0273-2289/05/125/0201/$30.00

Purification and Partial Characterization of an Extracellular Ribonuclease From a Mutant of *Aspergillus niger*

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Received August 23, 2004; Revised December 20, 2004; Accepted March 8, 2005

Abstract

A new extracellular ribonuclease (RNase) from a mutant of *Aspergillus niger*, named *A. niger* SA-13-20 RNase, was purified to homogeneity by $(NH_4)_2SO_4$ fractionation (50–85%), DEAE-cellulose anion-exchange chromatography, ultrafiltration and Sephacryl HR-200 chromatography. The enzyme was purified up to 54.4-fold with a final yield of 24.5%. There were differences in the molecular weight, pI value and some physico-chemical properties between *A. niger* SA-13-20 RNase and that from the parent strain. The enzyme is monomeric and its molecular weight and isoelectric point were 40.1 kDa and 5.3, respectively. The N-terminal amino acid sequence of *A. niger* SA-13-20 RNase was TIDTYSSDSP. The optimum pH, temperature and buffer concentration for the enzymatic reaction were 3.5, 65°C, and 0.175 *M*, respectively. Metal ions, such as K^+, NH_4^+, Mg^{2+}, and Ca^{2+} at the concentration of 1.0 m*M* had a slight activation effect on the enzyme activity and $(NH_4)_2SO_4$ activated the enzyme significantly. The enzyme was stable at pH lower than 8.5 and was easy to inactivate in strong alkali solution.

Index Entries: Ribonuclease; purification; *Aspergillus niger*; properties.

Introduction

Ribonucleases (RNases) are important analytical enzymes and widely distributed in nature. RNases from microorganisms are widely used in

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molecular biological study, food, and pharmaceutical industry. As an important analytical tool, they have played a major role in study on the structure and function of RNA *(1)*. In single-cell protein production, they are used to remove RNA in cell *(2)*. RNases have also been applied commercially to produce nucleotides for clinical use or for seasoning nucleotides *(3,4)*. Many RNases are highly cytotoxic. They have several important biological roles, such as antitumor and antiviral activity *(5–7)*. Therefore, RNases are considered as alternative chemotherapeutic drugs *(6,7)*.

Many RNases have been isolated and characterized *(8–11)*. It was reported early that *Aspergillus niger* NRC-A-233 (namely *A. niger* ATCC 26550) produced an extracellular RNase *(8)*. In view of this, we have initiated studies on the production of a RNase from *A. niger (12,13)*. There are some differences in the culture conditions and physiological properties of the mutant *A. niger* SA-13-20 and the parent strain *A. niger* ATCC 26550 *(13)*. Thus, the RNase from the mutant *A. niger* SA-13-20 may be different from the RNase from the parent strain *A. niger* ATCC 26550. To better understand the enzymatic behavior of the RNase from the mutant *A. niger* SA-13-20, we purified *A. niger* SA-13-20 RNase and some physico-chemical properties were investigated. The results show *A. niger* SA-13-20 RNase is a novel RNase.

Materials and Methods

Microorganism and Crude Culture

The fungus *A. niger* SA-13-20, a mutant resistant to sodium azide *(12)*, was maintained on malt agar slants at 4°C. The slant medium and the inoculum medium were the same as those reported in our previous paper *(12,13)*. Twenty-four hour inoculum was inoculated with a 12.5% (v/v) proportion into the sterilized fermentation medium *(13)* containing (g/L): glucose, 37.5; corn powder, 112.5; NH_4NO_3, 2.20; K_2SO_4, 0.11. Before sterilization, corn powder was partly hydrolyzed at pH 1.9 at 121°C for 1 h. Fermentation was carried out in the 5-l stirred tank bioreactor Biostat B (B. Braun Biotech International Diessel GmbH, Germany) with a working volume of 4 L at 30°C and pH 2.33 (controlled) for 72 h. The aeration rate and agitation rate were 1.67 vvm and 850 rpm respectively. After fermentation for 28 h, the dissolved oxygen concentration was controlled at 10% saturation by aerating oxygen.

The mycelium was removed by centrifugation at 10,000*g* at 4°C for 30 min and the supernatant solution was used as a source of enzyme.

Enzyme and Protein Assays

RNase activity was determined as described previously *(12)* using torula yeast type VI RNA (Sigma Chemical Co., St. Louis, MO) as substrate. One unit of enzyme activity was defined as the increase in absorbance at 260 nm of 1.00 units per 20 min under the conditions for assay.

Protein concentration was determined by using the method of Lowry et al. *(14)* or by measuring the absorbance of eluate at 280 nm.

Enzyme Purification

All purification steps were performed at 4°C. Crude enzyme solution (1000 mL) was brought to 50% $(NH_4)_2SO_4$ saturation and placed overnight. The precipitate was removed by centrifugation at 10,000*g* for 30 min and the supernatant solution was collected. This supernatant solution was adjusted to pH 5.0 and brought to 85% $(NH_4)_2SO_4$ saturation. After 1 d, the precipitate was obtained by centrifugation as above. This precipitate was dissolved in 25 m*M* sodium phosphate buffer (pH 7.2, containing 2.0 m*M* EDTA) and dialyzed overnight against the same buffer. The dialyzed enzyme solution (250 mL) was applied to a DEAE-cellulose column (3.5 × 60 cm) equilibrated with the stated sodium phosphate buffer. The bound protein was eluted with 2.4 L linear gradient of 0–0.60 *M* equilibration buffer. Fractions (10 mL) were collected and assayed for activity. The fractions with RNase activity were pooled together. The DEAE-cellulose pool (210 mL) was ultrafiltrated by an ultrafilter retaining a molecular weight of 30 kDa and above. The enzyme solution was partially purified further and concentrated to a much smaller volume in this step. Concentrated enzyme solution was applied to a Sephacryl HR-200 column (1.6 × 80 cm) equilibrated with the sodium phosphate buffer . The column was washed with the same buffer and 4.8 mL fractions were collected. The active fractions were pooled, then freeze-dried.

Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli *(15)* in a 12% (w/v) polyacrylamide slab gel. The proteins were stained using Coomassie Brilliant Blue R-250.

Isoelectric focusing (IEF) was carried out in the pH range of ampholytes 3.0–9.5 according to Robertson et al. *(16)*. The gel (7.8 cm in length) was sliced vertically into two halves. The piece with protein was stained to determined the protein location and the other piece was sliced vertically into 2-mm slices in order. The slices were immersed in 1.0 mL of 10 m*M* KCl solutions respectively in order overnight. The pH of each fraction was measured with a combination electrode.

N-Terminal Amino Acid Sequencing

The purified RNase was lyophilized and then was sent to Shanghai GeneCore Biotechnologies Co. Ltd., for N-terminal amino acid sequencing. The N-terminal amino acid sequence was analyzed by automatic Edman degradation using an Applied Biosystems Procise 492 cLC protein sequencer (Applied Biosystems, Foster City, CA).

Table 1
Results of Purification of *A. niger* SA-13-20 RNase

Purification steps	Volume (mL)	Protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (-fold)	Yield (%)
Culture filtrate	1000	3954	1.470×10^6	371.8	1.0	100
$(NH_4)_2SO_4$ precipitation	250	385.5	1.095×10^6	2840.9	7.6	74.5
DEAE-cellulose	210	38.4	4.733×10^5	12326	33.2	32.2
Ultrafiltration	65	20.6	3.734×10^5	18126	48.8	25.4
Sephacryl HR-200	76.8	17.8	3.602×10^5	20236	54.4	24.5

Effects of pH, Temperature, Buffer, and Cations on Enzyme Activity

The reaction pH was varied in the range 2.5–10.0 in the following 0.10 *M* buffer: sodium citrate/citric acid (pH 2.5–6.5) or sodium phosphate (pH 7.0–10.0). The reaction temperature was varied in the range 25–90°C. Citrate buffer (pH 3.5) concentration was examined in the range 0.025–0.20 *M*.

The effect of cation on the RNase activity was determined at pH 3.5 and 30°C in 0.175 *M* citrate buffer with the chloride salt at final concentration of 1.0 m*M* cation, viz. KCl, NH_4Cl, $MgCl_2$, $CaCl_2$, $BaCl_2$, $MnCl_2$, $CoCl_2$, $NiCl_2$, $CuCl_2$, $ZnCl_2$, $AlCl_3$, and $FeCl_3$.

Enzyme Stability

The enzyme solution was kept at 4°C for 2 d at different final pH in the range 2.5–10.0. The RNase activity was then determined.

The enzyme solution (pH 5.0) was incubated at different temperatures (40, 45, 50, and 55°C) for a period and an aliquot of the sample was taken out at 15-min intervals. An aliquot was cooled to 0°C quickly, and then used to determine the RNase activity.

Analysis was carried out in duplicate. The data given here are the average of the measurement and the relative errors were less than ±5%.

Results

Purification

The *A. niger* SA-13-20 RNase was purified by using the purification procedure described above and the results obtained are presented in Table 1.

After precipitation with ammonium sulfate, most non-RNase proteins were removed. The protein obtained was about 10% of the total protein amount, but RNase activity yielded was 74.5% of the total activity and purification was 7.6-fold.

During chromatography on DEAE-cellulose (Fig. 1), the bound RNase protein was eluted over a range of buffer gradient (0.40–0.50 *M*). Fractions with absorbance at 260 nm for the RNase activity higher than about 0.100 units were collected together (No. 179–199). This step caused a 33.2-fold purification and a 32.2% yield of RNase activity.

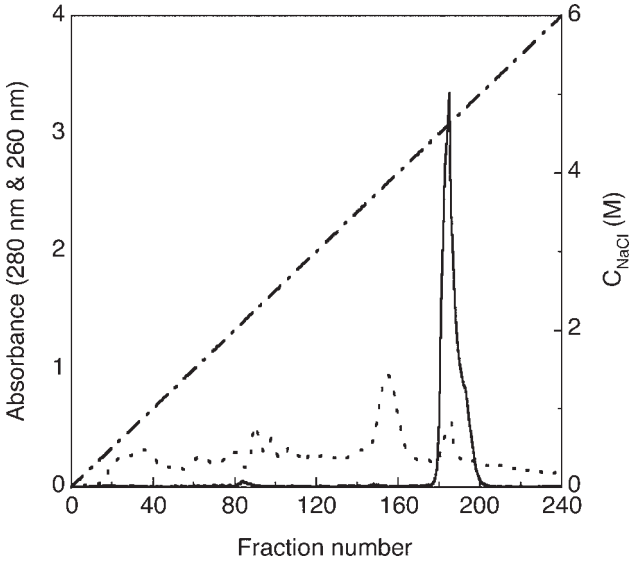

Fig. 1. Ion-exchange of RNase from *A. niger* SA-13-20 on a DEAE-cellulose column. Dotted line: absorbance at 280 nm for protein; solid line: absorbance at 260 nm for RNase activity; dash dotted line: concentration of NaCl.

After ultrafiltration, 210 mL enzyme solution from DEAE-cellulose chromatography was concentrated to 65 mL and partial non-RNase protein was removed further.

The final purification step was performed by gel filtration on Sephacryl HR-200. Two protein peaks were eluted out. The RNase activity appeared in the first protein peak and the activity peak were symmetrical (Fig. 2). The final yield of enzyme activity was 24.5% with a specific activity of 20236 units/mg and a 54.4-fold purification.

Molecular Weight and Isoelectric Point

SDS-PAGE electrophoretograms of protein samples obtained from each purification steps are shown in Fig. 3. A single band was detected after purification on the Sephacryl HR-200 column, which indicated that this RNase was purified to homogeneity by using the simpler scheme of purification than that previously reported *(8,9,17)*. The molecular mass of the purified RNase was estimated as 40.1 kDa from Fig. 3. The enzyme also exhibited a molecular mass of 40.1 kDa on Superose 12 HR gel filtration column suggesting the enzyme is monomeric.

The pI value of the purified enzyme was estimated by isoelectric focusing. The protein showed a single band in electrophoretograms and the pH of corresponding gel slice was 5.3, so the pI value of this RNase is 5.3.

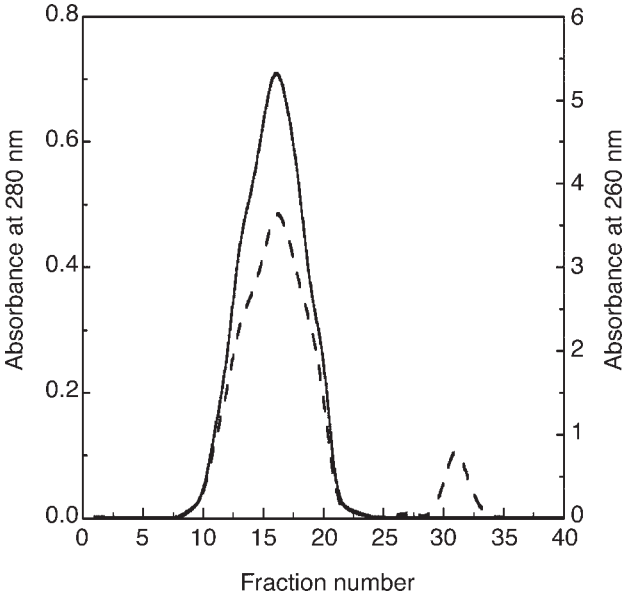

Fig. 2. Gel filtration of RNase from *A. niger* SA-13-20 on a Sephacryl HR-200 column. Dotted line: absorbance at 280 nm for protein; solid line: absorbance at 260 nm for *A. niger* SA-13-20 RNase activity.

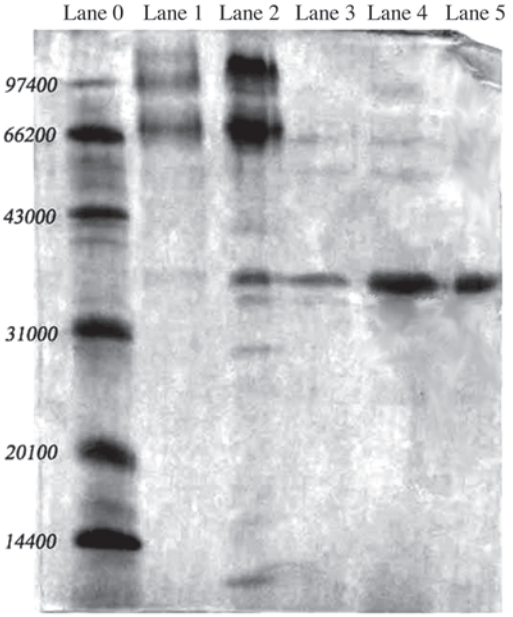

Fig. 3. SDS-PAGE electrophoresis of RNase from *A. niger* SA-13-20. Lane 0, protein molecular weight standards (hen egg white lysozyme, 14400; trypsin inhibitor, 20100; bovine carbonic anhydrase, 31000; rabbit actin, 43000; bovine serum albumin, 66200; rabbit phosphorylase b, 97400); lane 1, crude extract; lane 2, fraction obtained after fractionation with $(NH_4)_2SO_4$; lane 3, fraction obtained after DEAE-cellulose exchange; lane 4, fraction obtained after ultrafiltration; lane 5, fraction obtained after resolution by Sephacryl HR-200.

N-Terminal Amino Acid Sequence

The N-terminal amino acid sequence of the purified RNase was analyzed to the 10th amino acid residue by automatic Edman degradation. The N-terminal amino acid sequence of the purified RNase was TIDTYSSDSP. A homology search of the RNase using NCBI BLASTA showed 90% similarity to RNase M (TIDTCSSDSP) from *Aspergillus saitoi* (GeneBank, Accession No. P19791) and *Aspergillus phoenicis* (GeneBank, Accession No. JX0127). Only one amino acid (position 5) was different. But the molecular weight of *A. niger* SA-13-20 RNase (40.1 kDa) was larger than that of RNase M (26.6 kDa). These results indicate that *A. niger* SA-13-20 RNase is a novel RNase.

Optimum Conditions for Enzymatic Reaction

The maximum activity was observed at pH 3.5. The pH values corresponding to 50% activity were 2.8 and 4.2 and few activity was detected at pH higher than 6.0.

The RNase activity increased with the increase of reaction temperature to reach its maximum at 65°C and then decreased. Thus, the optimum temperature for the hydrolyzation of RNA was. 65°C. The RNase activity was 50% of the maximum at 53°C or 72°C and no activity was detected at 85°C.

Buffer concentration also affected the enzyme activity. The optimum concentration of citrate buffer was 0.175 *M* and the relative activities at 0.025 *M* and 0.25 *M* citrate buffer were 69 and 86%, respectively.

The effects of some cations (chlorides) at the concentration of 1.0 m*M* on the RNase activity were examined. K^+, NH_4^+, Ca^{2+} and Mg^{2+} at 1.0 m*M* caused an increase of the RNase activity about 10%. Cu^{2+}, Ni^{2+}, and Co^{2+} at 1.0 m*M* caused an inhibition of less than 10% in the RNase activity. Ba^{2+}, Mn^{2+}, Zn^{2+}, Al^{3+}, and Fe^{3+} at 1.0 m*M* didn't exhibit obvious effects on the RNase activity. The effects of the concentrations of K^+, NH_4^+, Ca^{2+}, and Mg^{2+} (chlorides) in range of 0–100 m*M* are shown in Fig. 4. Ca^{2+} and Mg^{2+} at higher concentration inhibited the RNase activity badly and they caused the loss of 56 and 67% in the RNase activity at 40 m*M*. The RNase activity was remained 73% in the present of K^+ at 100 m*M*. NH_4^+ always activated the RNase activity by about 13% and this activation was almost independent of NH_4^+ concentration. Experiments also showed that different ammonium salts also affected the RNase activity differently. Compared with absence of ammonium salt, the RNase activities in the present of NH_4Cl, NH_4NO_3, and $(NH_4)_2SO_4$ at NH_4^+ concentration of 0.40 *M* were 116, 67, and 187%, respectively.

Stability of Enzyme Activity

The RNase activity was stable at pH for storage lower than 8.5, but it decreased rapidly when pH for storage was higher than 9.6 and it reduced about a half at pH 10.0.

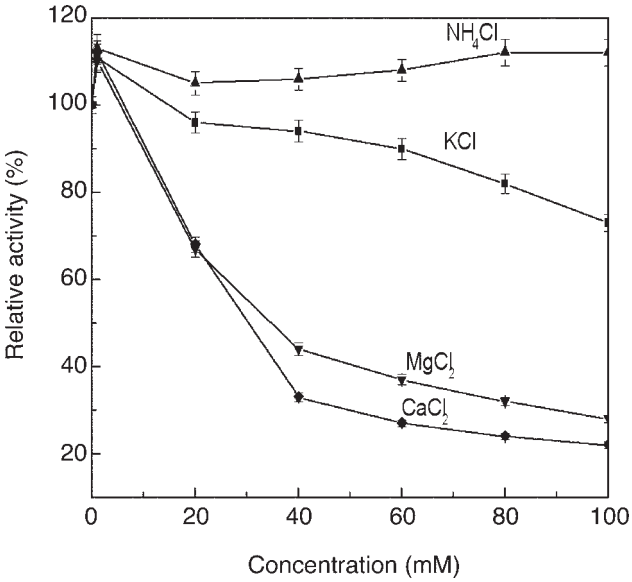

Fig. 4. Effect of chloride on *A. niger* SA-13-20 RNase activity.

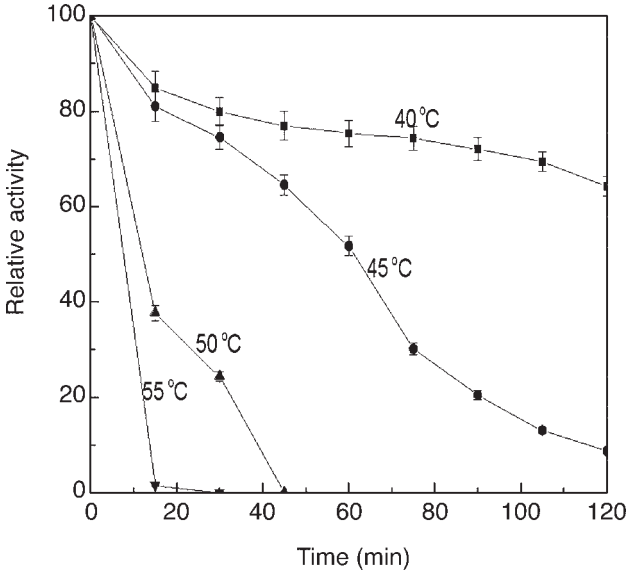

Fig. 5. Thermal stability of *A. niger* SA-13-20 RNase.

Taken the RNase activity of the enzyme solution without heating as 100%, the relative activity decreased with incubation time at different temperature is shown in Fig. 5. After incubation for 2 h at 40 and 45°C, the RNase activities were reduced by 35 and 90%. No activities were detected after 45-min incubation at 50°C and 15-min incubation at 55°C.

Discussion

Fractionation with ammonium sulfate was efficient to remove most of the non-ribonuclease proteins and reduce the volume of enzyme solution during purification of the RNase. Ultrafiltration played an important role in quick volume reduction. The final yield of 24.5% in this purification procedure is acceptable.

The molecular weight and pI value (40.1 kDa and 5.3, respectively) of the purified enzyme from *A. niger* SA-13-20 are different from those of the RNase from *A. niger* ATCC 26550 with 28.5 kDa and 2.8, respectively *(8)*. Although the mutant *A. niger* SA-13-20 was screened from *A. niger* ATCC 26550 *(12)*, it is sure that this RNase from *A. niger* SA-13-20 is not the same RNase from *A. niger* ATCC 26550. So this enzyme was named as *A. niger* SA-13-20 RNase. These two enzymes have the identical optimum pH value of 3.5, but have different optimum temperatures for the hydrolyzation of RNA, which are 65 and 50°C, respectively *(8)*. Compared in the aspect of effects of metals, Ba^{2+}, Cu^{2+}, and Ca^{2+} at 1.0 m*M* showed no obvious effect, a weak inhibition and a slight activation on the *A. niger* SA-13-20 RNase activity, respectively, but Ba^{2+} and Cu^{2+} at 1.0 m*M* inhibited *A. niger* ATCC 26550 RNase activity by 20.3 and 39.9%, respectively and Ca^{2+} at 1.0 m*M* activated it by 23.9% *(8)*. Herein, $(NH_4)_2SO_4$ activated *A. niger* SA-13-20 RNase activity significantly and it is a good precipitation reagent for the RNase protein because it does not cause loss of activity, but its effect on *A. niger* ATCC 26550 RNase were not reported.

Some other RNases had been isolated and characterized. The molecular weight and pI value of *A. niger* SA-13-20 RNase are similar to those of *A. saitoi* RNase with 38.8 kDa and 4.7 *(17)* or *A. oryzae* RNase T2 with 36.6 kDa and 5.0 *(17)*. The molecular weight is larger than that of *A. giganteus IFO 5818* RNase (17.0 kDa) *(9)*, *Trichoderma harzianum* RNase Th (10.3 kDa) *(18)*, or *Saccharomyces cerevisiae* RNase (29.0 kDa) *(19)*; however, pI value of *A. niger* SA-13-20 RNase is lower than that of these RNases *(9,18,19)*. The optimum pH (3.5) of *A. niger* SA-13-20 RNase is near to that of *Monascus* RNase (4.2) *(20)*, *Russulus virescens* RNase (4.5) *(21)*, or *A. saitoi* RNase (5.0) *(17)*. Its optimum temperature at 65°C is identical to that of *S. acidocaldarius* RNase *(22)*, and is near to that of *R. virescens* RNase at 60°C *(22)* or *Cantharellus cibarius* RNase at 70°C *(23)*.

A. niger SA-13-20 RNase is stable in a wide pH range (lower than 8.5), which is near to that for the stability of *Candida lipolytica* RNase (pH 3.5–9.0) *(24)*. *A. niger* SA-13-20 RNase was denatured completely after incubation at 55°C for 15 min, but *S. solfataricus* RNase retained 60% activity after incubation at 83°C for 15 min *(25)*. The optimum temperature for the enzymatic reaction of *A. niger* SA-13-20 RNase is higher than 55°C by 10°C. This indicates that its thermostability is enhanced after it is bound with its substrate.

Although there are differences in the molecular weight, pI value and some physico-chemical properties between *A. niger* SA-13-20 RNase and that from the parent strain, further studies (such as amino acid composi-

tions, base specificity, thermodynamic parameters, etc.) are needed to elucidate the properties of the enzyme.

Acknowledgments

We are grateful to National Natural Science Foundation of China and the Natural Science Foundation of Guangdong Province for their financial support.

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