the initial crystallization screen, crystals were grown in sitting drops at 295 K in Index condition No. 45 [0.1 *M* Tris–HCl pH 8.5, 25%(*w*/*v*) PEG 3350]. Crystals were observed after 4 d, but were very soft and stuck tightly to the bottom of the plate, making it hard to fish them out without damage. After optimization of the crystal-growth condition using a grid screen, varying the PEG molecular weight and concentration at 277 K, hexagonal prism-shaped crystals (approximately 0.1 × 0.05 × 0.05 mm according to the loop size) were obtained after about three weeks in the condition 0.1 *M* Tris–HCl pH 8.5, 10–15%(*w*/*v*) PEG 20 000 (Fig. 1*a*). Because these crystals were very fragile, they were cryoprotected using DMSO according to the 'no fail' *in situ* cryoprotection method (http://capsicum.colgate.edu/chwiki/tiki-index.php?page=Mounting+Protein+Crystals) and diffracted to around 6 Å resolution without cracking (Fig. 1*b*).

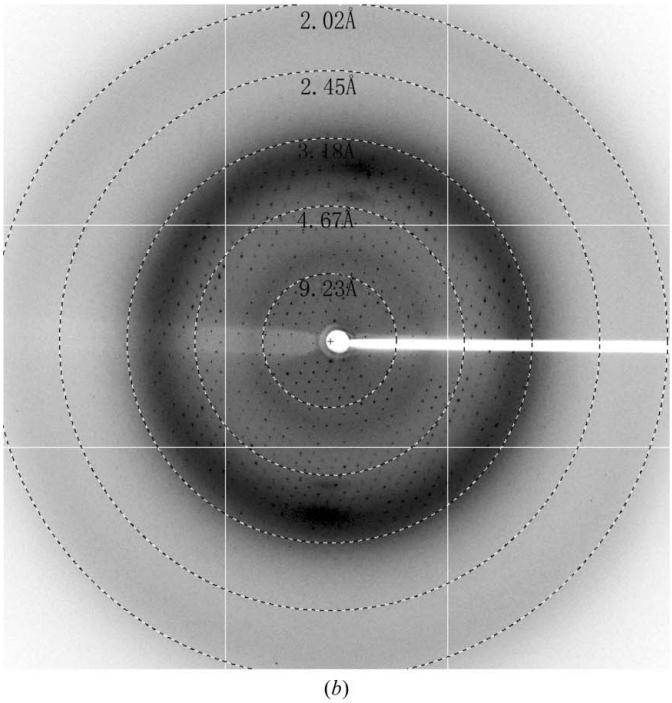
Using Additive Screen (Hampton Research) at 295 K, a better crystallization buffer consisting of 0.1 *M* Tris–HCl pH 8.5, 15%(*w*/*v*) PEG 20 000, 1 m*M* TCEP (where TCEP was an additive) was found to give clusters of thin plate-like crystals. Although the crystals diffracted to around 3 Å resolution at BSRF, they were not reproducible. N- and C-terminal truncations were then constructed based on the crystallizable sequence of GhKCH2MD. After deletion of three residues from the C-terminus, a fragment consisting of amino acids 371–745 could be reproducibly crystallized at 277 K in 1 d.

The key factors affecting crystal morphology and diffraction quality were explored using the new truncation fragment. Subsequent experiments showed that TCEP had no obvious effect on crystal quality, as was the case in the crystallization of the GCIP/HHM transcriptional regulator (Seto *et al.*, 2009), but could shorten the time of crystal growth. Accordingly, we set up a grid screen varying the pH (pH 7.0–8.5 in intervals of 0.5 pH units) against PEG concentration at 277 K and used streak-seeding with a series of equilibration times. We finally obtained much thicker single crystals (approximately 0.2 × 0.2 × 0.05 mm according to the loop size) using 0.1 *M* Tris–HCl pH 7.0, 15%(*w*/*v*) PEG 20 000, 1 m*M* TCEP with streak-seeding after equilibration for 24 h (shown in Fig. 2*a*). We also tried HEPES buffer and obtained the same results as with Tris buffer. The crystals obtained at pH 7.0 were rigid thin plates with an irregular edge on one side, differing greatly from the crystals obtained at pH 8.5. The crystals were cryoprotected with 20%(*v*/*v*) ethylene glycerol, flash-cooled and stored in liquid nitrogen.

A data set was collected and processed to 2.8 Å resolution at SSRF. The diffraction images are shown in Fig. 2(*b*). The crystal belonged to space group $P2_12_12_1$. The Matthews coefficient V_M (Matthews, 1968) of 2.27 Å^3 Da^{-1} suggested that there are two molecules in the asymmetric unit. A preliminary structural solution was obtained by molecular replacement with *BALBES* (Long *et al.*, 2008) using the crystal structure of the human kinesin-like protein KIFC1 (46% sequence identity and 71% sequence similarity; PDB entry 2rep; Structural Genomics Consortium, unpublished work) as a template. The best molecular-replacement solution showed good crystal packing without any steric clashes between symmetry-related molecules. Initial refinement gave R_{work} = 0.409% (R_{free} = 0.503%). Further improvement of the crystal diffraction resolution and model building are now in progress.



(*a*)



(*b*)

Figure 2
(*a*) Crystals of GhKCH2MD grown using 0.1 *M* Tris–HCl pH 7.0, 15% PEG 20 000, 1 m*M* TCEP. (*b*) Diffraction pattern of a crystal at pH 7.0.

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