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# Purification, crystallization and preliminary X-ray crystallographic analysis of nucleoside diphosphate kinase from rice

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Nucleoside diphosphate kinase (NDK) catalyses the transfer of the $\gamma$-phosphoryl group from a nucleoside triphosphate (NTP) to a nucleoside diphosphate (NDP). NDK is involved in and required for coleoptile elongation in rice. The level of the enzyme changes during seed germination and the early stages of seedling growth. Recombinant rice NDK (rNDK) has been crystallized using the hanging-drop vapour-diffusion method. rNDK crystals diffracted to a resolution of 2.50 Å and belong to space group $P2_12_12_1$, with unit-cell parameters $a$ = 70.98, $b$ = 182.26, $c$ = 188.30 Å. Preliminary analysis indicates there to be 12 rNDK molecules in each asymmetric unit, with a solvent content of 47.2%.

## 1. Introduction

Nucleoside diphosphate kinase generally catalyses the transfer of the $\gamma$-phosphoryl group from a nucleoside triphosphate to a nucleoside diphosphate,

$$N_1TP + N_2DP \longleftrightarrow N_1DP + N_2TP.$$

The enzyme functions *via* a ping-pong mechanism. During the first half of the reaction, the $\gamma$-phosphoryl group from the NTP donor is transferred to an invariant histidine residue. After release of the donor, the accepted NDP binds at the same site and the phosphoryl group is transferred to yield the NTP product. Nucleoside diphosphate kinases (NDKs) are found in almost all living organisms and play a critical role in cell metabolism by maintaining pools of nucleoside triphosphates for the synthesis of DNA and RNA (Parks & Agarwal, 1973). It is also known that polymerization of the protein is essential for activity and regulation. It is generally assumed that NDKs exist as hexamers in eukaryotes and as tetramers in bacteria (Giartosio *et al.*, 1996). The NDK from the Gram-positive organism *Bacillus halodenitrificans* shows a hexameric molecular complex in both crystals and solution (Chen *et al.*, 2003).

The involvement of NDK in cell growth and differentiation has been reported in animal systems (Steeg, Bevilaqua, Kopper *et al.*, 1988; Steeg, Bevilaqua, Pozzatti *et al.*, 1998; Rosengard *et al.*, 1989; Biggs *et al.*, 1990). NDK is also involved in tumour metastasis (Leone *et al.*, 1991) and regulates synaptic vesicle recycling (Krishnan *et al.*, 2001). The enzyme has been implicated in mediating hormone action by activating guanine nucleotide-binding proteins (Kimura & Shimade, 1988, 1990; Bominaar *et al.*, 1993). NDK is thought to play a crucial role in the differentiation process: it forms molecular complexes with $\beta$-tubulin and the level of the complexes dramatically increases during the process (Lombardi *et al.*, 1995). It has also been showed that human and *Escherichia coli* NDK can bind, cleave and repair DNA, which would fit well with the function of NDK in the development of cancer (Postel, 1999; Levit *et al.*, 2002).

Previous research results have shown that NDK participates in photoreceptor operation in fungi and plant systems (Ogura *et al.*, 2001), coleoptile elongation in rice (Pan *et al.*, 2000), microorganism growth and signal transduction (Chakrabarty, 1998). Biochemical studies suggest that NDK controls exchange and conversion among chemicals with high energy in cells, such as GTP synthesis for signal transduction (Ellis & Miles, 2001). For the process of rice sprouting, a special phenomenon is observed in which the rice can still sprout restrictedly to grow a leaf sheath under anaerobic conditions in which the rice plant is only capable of carrying out molecular respiration; therefore, the production of ATP molecules will be very limited. Thus, NDK must be very active in generating NTP to supply other necessary physiological activities. It is also possible that NDK interacts with G-protein according to our previous study on the bacterial system, despite there being only 48% sequence homology between the bacterial and rice enzymes.

Several NDK genes have been cloned in plants, such as spinach (Nomura *et al.*, 1992; Zhang *et al.*, 1993), tomato (Harris *et al.*, 1994), pea (Finan *et al.*, 1994), oat (Sommer & Song, 1994) and rice (Yano *et al.*, 1993, 1995), but the structure–function relationship of this protein during plant growth and development remains puzzling. We have isolated the cDNA clone

encoding NDK from rice (*Oryza sativa*; Chang, 2003) and established a successful protein-expression and purification procedure to obtain a large quantity of enzyme for crystallization. Moreover, we have also found that rice NDK (rNDK), with a molecular weight of 17 kDa, is involved and required for coleoptile elongation during seed germination and in the early stages of seedling growth. To further understand the biological significance of NDK in rice development, it is important and necessary to elucidate its detailed three-dimensional structure–function relationship. Here, we report the cloning, overexpression, purification, crystallization and preliminary X-ray characterization of rNDK. To our knowledge, this would be the first NDK crystal structure from a plant system.

## 2. Materials and methods

### 2.1. Cloning, expression and purification

Recombinant proteins were prepared by expression in *Escherichia coli* with the recombinant gene rNDK which had been inserted into a bacterial expression vector containing an N-terminal HAT tag pHAT12 (Clonthec).

The rice (*O. sativa*) NDK coding sequence was first amplified by polymerase chain reaction (PCR). The cDNA prepared by reverse transcriptase reaction with mRNA purified from elongating rice coleoptiles was used as the template, with the forward primer 5′-ATGGAGCAGTCCTTCATC-ATG-3′ and the reverse primer 5′-CTAA-GACTCATAGATCCAAGG-3′. The PCR products were then cloned into the pT-Adv vector (Advantage Tage PCR Cloning Kit, Clontech). The inserted fragments of the recombinant pT-Adv plasmids were purified from the ampicillin-resistant colonies and then checked by nucleotide sequencing. The rice NDK fragments with the correct nucleotide sequence were reamplified by PCR. The PCR product was then inserted into the expression vector pHAT12 on its modified *Kpn*I site by blunt-end ligation. To obtain this modified *Kpn*I site, the protruding 3′ sequence was converted to a blunt end using Klenow DNA polymerase (New England Biolabs) after *Kpn*I digestion. The ligation mixture was then transformed into *E. coli* JM109 and incubated on LB agar plates containing 50 µg ml$^{-1}$ ampicillin at 310 K overnight. The rNDK clones were picked and their plasmids screened by agarose-gel electrophoresis after restriction cut by *Bam*HI and *Eco*RI. The putative plasmid containing the rNDK fragment was then further confirmed by nucleotide sequencing.

For expression of rNDK, a single colony of the bacteria was picked and placed into 20 ml Luria–Bertani (LB) broth containing 50 µg ml$^{-1}$ ampicillin for incubation overnight. The overnight culture was then transferred into 1 l fresh LB medium (with 100 µg ml$^{-1}$ ampicillin). The culture was grown at 310 K with medium-speed shaking. After incubation for 30 min, 1 m*M* IPTG was added to the culture and incubation was continued for another 5 h. The cells were then harvested by centrifugation (6000 rev min$^{-1}$) at 277 K for 10 min. The medium was discarded and the cell pellet was resuspended with 25 ml Pierce bacterial extraction buffer and incubated and mixed gently at 277 K for 10 min. The suspension was collected by centrifugation at 277 K for 10 min (6000 rev min$^{-1}$). The soluble protein extract was then passed through a 10 cm long Talon-$Co^{2+}$ column which was pre-equilibrated with 50 ml PBS buffer. The column was washed with 30 ml extraction buffer (1/10×) and the purified protein was then eluted with 20 ml of elution buffer containing 100 m*M* imidazole. The protein sample was dialyzed in PBS buffer (phosphate-buffered saline; pH 7.0) overnight. The yield of the protein was approximately 5 mg and the purity was better than 95% as analysed by 12.5% SDS–PAGE (Fig. 1) and UV–visible spectra.

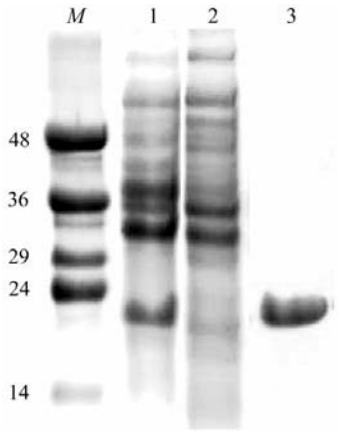


**Figure 1**
Coomassie-blue stained 12.5% SDS gel of pools from rNDK purification. Lane *M*, molecular-weight markers in kDa; lane 1, pool from soluble protein extract; lane 2, wash collection from Talon-$Co^{2+}$ column; lane 3, purified rNDK after Talon-$Co^{2+}$ column.

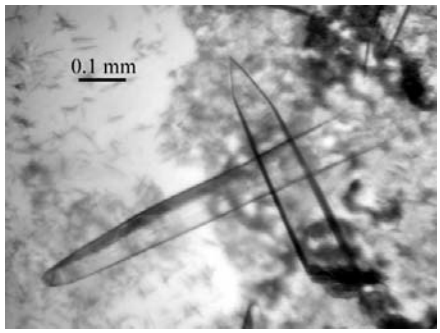


**Figure 2**
Single crystals of rice NDK grown by the hanging-drop method.

### 2.2. Crystallization

Before crystallization trials, rNDK was ultracentrifuged to a concentration of 6.1 mg ml$^{-1}$ in 0.1 *M* PBS buffer pH 7.0. Crystallization was achieved by the hanging-drop vapour-diffusion method at 291 K. A shower of small rod-shaped crystals were obtained from a condition containing PEG 4000 and calcium acetate as precipitant within 5 d of the initial setup. This condition was further refined to produce larger rNDK crystals using 2 µl hanging drops of equal volumes of protein solution and a reservoir solution containing 18% PEG 4000, 0.1 *M* imidazole buffer pH 8.0. These diffraction-quality crystals were used for X-ray data collection (Fig. 2).

### 2.3. X-ray data collection and processing

The protein crystals were initially screened and characterized using a synchrotron-radiation X-ray source at the protein crystallographic beamline BL17B2 equipped with an R-AXIS IV++ detector (Rigaku/MSC) at the National Synchrotron Radiation Research Center (NSRRC) in Taiwan. The complete data collection was carried out at beamline BL12B2 equipped with a Quantum 4R CCD (ADSC) detector at SPring-8 in Japan. The crystal was transferred into a cryoprotectant solution containing 20% glycerol, mounted on a 0.2–0.3 mm fibre loop (Hampton Research) and then flash-cooled in liquid nitrogen at 100 K. For the complete data collection, 270° of data were measured with 0.5° oscillations using an X-ray wavelength of 1.00 Å with an exposure time of 40 s and a crystal-to-detector distance of 230 mm at 100 K in a nitrogen stream produced by an X-Stream cryosystem (Rigaku/MSC). The data were indexed, integrated, scaled and merged using the programs *HKL*1.91, *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

## 3. Results and discussion

Sword-shaped rNDK crystals appeared in a week and continued growing to final

**Table 1**
Diffraction statistics of rice NDK.

Values in parentheses are for the highest resolution shell (2.59–2.50 Å).

| | |
|---|---|
| Wavelength | 1.00 Å (SPring-8, BL12B2)/1.127 Å (NSRRC, BL17B2) |
| Temperature (K) | 100 |
| Resolution range (Å) | 25.0–2.50 |
| Space group | $P2_12_12_1$ |
| Unique reflections | 81623 |
| Completeness (%) | 95.3 (75.1) |
| $I/\sigma(I)$ | 8.6 (4.8) |
| Average redundancy | 7.37 |
| $R_{sym}$† (%) | 7.4 (21.2) |
| Mosaicity | 0.76 |
| Unit-cell parameters (Å) | $a$ = 70.98, $b$ = 182.26, $c$ = 188.30 |
| No. of molecules per AU | 12 |
| Matthews coefficient (Å$^3$ Da$^{-1}$) | 2.42 |
| Solvent content (%) | 47.2 |

† $R_{sym} = \sum_h \sum_i [|I_i(h) - \langle I(h)\rangle|/\sum_h \sum_i I_i(h)]$, where $I_i$ is the $i$th measurement and $\langle I(h)\rangle$ is the weighted mean of all measurements of $I(h)$.

dimensions of 0.1 × 0.1 × 0.5 mm within one month in an incubator at 291 K. The protein crystals are very sensitive to the change of precipitant concentration when being transferred to the cryoprotectent solution containing 20% glycerol. Crystals were carefully screened for quality and selected for data collection as some of them had fairly large mosaicities of over 1.0°, which caused an overlap of diffraction spots owing to the long axes $b$ and $c$, which were >180 Å. Radiation damage was also observed after long exposure during data collection, which caused a decrease in $I/\sigma(I)$ and an increase in $R_{sym}$. Thus, although data were colllected over a total rotation range of 360°, only a 270° range was selected for data processing after inspection of data statistics with regard to crystal decay.

Analysis of the diffraction pattern indicated that the crystals exhibited orthorhombic symmetry, with unit-cell parameters $a$ = 70.98, $b$ = 182.26, $c$ = 188.30 Å. Systematic absences suggested that the space group was $P2_12_12_1$. The merged data set was 95.3% complete with an internal agreement ($R_{sym}$) of 7.4% for the resolution range 25.0–2.50 Å. The locked self-rotation function with data in the resolution range 15–4 Å, calculated using the program *GLRF* (Tong & Rossmann, 1990), showed non-crystallographic threefold symmetry (data not shown). This result clearly indicated that the rNDK structure is composed of a multiple of three homologous subunits, *i.e.* trimers, in an asymmetric unit. Three possible multimers ($n$ trimers, $n$ = 3, 4, 5) were considered. Assuming nine, 12 and 15 molecules per asymmetric unit, the Matthews coefficient is estimated to be 3.22, 2.42 and 1.93 Å$^3$ Da$^{-1}$, corresponding to a solvent content of 60.4, 47.2 and 33.9%, respectively (Matthews, 1968), which are all within the normal range for protein crystals. Details of the data statistics are given in Table 1.

Initial attempts to solve the crystal structure of rNDK were performed by molecular replacement. The previously determined structure of NDK from *B. halodenitrificans* (PDB code 1nb2; Chen *et al.*, 2003), which shows 48% sequence identity, served as the search model. A molecular-replacement solution was obtained using the *AMoRe* program (Navaza, 1994) and confirmed there to be 12 molecules in one asymmetric unit. After rigid-body refinement in the resolution range 25–3.0 Å, the $R$ factor was 40.4%. Model building and refinement of the structure to 2.50 Å is in progress and structural details will be described in a separate paper.

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