Kasetsart J. (Nat. Sci.) 40 (Suppl.) : 17 - 25 (2006)

Extracellular Halophilic Ribonuclease from a Halotolerant *Pseudomonas* sp. : Purification and Characterization

Werasit Kanlayakrit*, Anan Boonpan, Takahiro Ikeda and Somporn Tojai

ABSTRACT

A halotolerant bacterium, *Pseudomonas* sp. No. 3241, isolated from Thai fish sauce obtained in Suratthani province, produced an extracellular ribonuclease when cultivated aerobically without NaCl in Sehgal and Gibbons complex medium (SGC medium). Ribonuclease was purified by ethanol precipitation, Sephadex G-150 gel filtration and DEAE Toyopearl 650M anion-exchange chromatography. The purity and molecular weight were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The result showed that the molecular weight of ribonuclease from halotolerant *Pseudomonas* sp. No. 3241 was 61,000 daltons. Purified ribonuclease was optimum at pH 10.0 and at temperature of 40°C. Purified ribonuclease was stable between pH 6.0 and 10.0 and at temperatures between 30 and 40°C. This enzyme had marked halophilic enzyme properties that required an optimum NaCl concentration of 3.0 M (18%).

Key words: halophilic enzyme, ribonuclease, halotolerant bacteria, *Pseudomonas* sp., purification

INTRODUCTION

As halophilic means "salt loving", halophilic enzymes or bacteria by definition require NaCl for activity or growth. In contrast, halotolerant forms do not require NaCl for growth (Garabito *et al*., 1998). Nuclease comprises deoxyribonuclease (DNase) and ribonuclease (RNase). DNase catalyses deoxyribonucleic acid (DNA) to 5′-deoxyribo-nucleotides (5′-GMP, 5′-AMP, 5′-CMP and 5′-TMP) or 2′,3′-nucleotides. RNase, on the other hand, catalyses ribonucleic acid (RNA) to 5′-ribo-nucleotides (5′-GMP, 5′-AMP, 5′-CMP and 5′-UMP) or 2′,3′-nucleotides (Kuninaka, 1986). Nucleotide-5′-monophosphates, especially 5′-IMP and 5′-GMP are flavour enhancers that interact synergistically with monosodium glutamate (MSG) and nucleotide-5′-monophosphate (Kuninaka *et al.*, 1961).

Kamekura and Onishi (1974) first reported production of a halophilic nuclease by the moderately halophilic bacterium, *Micrococcus varians* var. *halophilus*. This enzyme has marked halophilic properties, requiring an optimal level of 2.9 M NaCl. Furthermore, Onishi *et al.* (1983) reported a halophilic nuclease from a moderately halophilic *Bacillus* sp. N23-2 that was later identified by Ventosa (1991) as *Bacillus halophilus*. Maeda and Taga (1976a, b) also studied an extracellular deoxyribonuclease from *Vibrio* sp. strain No. 2 that was isolated from seawater, but they did not report any halophilic nuclease properties. These halophilic and marine

Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Bangkok 10900, Thailand.
* Corresponding author, e-mail: fagiwrk@ku.ac.th

Received date : 18/04/05 Accepted date : 30/06/05

bacterial nucleases showed both ribonuclease and deoxyribonuclease activities in the presence of salt (Kamekura and Onishi, 1974; Onishi *et al.* 1983; Maeda and Taga, 1976b). As for halophilic ribonuclease production by halotolerant bacteria, only ribonuclease of *Pseudomonas* sp. No. 3241 has been reported (Kanlayakrit *et al*., 2001). This specie is of interest for its potential use in industrial applications. However, little is known about the characteristics of the purified ribonuclease from this bacterium. This paper describes the purification and some properties of the halophilic ribonuclease produced from halotolerant *Pseudomonas* sp. No. 3241.

MATERIALS AND METHODS

1. Bacterial strain and media

The bacterium used in this study was a halotolerant bacterium isolated from fermented fish sauce in Suratthani province, Thailand. This bacterium was identified as *Pseudomonas* sp. No. 3241 by Kanlayakrit *et al.* (2001). The stock culture was maintained on agar slant of Sehgal and Gibbons complex medium (SGC medium) (Sehgal and Gibbons, 1960) containing 2.0 M NaCl at 4°C. SGC medium contains 7.5 g/l casamino acids (Difco Laboratories), 10 g/l yeast extract (Merck), 2.0 g/l KCl, 3.0 g/l sodium citrate, 20.0 g/l $MgSO_4.7H_2O$ and 0.01 g/l $FeCl_2.nH_2O$. The medium was adjusted to pH 7.0 by 0.5 M NaOH.

2. Ribonuclease production

The SGC medium was supplemented with twice of the amount of nitrogen sources (15g/l casamino acids, 20 g/l yeast extract) and lacking of NaCl was used for enzyme production (Kanlayakrit *et al.*, 2001). Inocula (150 ml) of a 18 hr culture grown in 2.0 M NaCl SGC medium were added to 2-liters mini jar fermenter (Eyela Mini Jar Fermenter Model M100, Japan). Modified SGC medium for ribonuclease production contained 1,350 ml of SGC production medium. Fermentation conditions were maintained at 30°C, 600 rpm agitation and air supply at 1.0 vvm for 48 hr. The pH of the broth was not controlled during this process. After cultivation, the culture broth was centrifuged at 8,000 ×g for 20 min and the supernatant was used as the crude enzyme solution.

3. Ribonuclease activity assay

Halophilic ribonuclease activity was measured by a modified method of Kamekura and Onishi (1974). Each 1 ml of assay substrate contained 1 mg/ml ribonucleic acid (from *Torula* yeast), 1.8 mM NaCl, 0.04 mM Tris-HCl buffer (pH 8.0), and 0.01 mM $MgSO_4 .7H_2O$. This was mixed with 1 ml enzyme solution (dialysed against 0.01 M Tris-HCl buffer, pH 8.0, containing 2.0 M NaCl at 4 °C) and incubated at 40°C for 2 hr. Enzyme activity was terminated with 6 ml 99.9% cold ethanol for 20 min at 0°C. The precipitate was removed by centrifugation at 6,000 ×g at 4°C for 20 min. The supernatant (1 ml) was diluted 5 times with distilled water and absorbance was measured at 260 nm. A blank measure was carried out using 1 ml of substrate solution alone, followed by incubation at 40°C for 2 hr. This was then supplemented with 1 ml of enzyme solution followed immediately with 6 ml 99.9% cold ethanol. One unit of the ribonuclease activity was defined as the amount of enzyme catalyzing an increase of 1.0 in absorbance at 260 nm under the above conditions.

4. Purification of ribonuclease

All purification steps were carried out at 5°C. In all cases, the Tris-HCl buffer referred to 0.01 M Tris-hydroxychloride buffer, pH 8.0. The crude enzyme solution obtained from the mini jar fermenter was mixed slowly with an equal volume of 99.9% cold ethanol at 0°C on ice. After standing overnight, the mixture was centrifuged at 10,000 ×g for 20 min. The precipitate was dissolved in a

minimum volume of Tris-HCl buffer, followed by dialysis against Tris-HCl buffer for 24 hr and then concentrated by lyophilization. A 200 mg of lyophilized enzyme was dissolved in 5.0 ml Tris-HCl buffer and applied to a Sephadex G-150 gel filtration column (2.0 by 100 cm) equilibrated with Tris-HCl buffer. The eluted enzyme was concentrated and rechromatographed on the Sephadex G-150 gel filtration column (2.0 by 100 cm) equilibrated with Tris-HCl buffer. The eluted enzyme from rechromatographed was concentrated and applied to DEAE Toyopearl 650M anion exchange column (2.0 by 50 cm) equilibrated with Tris-HCl buffer. After being washed, the ribonuclease was eluted by a linear gradient of 0-1.0 M NaCl. The active fractions were combined and dialysed against Tris-HCl buffer. The purified enzyme was concentrated by lyophilization and stored at 5°C.

5. Gel electrophoresis

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of protein was performed by a modified procedure of Laemmli (1970). A 12% (w/v) polyacrylamide gel was used in this experiment and high molecular weight standard mixture (Sigma) was used as the standard protein for determination of the molecular weight of the enzyme.

6. Analytical methods

The protein concentration during most stages of enzyme purification was determined by the Folin-Phenol method (Lowry *et. al.*, 1951) with bovine serum albumin (Sigma, USA) as a standard. The protein concentration of each fraction separated by column chromatography was estimated by measuring the absorbance at 280 nm (UV-VIS spectrophotometer, UV-120 SHIMADZU, Japan)

7. Characterization of purified ribonuclease

The optimum pH for ribonuclease purification was determined at 40°C for 2 hr in 0.04 M buffers of various pH values (pH 4.0-12.0) containing 1.8 M NaCl. To determine the pH stability, the purified ribonuclease (pH 8.0) was mixed with buffer at a ratio of 1:1. The mixture was kept at 4°C for 24 hr in 0.04 M buffers of various pH values (pH 4.0-12.0) containing 1.8 M NaCl. Subsequently, pH was adjusted to 8.0 by adding 1 M Tris-HCl, pH 8.0, and residual ribonuclease activity was measured.

The optimum pH and pH stability were determined using the following buffers: 0.04 M Acetate buffer (pH 4.0-5.0), 0.04 M Tris-acetate buffer (pH 6.0-7.0), 0.04 M Tris-HCl buffer (pH 7.5-9.0), 0.04 M Glycine-NaOH buffer (pH 9.5-12.0). The optimum temperature for ribonuclease purification was determined at various temperatures (30-70°C) for 2 hr in 0.04 M Tris-HCl buffer, pH 8.0, containing 1.8 M NaCl.

To determine temperature stability, purified ribonuclease solutions prepared in 0.04 M Tris-HCl buffer, pH 8.0, containing 2.0 M NaCl were kept for 30 min at various temperatures (30-70°C). The mixture was then cooled to 4°C and residual ribonuclease activity was measured.

The effect of NaCl on the purified ribonuclease activity was determined by measuring the activity at various NaCl concentrations (0-4.0 M NaCl) for 2 hr at 40°C in 0.04 M Tris-HCl buffer, pH 8.0.

RESULTS

1. Enzyme purification

The procedures for the purification of ribonuclease were described in detail as followed, provided all purification steps were carried out at 5°C and Tris-HCl buffer refered to 0.01 M Tris-hydroxychloride (pH 8.0).

(I) Cell-free filtrate

The culture broth was subject to centrifugation at 8,000 ×g for 20 min to remove cells, yielding about 1.5 liters of clear solution with

a protein concentration of 3.60 mg/ml and a specific activity of 3.22 U/mg of protein.

(II) Ethanol precipitation

The cell-free filtrate was mixed slowly with an equal volume of 99.9% cold ethanol at 0°C on ice and kept overnight. The precipitation obtained was collected by centrifugation and dissolved in 150 ml of Tris-HCl buffer, followed by dialysis against Tris-HCl buffer for 24 hr.

(III) First Sephadex G-150 gel filtration

The enzyme solution was concentrated by lyophilization. A 200 mg of lyophilized enzyme was dissolved in 5.0 ml Tris-HCl buffer and applied to Sephadex G-150 gel filtration column (2.0 by 100 cm) equilibrated with Tris-HCl buffer and fractions of 4.0 ml were collected. In the peak fraction of eluted enzyme did not coincide with protein concentration and ribonuclease activity (Figure 1 A).

(IV) Second Sephadex G-150 gel filtration

The active fraction was concentrated by lyophilization. A 200 mg of lyophilized enzyme was dissolved in 5.0 ml Tris-HCl buffer and fractionated on a second Sephadex G-150 column by the same procedure. In the peak fraction, eluted enzyme did not coincide with protein concentration and ribonuclease activity (Figure 1 B).

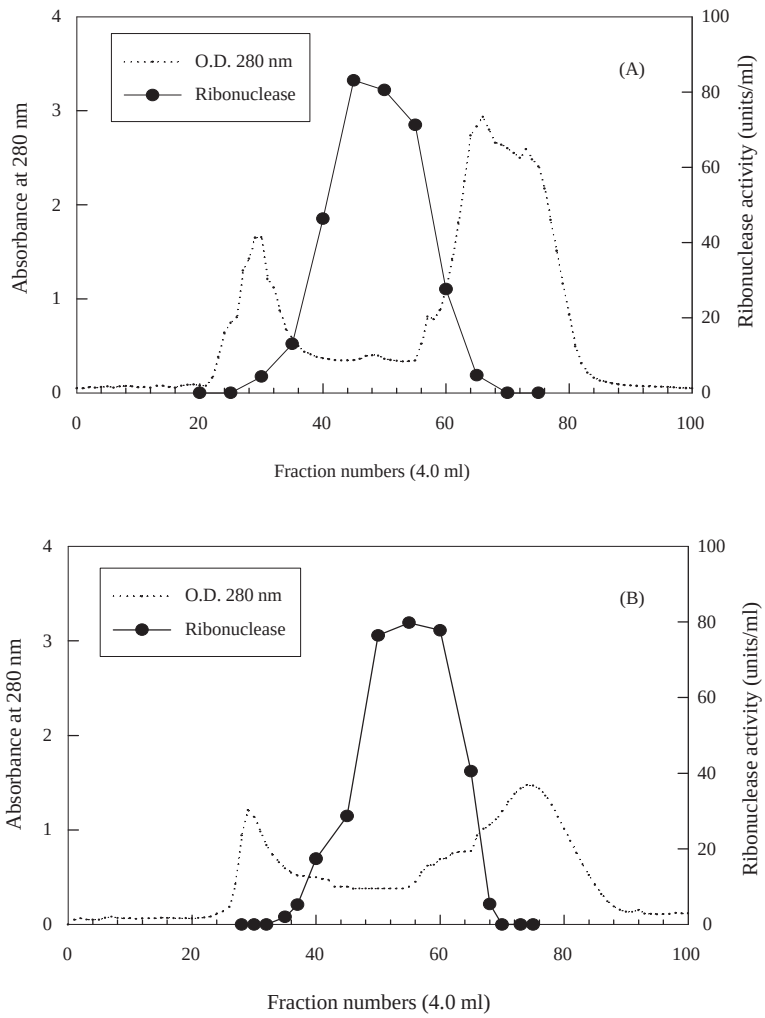

Figure 1 1st (A) and 2nd (B) elution patterns of ribonuclease on Sephadex G-150 gel filtration.

(V) DEAE Toyopearl 650M anion exchange chromatography

The active fraction was concentrated by lyophilization. A 200 mg of lyophilized enzyme was dissolved in 5.0 ml Tris-HCl buffer and passed through a DEAE Toyopearl 650M anion exchange column (2.0 by 50 cm) equilibrated with Tris-HCl buffer. Ribonuclease was adsorbed into the column. A linear gradient elution was carried out in Tris-HCl buffer containing 1.0 M NaCl. Fractions of 4.0 ml were collected, and the enzyme was eluted on 0.4 to 0.5 ionic strength gradients (Figure 2).

The purification steps summarized in Table 1 disclosed 78 folds purification over the culture filtrate with a 7.57% recovery of activity.

Enzyme obtained from DEAE Toyopearl 650M anion exchange column was examined by SDS-PAGE. The ribonuclease gave a single band on the polyacrylamide gel (Figure 3). The molecular weight of the ribonuclease was determined by comparison of mobility (R_f) of ribonuclease and standard proteins of known molecular weight on the acrylamide gel. The semilogalithmic plot of the molecular weight of standard protein versus their relative mobilities showed that the molecular weight of the ribonuclease was 61,000 daltons.

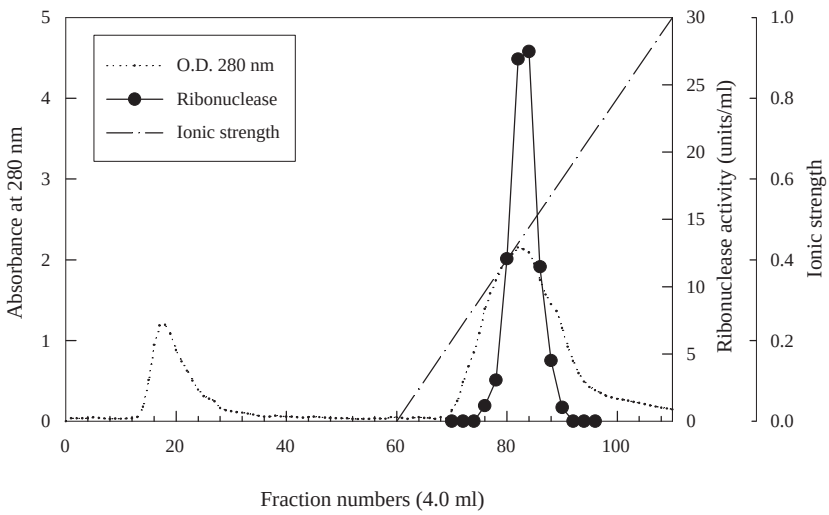

Figure 2 Elution pattern of ribonuclease on DEAE Toyopearl 650M anion exchange chromatography.

Table 1 Purification of the ribonuclease of halotolerant *Pseudomonas* sp. No. 3241.

Purification step	Volume (ml) (mg)	Total protein (units)	Total activity protein)	Specific act. (units/mg	Purification folds	Yield %
Culture filtrate	1500	5400	17400	3.22	1	100
50% Ethanol precipitate	150	84.00	14527.50	172.94	53.70	83.49
Dialysis	165	82.50	14290.65	173.22	53.79	82.13
1st Sephadex G-150	130	58.50	11008.40	188.17	58.43	63.29
2nd Sephadex G-150	120	43.20	9626.40	222.83	69.20	55.32
DEAE Toyopearl 650M	48	5.28	1317.60	249.54	77.49	7.57

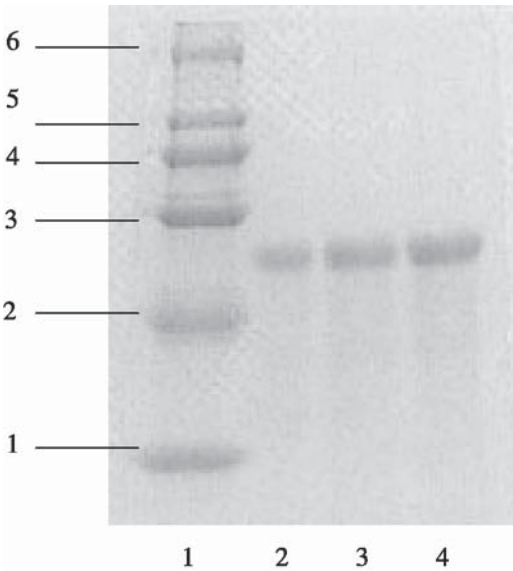

Figure 3 SDS-PAGE of the ribonuclease. Lane1: standard protein,1: carbonic anhydrase (29,000 daltons), 2: egg albumin (45,000 daltons), 3: bovine albumin (66,000 daltons), 4: phosphorylase b (97,000 daltons), 5: β-galactosidase (116,000 daltons), 6: myosin (205,000 daltons), lane 2,3 and 4: purified ribonuclease.

2. Characterization of purified ribonuclease

Enzyme characteristics were studied using purified ribonuclease obtained from DEAE Toyopearl 650M anion exchange column chromatography. The purified ribonuclease activity had an optimal pH and temperature of 10.0 and 40 °C, respectively (Figure 4). The purified ribonuclease was stable between pH 6.0 and 10.0 and at temperature between 30 and 40 °C (Figure 5). The effect of NaCl on activity of the purified ribonuclease was presented in Figure 6. This enzyme had marked halophilic properties, which required an optimal NaCl level of 3.0 M (18%). From these properties, it was concluded that purified ribonuclease from halotolerant *Pseudomonas* sp. No. 3241 is a form of halophilic ribonuclease.

DISCUSSION

There are only a few reports of halophilic nuclease production by halophiles, including *Micrococcus varians* var. *halophilus* (Kamekura and Onishi, 1974) and *Bacillus halophilus* (Onishi *et al*., 1983; Ventosa, 1991). In these experiments, halophilic nucleases were produced from

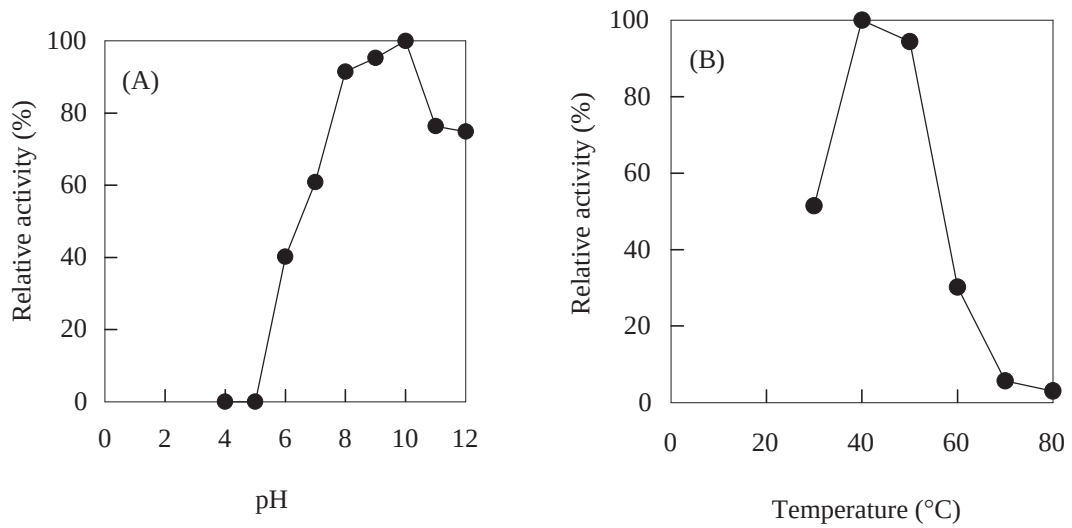

Figure 4 Effect of pH (A) and temperature (B) on activity of the purified ribonuclease from halotolerant *Pseudomonas* sp. No. 3241.

moderately halophilic bacteria, but not from halotolerant bacteria. In the previous study halophilic ribonuclease from a halotolerant *Pseudomonas* sp. No.3241, found by Kanlayakrit *et al*. (2001), is the first report on a halophilic ribonuclease. The authors found that a halotolerant *Pseudomonas* sp. No.3241 produced an extracellular ribonuclease during growth in medium without NaCl and enzyme had marked halophilic properties, which required an optimal NaCl level of 3.0 M. The purification and properties of halophilic ribonuclease were described here.

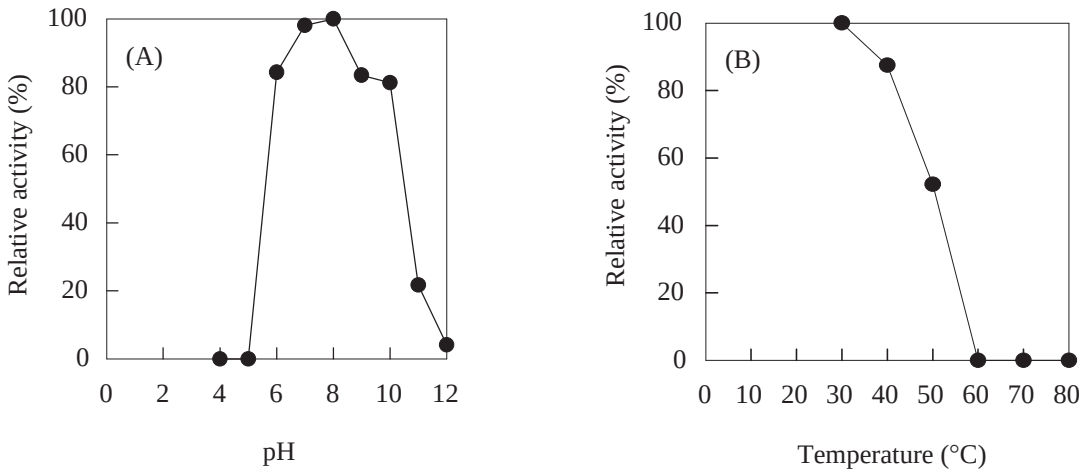

Figure 5 pH (A) and temperature (B) stability of the purified ribonuclease from halotolerant *Pseudomonas* sp. No. 3241.

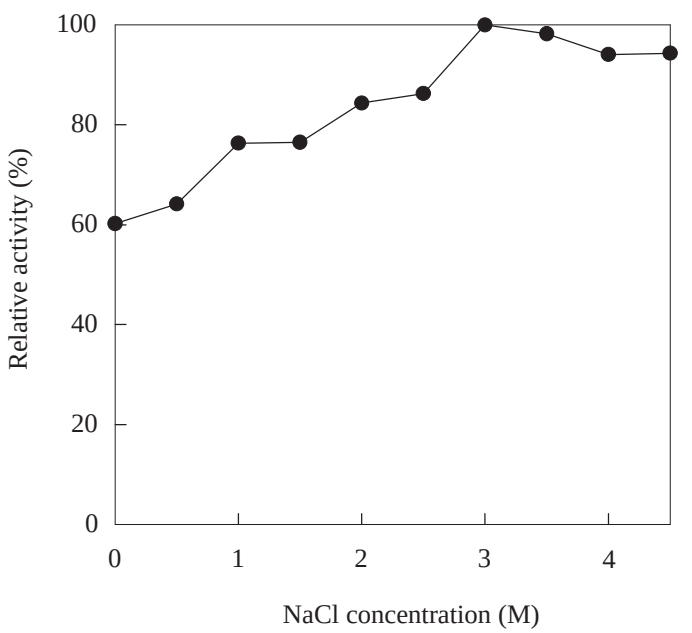

Figure 6 Effect of NaCl on activity of the purified ribonuclease from halotolerant *Pseudomonas* sp. No. 3241.

As shown in Figure 1, the peak fraction of eluted enzyme did not coincide with protein concentration and ribonuclease activity. These showed that a small portion of the contaminating protein was removed from the ribonuclease fraction in this step, thus Sephadex G-150 gel filtration column was not suitable and low efficiency for ribonuclease purification. However, ribonuclease was further purified using DEAE Toyopearl 650 M anion exchange column chromatography with a linear gradient of 0-1.0 M NaCl in 0.01 M Tris-HCl buffer (pH 8.0), the results showed that ribonuclease was adsorbed into the column and the enzyme was eluted on 0.4 to 0.5 ionic strength gradients (Figure 2). In this step a large portion of the contaminating protein was removed from the ribonuclease fraction. The purified enzyme gave a single band on polyacrylamide gel electrophoresis (Figure 3). The results suggested that the purification procedures in this study is suitable for ribonuclease purification.

Enzyme activity (units/ml) was increased with further purification steps and yield of total units was low, through the overall purification folds was increased to 77.49 times compared to the enzyme of culture filtrate (Table 1). These results indicated that ribonuclease activity was lost during purification and the loss of activity caused by various steps during purification, so the more efficient procedure for ribonuclease purification should be studied in order to decrease purification step and give higher enzyme activity and yield of total units.

The properties of purified ribonuclease of halotolerant *Pseudomonas* sp. No. 3241 resemble purified nucleases of *Bacillus* sp. (Onishi *et al*., 1983) and *Micrococcus varians* subsp. *halophilus* (Nuclease H) (Kamekura and Onishi, 1974, 1978) in many respects such as optimal pH and temperature for the enzyme activity and halophilic properties. The purified ribonuclease of halotolerant *Pseudomonas* sp. No. 3241 required 3.0 M NaCl for maximal activity, whereas the nuclease of *Bacillus* sp. required 1.4-3.2 M NaCl and nuclease H required 2.9 M NaCl for maximal activity. These results indicated that the ribonuclease of halotolerant *Pseudomonas* sp. No. 3241 had marked halophilic properties. The purified ribonuclease of halotolerant *Pseudomonas* sp. No. 3241 showed a single band on the polyacrylamide gel with molecular weight of 61,000 daltons, the molecular weight was considerably smaller than other halophilic nucleases described by Onishi *et al*.(1983) and Kamekura and Onishi.(1978), who reported 138,000 and 99,000 daltons, respectively.

CONCLUSION

The properties of purified halophilic ribonuclease from *Pseudomonas* sp. No. 3241 has several advantageous features for industrial applications. The purified halophilic ribonuclease activity was highest in the presence of 3.0 M (18%) NaCl, these fact suggests a possible application for purified halophilic ribonuclease in the commercial production of 5′-GMP by enzymatic hydrolysis of RNA with low risk of microbial contamination. In addition, studied on halophilic ribonuclease from *Pseudomonas* sp. No. 3241 may lead to further application in fish sauce production for flavor improvement.

LITERATURE CITED

Garabito, M.J., M.C. Maquez and A. Ventosa. 1998. Halotolerant *Bacillus* diversity in hypersaline environments. **Can. J. Microbiol.** 44: 95-102.

Kamekura, M. and H. Onishi, 1974. Halophilic nuclease from a moderately halophilic *Micrococcus varians*. **J. Bacteriol.** 119: 339-344.

Kamekura, M. and H. Onishi, 1978. Properties of the halophilic nuclease of a moderate

halophile, *Micrococcus varians* subsp. *halophilus*. **J. Bacteriol.** 133: 59-65.

Kanlayakrit, K., T. Ikeda, S. Tojai, P. Bovornreungroj and A. Boonpan. 2001. Production and characterization of extracellular halophilic ribonuclease from halotolerant *Pseudomonas* sp. **Bulletin of National Pintung University of Science and Technology.** 10: 281-289.

Kuninaka, A. 1986. Nucleic acids, nucleotides and related compounds, pp. 3-39. *In* H.J. Rehm, G. Reed, H. Pape and H.J. Rehm (eds.). **Biotechnology. Vol. 4, Microbial products II**. VCH Verlagsgesellschaft. Weinheim.

Kuninaka, A., M. Kibi, H. Yoshino and K. Sakaguchi. 1961. Studies on 5′-phosphodiesterases in microorganisms. Part 2. Properties and application of *Pencillium citrinum* 5′-phosphodiesterase. **Agric. Biol. Chem.** 25: 693-701.

Laemmli, U.K. 1970. Cleavage of structural during the assembly of the head of bacteriophage T4. **Nature (London).** 277: 680-685.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall. 1951. Protein measurement with the folin phenol reagent. **J. Biol. Chem.** 193: 265-275.

Maeda, M. and T. Taga. 1976a. Extracellular nuclease produced by a marine bacterium. I. Extracellular deoxyribonuclease formation by a marine *Vibrio* sp. **Can. J. Microbiol.** 22: 1437-1442.

Maeda, M. and T. Taga. 1976b. Extracellular nuclease produced by a marine bacterium. II. Purification and properties of extracellular nuclease from a marine *Vibrio* sp. **Can. J. Microbiol.** 22: 1443-1452.

Onishi, H., T. Mori, S. Takeuchi, K. Tani, T. Kobayashi and M. Kamekura. 1983. Halophilic nuclease of a moderately *Bacillus* sp.: production, purification and characterization. **Appl. Environ. Microbiol.** 45: 24-30.

Sehgal, S.N. and N.E. Gibbons. 1960. Effect of some metal ions on the growth of *Halobacterium cutirubrum*. **Can. J. Microbiol.** 6: 165-169.

Ventosa, A. 1991. Taxonomy of new species of moderately halophilic eubacteria, pp. 45-51. *In* F.R.Valera, (ed.). **General and Applied Aspects of Halophilic Microorganisms.** Plenum Press. New York.