Harvest Active Recombinant Rho Kinase from *Escherichia coli*

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Rho kinase (ROCK) inhibitors are effective candidates for treating nerve or myocardial injury, erectile dysfunction, and other cardiovascular diseases. Purified ROCK is a foundation for ROCK inhibitors screening and for its function research *in vitro*. This article established an easy way to harvest active recombinant ROCK catalytic domain (ROCK-CD) of rat in *Escherichia coli* (*E. coli*). The cDNA of ROCK-CD was amplified by RT-PCR, and subcloned to pET28a(+) vector to express the protein in *E. coli* BL(21) as inclusion bodies. The protein was purified by HiTrap chelating column, and its refolding was achieved by gradient dilution from guanidine hydrochloride solution, and desalinated by ultrafiltration. The result of DNA sequencing and protein sequence analysis indicate there were three amino acid residua of mutation, but the activity was not significantly affected. The activity of the recombinant protein was confirmed by ROCK II activity fluorescence polarization kit. Therefore, this is an easy and rapid procedure to harvest a large quantity of activity recombinant ROCK-CD at low cost.

Key words rho-associated kinase; activity assay, recombination

Rho kinase, known as Rho-associated coiled-coil forming protein serine/threonine kinase (ROCK),¹⁾ is one of the central regulatory molecules for cytoskeleton control and cell adhesion process.²⁾ ROCK also involves in other cell functions such as apoptosis³⁾ and tumor invasiveness.⁴⁾ Rho kinase is thought to play an important role in a variety of cellular functions such as stress fiber formation, focal adhesion formation, cell aggregation, cell morphology, cytokinesis, cell migration, and Ca²⁺-sensitization in the smooth muscle.^{2,5)} Recent report showed that Rho kinase involves in regulation of the amyloid precursor protein processing.⁶⁾ ROCK inhibitors are beneficial candidates for a wide range of diseases such as hypertension, inflammation, cancer,⁴⁾ erectile dysfunction,⁷⁾ and injury caused by ischemia and reperfusion.⁸⁾

There are two types of ROCK: ROCK-I (ROCK- β) mainly found in hepatocytes, and ROCK-II (ROCK- α) in central nerve system (CNS). ROCK is activated by binding with the activated form of a low molecular weight G protein, Rho.⁹⁾ Purified ROCK is important foundation for understanding the full function of ROCK, and is very useful in ROCK inhibitors screening. Katoh *et al.*¹⁰⁾ have conformed that 1–543 amino acid residua have catalytic function; further research suggested the kernel catalytic domain of ROCK (ROCK-CD) may be 92–354 amino acid residua.¹¹⁾ Turner *et al.*¹²⁾ reported a way to harvest recombinant human ROCK-CD from Sf-21 cell, which became a main purified ROCK source currently. However, this procedure is time-consuming and at a relatively high cost; which makes its price relatively expensive. It is needed to improve a procedure to harvest the ROCK-CD at low price, at high quality, and at large quantity; harvesting active ROCK-CD from *Escherichia coli* (*E. coli*) may be the choice met the request.

MATERIALS AND METHODS

RT-PCR The cDNA encoding the amino acids 5–552 of rat ROCK-II was cloned with polymerase chain reaction (PCR) techniques. Primers were designed according to rat ROCK-II sequence NM_009072 in GenBank.¹¹⁾ Briefly, Rat

brain total RNA was extracted by TRIzol kit (Invitrogen, U.S.A.), cDNA was reverse transcribed by reverse transcriptase from rat brain total RNA. A partial cDNA fragment of rat ROCK-II, 1644 nt (13–1656) was amplified using Taq DNA Polymerase (Takara Biotech, Japan) with 10 pM of each primer (cycling conditions: 94 °C for 60 s, 55 °C for 60 s, 72 °C for 90 s, 30 cycles, finally kept at 72 °C for 20 min). Primer A was 5'-ATGAGCGGATCCCCGCCGACGGGA-AAAT-3' and primer B was 5'-ACCTCTCTCGAGTATCTGAGAGCTCTGGT-3' (the underline in primer A is *Bam*HI site; and in primer B is *Xho*II site).

The PCR-amplified fragment was subcloned into pGEM-T Easy Vector (Promega, U.S.A.). Then, the cDNA of ROCK-CD was sequenced by the vector and confirmed as the encoding 5–552 amino acids.

Positive Clone Screening ROCK-CD cDNA in pGEM-T Easy Vector was digested with *Bam*HI and *Xho*I and ligated into pET28a(+) expression vector (Novagen, U.S.A.). The pET28a(+) contains a (His)₆-tag at its N-terminus. The expressed protein is a 67.4 kDa fusion protein by calculation.

Two micrograms of ligated pET28a(+) was transferred to 2 ml *E. coli* BL(21) with the help of CaCl₂ treatment. Promising clones were selected by 30 µg/ml kanamycin.

Promising clones were induced by 1 mM isopropyl- β -D-thiogalactoside (IPTG); clones that expressed proteins were screened by routine 10% SDS-PAGE. The promising clones that were able to express a new 67.4 kDa protein were recognized as positive clones. The (His)₆-tag of the protein was also used for identification and purification by HiTrap chelating column (Amersham Biosciences, Sweden).

Positive Clone Culture Positive BL(21) clones were cultured in LB culture media (tryptone 10 g/l; yeast extraction 5 g/l; 10 g/l of NaCl) containing 30 µg/ml kanamycin, at 37 °C, 200 rpm in a rocking incubator. When the absorbance (600 nm, 1 cm) of the culture media is about 0.8, different doses of IPTG were exposed to, and the temperature and rotation speed were adjust to room temperature and 100 rpm respectively. After the clones were induced for 0–12 h, the bacteria were isolated by centrifugation (4 °C, 12000×g, 5

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min).

Protein Extraction and Purification The ROCK-CD protein of rat (rROCK) was expressed as inclusion bodies, and the extraction was carried out as follow. Briefly, the bacteria from 100 ml were suspended in 4 ml Suspense Buffer (20 mM TrisHCl, pH 7.5), and was sonicated (300 W, 10 s sonication, 10 s interval, 30 cycles) on ice. The precipitation was got by centrifugation ($12000\times g$, 4°C, 10 min) and re-suspended in 4 ml Resuspense Buffer (20 mM TrisCl, 0.2 M NaCl, 2 M urea, 1 mM 2-mercaptoethanol, 2% Triton X-100, pH 7.5). The second sonication (300 W, 10 s sonication, 10 s interval, 20 cycles) was carried out and the precipitation was obtained by centrifugation ($12000\times g$, 4°C, 10 min). Purified inclusion bodies were obtained by rinsing the precipitation twice with Resuspense Buffer without Triton X-100. The inclusion bodies were dissolved in 4 ml Degeneration Buffer (20 mM TrisCl, 0.2 M NaCl, 0.05 M imidazole, 6 M guanidine hydrochloride, 1 mM 2-mercaptoethanol, pH 7.5) by vortex at room temperature for about 15 min. After removed remaining particles by passing the sample through a 0.22 μ m filter, the purification procedure followed.

HiTrap chelating 1 ml column was washed with 5 ml purified water using a 5 ml syringe. Load 0.5 ml 0.1 M NiSO₄ and continue to wash with 5 ml purified water. Equilibrate the column with 5 ml Degeneration Buffer. Then Load the sample and wash the column with 10 ml Degeneration Buffer and with 5 ml Elution Buffer (20 mM TrisCl, 0.2 M NaCl, 0.5 M imidazole, 1 mM 2-mercaptoethanol, pH 7.5). The Elution Buffer (containing rROCK-CD) was fractionally collected and stored at -20°C for detection and refolding.

Refolding of rROCK-CD Let the Elution Buffer thawed at room temperature. rROCK-CD protein was separated by centrifugation ($12000\times g$, 4°C, 10 min). Two milligrams precipitation was dissolved in 200 μ l Degeneration Buffer without imidazol. The refolding was accomplished by diluting the solution to about 2 ml with purified water very slowly on ice. Guanidine hydrochloride was removed by ultrafiltration (10 kDa ultrafiltration tub, Millipore). rROCK-CD solution was adjusted to 1 mg/ml (20 mM TrisHCl, 20 mM MgCl₂, 1 mM 2-mercaptoethanol, 0.56% NaN₃, pH 7.2) and stored at -70°C. The protein concentration was determined by Coomassie brilliant blue assay kit (Nanjing Jiancheng Bio-engineering Institute, China).

rROCK-CD Activity Assay The activity of the protein was verified by ROCK assay fluorescence polarization (FP) kit (Molecular Devices Company, U.S.A.). This kit uses 200 nM S6-derived fluorescence peptide as its substrate; the phosphorylated peptide can bind to a macromolecule, and the fluorescence varies from low FP to high FP according to the amount of the phosphorylated substrate. The FP was detected by Tecan Reader (Megallan, Swiss). Comparison of rROCK-CD activity was made with standard 63.3 kDa human ROCK-CD (hROCK-CD) (purchased from Upstate Company, U.S.A.). The steady-rate constants, K_m and K_{cat} , and IC_{50} of Y-27632 (purchased from Alexis Biochemicals, U.S.A.) were determined also by the assay kit followed the methods of ref. 13. IC_{50} of Y-27632 was calculated by Bliss Method.¹⁴⁾

RESULTS

RT-PCR of rROCK-CD The RT-PCR product was de-

Fig. 1. Result of RT-PCR, and Protein Analysis

RT-PCR fragment was detected by 0.8% agarose electrophoresis, the fragment is 1644 nt (a). The cDNA sequence indicated that RT-PCR generated three amino acid residua mutation (P₁₈→A, L₄₉₀→F, S₄₉₃→N). The mutated amino acid residua were underlined, beneath which were original amino acid residua. Sequence before or after rROCK-CD sequence was fusion protein segment from pET28a(+) vector.

Fig. 2. Positive Clone Screening by 10% SDS-PAGE

Six positive clones were screened. Clones with rROCK-CD⁺ pET28a(+) vector was able to express a new protein about 67.4 kDa (83, 82, 81, 43, 42, 41) induced by IPTG, but clone with rROCK-CD-pET28a(+) can't (Blk). The target protein was shown in the white square.

tected by 0.8% agarose electrophoresis (Fig. 1a), which indicated the product could be the sequence of 1644 nt. The result of sequencing indicated that there are three sites (P₁₈→A, L₄₉₀→F, S₄₉₃→N) of mutation in amino acid sequence from visual translation (Fig. 1b). The recombinant protein (including fusion fragment from vector) is about 67.4 kDa by calculation (Fig. 1b).

Positive Clone Selection Promising clones were induced by IPTG; their protein were extracted and detected by 10% SDS-PAGE. Figure 2 indicated that six positive clones appeared, since a new protein of about 67.4 kDa were induced compared to the negative clone. In order to verify the result, the vector in the *E. coli* was extracted and amplified by PCR with the same pair of primers under the same conditions, and the result of PCR was the same as showed in Fig. 1a.

Optimization of rROCK-CD Expression and Extraction In order to increase the yields and save the time of the whole procedure, conditions of expression and extraction were optimized. Figure 3a showed that it takes only 3–6 h for the positive clones to express the maximal yield of the protein at room temperature. And the experiment (Fig. 3b) indicates that the positive clones exposed to 0.1–0.5 mM IPTG were enough to induce the maximal yield.

Refolding of rROCK-CD The Elution Buffer contained rROCK-CD as high as 1 mg/ml. 10% SDS-PAGE detection indicated that there are almost no contaminated proteins in the final product (Fig. 4a), and the purity was thought to be

of no less than 95%. More than 2 mg of final product can be obtained from 1000 ml culture media.

rROCK-CD Activity Assay The activity of refolded rROCK-CD was assayed by ROCK assay FP kit. The result in Fig. 4b showed that the activity of the rROCK-CD was similar to that of hROCK-CD. The steady-state rate constants of rROCK-CD were measured by ROCK activity assay FP kit compared with hROCK-CD. The constants were similar to those of hROCK-CD showed in Table 1.

Because urea and guanidine hydrochloride were applied in this procedure, it is needed to elucidate what concentration of the two denaturing agents are tolerable in its work solution.

Fig. 3. Conditions Optimization of rROCK-CD Expression and Extraction

(a) The protein induced by 1 mM IPTG in positive clone for different time. A positive clone induced for 0, 3, 6, 9, or 12 h, but when induced for 3 or 6 h, the yield attained maximum. (Blk is abbreviation for “blank”; i for “induced by IPTG”) (b) Protein induced by IPTG of different doses (0–1.0 mM). Positive clone exposed to about 0.1–0.5 mM IPTG is enough to express the protein. The target protein was shown in the white squares.

Fig. 4. ROCK Purity Detection and Result of Recombinant ROCK Activity

(a) rROCK-CD detected by 10% SDS-PAGE after purified by HiTrap chelating column, desalinated by ultrafiltration. (b) rROCK-CD was assayed with ROCK activity assay FP kit compared to hROCK-CD (standard ROCK-CD) (mean $\pm$ S.D., $n=3$). Condition: 37 °C, 2 μ g/ml enzyme, 200 nM S6-derived peptide, 10 μ M ATP.

Table 1. Steady-State Rate Constants of rROCK-CD for S6-Derived Peptide Compared with hROCK-CD

Enzyme	K_{cat} (min^{-1})	K_m (μM)	K_{cat}/K_m ($\text{min}^{-1} \mu\text{M}^{-1}$)
rROCK-CD	73.8 ± 5.8	1.05 ± 0.14	70.3 ± 0.8
hROCK-CD	77.3 ± 9.0	1.06 ± 0.20	72.9 ± 1.8

Kinetic parameters were calculated from the Michaelis-Menten equation using Lineweaver-Burk analysis.¹³⁾ Conditions: 37 °C, 25 pM enzyme, 200 nM S6-derived peptide, 15 min reaction (mean $\pm$ S.D., $n=3$).

Fig. 5. Urea or Guanidine Hydrochloride Inhibition on rROCK-CD

rROCK-CD is sensitive to urea in a dose-dependence manner, and is more sensitive to guanidine hydrochloride. Conditions: 37 °C, 25 pM enzyme, 200 nM S6-derived peptide, 10 μ M ATP, 30 min reaction (mean $\pm$ S.D., $n=3$).

Figure 5 showed that rROCK-CD is sensitive to urea or guanidine hydrochloride, but at low concentration (below 1 mM), guanidine hydrochloride almost has little effects on rROCK-CD, so does urea.

Inhibition of rROCK-CD ROCK selective inhibitor, Y-27632, inhibited activity of both rROCK and hROCK in a dose-dependent manner in the activity assay system, the pattern of them and IC_{50} ($0.26 \pm 0.02 \mu M$ for rROCK-CD, and $0.35 \pm 0.02 \mu M$ for hROCK-CD) were similar showed in Fig. 6.

DISCUSSION

ROCK is a central enzyme associated in many cell functions, the catalytic domain which was recognized to be the first 543 amino acid residua.¹⁰⁾ Other domains (including Rho Binding Domain) in ROCK regulate the catalytic activity. ROCK inhibitors, like Y-27632 and fasudil,¹⁵⁾ can selectively bind to the catalytic domain and inhibit its activity. ROCK inhibitors were regarded as promising choice to treat nerve injury,¹⁶⁾ myocardial injury,⁸⁾ erectile dysfunction,⁷⁾ and other diseases. Therefore, purified ROCK-CD, as an executive domain, is very useful in screening ROCK inhibitors and studying ROCK function *in vitro*.

Turner *et al.*¹²⁾ have developed a method to harvest recombinant ROCK-CD of human (hROCK-CD) from Sf-21 cell, but the cost is relatively high and the procedure is time-consuming. Katoh *et al.*¹⁰⁾ had tried to recombine rat ROCK-CD in *E. coli* in their experiment, but there are no further reports could verify whether this method is feasible. Usually, recombinant eukaryotic protein expressed in prokaryote like *E. coli* would give rise to mis-folding, although this kind of expression give a very high yield. Refolding recombinant protein in *E. coli* as inclusion bodies sometimes was proved to be a formidable task because of the low refolding efficiency.

Fortunately, the purification and refolding of rROCK-CD are not as difficult as we thought. The yield of (His)6-tagged rROCK-CD expressed in *E. coli* is more than 2 mg in 1000 ml culture as inclusion bodies, the yield is about once more than that of Turner *et al.*¹²⁾ Our data also indicated that there are almost no contaminated proteins in the final product (Fig. 4a). The refolding procedure was proved to be simple and effective. The gradient dilution for refolding should keep very slow, especially at the beginning; and should dilute to no less than 10 times. There are several successful examples which used the similar procedure from inclusion bodies.^{17,18)}

The activity curve in Fig. 5 showed similar property of rROCK-CD and hROCK-CD. Usually, the parameters of kinase were obtained by isotope. Present study used FP assay kit to determine the parameters. The classical selective substrates for ROCK are myosin light chain,¹⁹⁾ and myosin light chain phosphatase,²⁰⁾ which regulate actin contraction. Trauger's report²¹⁾ suggested that S6-derived peptide is also one of selective substrates for ROCK, and conformed by Turner *et al.*¹²⁾ The constants of hROCK (Table 1) and IC_{50} of Y-27632 were similar to Turner's report,¹²⁾ which suggested the FP assay system is effective and feasible to determine ROCK activity. Compared with hROCK-CD, the parameters of rROCK-CD (including activity, K_m , K_{cat} , and IC_{50} of Y-27632) based on the activity assay system were also similar; which indicated that the purified rROCK-CD could

Fig. 6. rROCK-CD Activity Inhibited by Y-27632

Y-27632 inhibited rROCK-CD and hROCK-CD in a dose-dependent manner. The inhibition curves were similar. The IC_{50} were calculated by Bliss method,¹⁴⁾ $0.26 \pm 0.02 \mu M$ for rROCK-CD and $0.35 \pm 0.02 \mu M$ for hROCK-CD in the assay system. Conditions: 37 °C, 25 pM enzyme, 200 nM S6-derived peptide, 10 μM ATP, 30 min reaction (mean $\pm$ S.D., $n=3$).

be used for drug screening or further activity research.

In order to refold the protein as inclusion bodies, urea or guanidine hydrochloride is usually applied. Present study showed rROCK-CD is sensitive to both of them. Urea and guanidine hydrochloride can be removed by ultrafiltration. Our data (Fig. 5) indicated that, if urea or guanidine hydrochloride was controlled below 1 mM in work solution, the interference is omissible (inhibition <10% or 5%); which indicate that the degenerative reagent needn't remove thoroughly on some occasions.

In the present study, RT-PCR introduced three amino acid residua mutation ($P_{18} \rightarrow A$, $L_{490} \rightarrow F$, $S_{493} \rightarrow N$). For these mutated amino acids were far away from the kernel sequence of rROCK catalytic domain (92—354 amino acid),¹¹⁾ if there were discount of activity of rROCK-CD, it would not be significant; which confirmed by activity assay.

Taken together, an easy and rapid procedure was established to harvest a large quantity of active recombinant rROCK-CD at low cost from *E. coli*; the convenience of the procedure is obvious.

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