International Scholars Journals

Advanced Journal of Microbiology Research ISSN 2241-9837 Vol. 13 (2), pp. 001-007, February, 2019. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Isolation and characterization of a cold-active amylase from marine *Wangia* sp. C52

Jianguo Liu[1]*, Zhiqiang Zhang[1], Hongyue Dang[1], Jianren Lu[2] and Zhanfeng Cui[3]

[1]Center for Bioengineering and Biotechnology, China University of Petroleum (East China), Qingdao 266555, People's Republic of China.
[2]School of Physics, Manchester University, Manchester M60 1QD, UK.
[3]Department of Engineering Science, University of Oxford, Oxford OX1 3PJ, UK.

Accepted 7 January, 2019

Amylase activity was detected in the culture medium of marine Wangia sp. C52, which was isolated from the Southern Okinawa Trough deep-sea sediment. In the present study, a cold-active amylase was purified to homogeneity from the culture broth by ammonium sulfate precipitation and gel filtration chromatography. The molecular mass of the amyalse was 58 kDa, and its isoelectric point was close to 5.6. The optimal pH and temperature were 30°C and 6.0, respectively. In the presence of Ca2+ and Co2+, the enzyme activity was stimulated while Cu2+, Hg2+, Mn2+, Zn2+, Fe3+, Al3+, EDTA, EGTA and SDS reduced the activity. Km and Vmax values of the purified enzyme for soluble starch were 2.08 ± 0.3 mg/ml and 1.26 ± 0.02 mg/ml/min, respectively. The final purified enzyme had -helix of 25%, -sheet of 26% and random coil of 49%, consistent with the theoretical values. This showed that the purified amylase folded with a reasonable secondary structure.

Key words: Cold-active amylase, *Wangia* sp., purification, enzyme characterization.

INTRODUCTION

Cold-active amylase is one of the earliest studied psychrophilic enzymes, which is widely used as the model enzyme for the purpose of studying protein adaptation to cold at a molecular level (Aghajari et al., 1998; D'Amico et al., 2003). At present, this extensively studied enzyme is mainly produced by polar microorganisms including *Alteromonas* (Feller et al., 1992), *Nocardiopsis* (Zhang and Zeng, 2008), *Janthinobacterium* (Mannisto and Haggblom, 2006), *Duganella* (Mannisto and Haggblom, 2006), and *Cytophaga* (Nichols et al., 1999). However, little attention has been put on the enzyme from deep-sea microorganisms. The ocean covers 71% of the earth's surface with an average depth of 3800 m, and the deep-sea is the largest low-temperature environment in the earth, which must contain abundant resources of psychrophilic microorganisms and enzymes (Polymenakou et al., 2009).

In our former research, a total of 98 heterotrophic bacteria were isolated from the deep-sea sediments in the Southern Okinawa Trough, and the diversity of their extracellular hydrolytic enzymes was also studied (Dang et al., 2009). The results showed that the strain C52 could produce large amount of cold- active amylase, and even had obvious amylase activity at 4°C. The strain C52 was identified as *Wangia* sp., which was a new genus of the Flavobacteriaceae family described in 2007 for the first time (Qin et al., 2007).

In the present work, a cold-active amylase was purified

*Corresponding author. E-mail: jianguoliu@upc.edu.cn. Tel/fax: +86 532 86981566.

Abbreviations: CD, Circular dichroism; **IEF,** isoelectric focusing; **SDS-PAGE,** sodium dodecyl sulfate-polyacrylamide gel electrophoresis; **SDS,** sodium dodecyl sulfate; **PMSF,** phenylmethane-sulfonyl fluoride; **EDTA,** ethylenediaminetetraacetate; **EGTA,** [ethylenebis(oxonitrilo)]tetra-acetate; **DTT,** 1,4-dithiothreitol; **p*I*,** isoelectric point.

from marine *Wangia* sp. C52 by ammonium sulfate precipitation and gel filtration chromatography. The resulted amylase product was then analyzed by circular dichroism (CD), isoelectric focusing (IEF) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), to confirm its molecular secondary structure, isoelectric point and molecular weight. This is the first report on purification and characterization of a cold-active amylase from *Wangia* sp.

MATERIALS AND METHODS

The following chemicals were obtained from Sigma and used as supplied: phenylmethane-sulfonyl fluoride (PMSF), sodium dodecyl sulfate (SDS), ethylenediaminetetraacetate (EDTA), [ethylenebis(oxonitrilo)]tetra-acetate (EGTA), 1,4-dithiothreitol (DTT) and bovine serum albumin (BSA). Glucose, tryptone, yeast extract and agar were purchased from Oxoid Ltd. (Basingstoke, England). The other chemicals used in the preparation were all purchased from Sinopharm Chemical Reagent Corporation and were of analytical grade or higher grade.

Microorganism and culture media

The heterotrophic bacteria were isolated from the Southern Okinawa Trough deep- sea sediment (Dang et al., 2009) and identified as *Wangia* sp. (China Center for Type Culture Collection, AB 209217). The strain was maintained on LB agar medium containing 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 20 g/L agar and kept at 4°C. One loop of the bacteria strain cells was transferred to 50 ml of LB medium as seed culture, which was prepared with distilled water in 250 ml flask. After aerobically cultivated for 48 h (20°C), a 4% (v/v) inoculum was transferred to 50 ml of production medium in orbital shakers at 180 rpm and then cultivated for 120 h at 20°C. The production medium was consisted of 6.38 g/L starch, 33.84 g/L tryptone, 3.00 g/L yeast extract, 30.00 g/L NaCl, 0.60 g/L $MgSO_4$, 0.56 g/L $CaCl_2$ and adjusted to pH 7.18 with 1.0 M HCl and 1.0 M NaOH. Two hundred milliliter of cell-free supernatant was harvested by centrifugation at 5000×*g* at 4°C for 10 min and used as crude enzyme solution for purification.

Amylase purification

The crude enzyme solution was subjected to ammonium sulfate precipitation, and the protein precipitating at 70% saturation was resuspended in 50 mM citrate buffer (pH 6.0) and dialyzed against the same buffer for 18 h. The dialyzed solution was then applied to a Sephadex G-75 column and eluted with 50 mM citrate buffer (pH 6.0). The active fractions were collected and concentrated using Amicon Ultra centrifugal units (from Sigma, 5 kDa molecular weight cut-off, Cat. No. Z648019). Finally, the resulting enzyme preparation was desalted by dialysis. All the purification experiments were performed at 4°C.

Enzyme assay and protein determination

The activity of amylase was measured by modified Bernfeld method (Berfeld, 1955). In brief, 1.5 ml of 1% (w/v) soluble starch solution made in 50 mM citrate buffer (pH 6.0) mixed with 0.5 ml of appropriately diluted crude enzyme extract. The reaction mixture was incubated at 35°C for 20 min. The liberated reducing sugars were estimated by the dinitrosalicylic acid test (Miller, 1959). The colors developed were read at 540 nm on a UV-2450 (SHIMADZU, Japan). Calibration curve was constructed using glucose as the standard. To subtract the reducing sugars caused by the medium from experimental results, the experiments without the addition of starch were conducted and the measured sugar content was deducted from the assay. One unit (U) of amylase was defined as the amount of enzyme releasing 1.0 µg of glucose equivalent per minute under the assay conditions. The protein concentration was measured with bovine serum albumin as a standard (Sedmak and Grossberg, 1977). All measurements in this experiment were made in triplicate.

Electrophoresis

The isoelectric point (p*I*) of amylase was examined with the Rotofor Isoelectric Focusing cell (Bio-Rad, U.S.A.) using Bio- Lyte ampholyte (pH 3.9 to 9.5, Cat. No. 163-1112) to produce the necessary pH gradient. The amylase sample (18 ml) was loaded into the Rotofor cell and constant power (10 W) was applied with the system cooled to 4°C. Initial voltage was 570 V and a plateau of 1100 V was reached after 4 h. A total of 20 fractions were collected into separate vials, using a vacuum source attached through plastic tubing to an array of 20 needles. The amylase assay was measured for each of the Rotofor fractions, and pH values determined.

The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed under reducing conditions using NuPAGE 4 to 10% Bis-Tris mini gels (Invitrogen, USA), following the manufacturer's instructions. Protein bands were detected by staining with Brilliant Blue Coomassie G-250.

Circular dichroism determination of secondary strucutre of amylase

Circular dichroism (CD) experiments were preformed on PMS 450 spectropolarimeter (Biologic, France) with a 1 mm path length cell at 25°C. The purified amylase sample was concentrated to 1.0 g/L and the reference solvent was ultrapure water. The CD spectrum was recorded over a wavelength range of 190 to 250 nm with 1 nm resolution and 5 s of average time. Ultrapure water was used as a blank to correct the baseline. Results were expressed as the molar mean residue ellipticity ( ) at a given wavelength.

Kinetic studies

To obtain K_m and V_{max} for soluble starch, 2 ml of 0.2, 0.5, 1, 1.5, 2, 3, 4, 5 and 6% (w/v) soluble starch in 50 mM citrate buffer (pH 6.0) was mixed with 2 ml of the purified amylase (the final enzyme concentration was 1.0 U/ml), respectively, and the mixture was incubated at 35°C for 20 min and the reaction was stopped immediately by heating at 100°C for 10 min. K_m and V_{max} values were obtained from Lineweaver-Burk plot and expressed as the mean of the three different experiments.

Effect of pH and temperature on activity of the amylase

The influence of pH on amyalse activity was determined at 35°C in 50 mM citrate buffer (pH 4.0 to 5.0) and 50 mM sodium phosphate buffer (pH 6.0 to 8.0). All pH values were adjusted at room

Table 1. Summary of the purification steps of amylase from marine *Wangia* sp. C52.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Crude enzyme solution	676	108	6	1.0	100
Ammonium sulfate precipitation	458	35	13	2.2	68
Gel filtration chromatography	379	2.6	146	24.3	56

temperature and activity was estimated as a percentage of the maximum. Temperature optimum was determined in 50 mM sodium phosphate buffer (pH 6.0) over a temperature range of 0 to 60°C for 30 min.

Thermostability

Thermostability of amylase was examined in the range of 10 to 50°C. The purified enzyme was incubated in 50 mM citrate buffer (pH 6.0) for 1 h. The remaining enzyme activity was then measured as previosly described.

Effect of chemical reagents on amylase activity

The effects of metal ions on amylase were examined by determining the activities after 1-h incubation at 4°C in 50 mM citrate buffer (pH 6.0) containing various metal ions at 5 mM. The activity assayed in the absence of metal ions was defined as control. The metal ions tested include KCl, $CuCl_2 \cdot 2H_2O$, $CaCl_2$, $HgCl_2$, $MnCl_2$, $CoCl_2 \cdot 6H_2O$, $MgSO_4$, $ZnCl_2$, $FeCl_3$ and $AlCl_3$.

The effects of protease inhibitors (SDS, EDTA, EGTA, PMSF and DTT) on amylase activity were measured in the reaction mixture as described previously with various inhibitors at 5 mM. The purified enzyme was pre-incubated with the respective compound for 10 min at 4°C, followed by the standard enzyme assay as described previously. The relative activity assayed in the absence of the protein inhibitors was regarded as 100%.

RESULTS AND DISCUSSION

Enzyme purification

The purification of amylase is summarized in Table 1. Total amylase activity from the culture fluid of *Wangia* sp. C52 was 676 U. The effect of the ammonium sulfate precipitation was to reduce the total enzyme activity by 32% but gave an improvement of specific enzyme activity of 2.2 times. The effect of gel filtration chromatography was to decrease the total protein sharply from 35 to 2.6 mg, but only to decrease the total enzyme activity by 17%. The overall yield of 56% was achieved. All these results indicate that the amylase remained correctly folded in all the purification processes.

Electrophoresis

The isoelectric point of the amylase was found to be 5.5 to 5.8. To determine the molecular structure and molecular weight of amylase, the SDS-PAGE analysis was performed under reducing conditions (Figure 1). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis showed a single band near 58 kDa. This molecular weight falls within the range of values (54 to 68 kDa) reported for purified raw- starch-digesting amylase from other sources (Ueda et al., 2008).

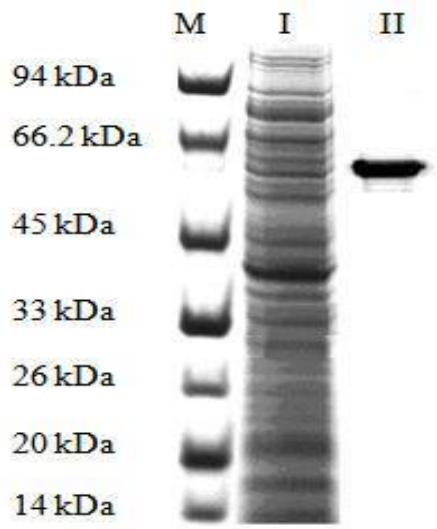

Figure 1. SDS-PAGE analysis of purified amylase. M, molecular mass marker; I, crude enzyme solution; II, purified and concentrated amylase sample.

Secondary structure analysis

Circular dichroism (CD) is a valuable method for the analysis of protein secondary structure, and the far- UV CD spectroscopy from 190 to 250 nm was used to estimate the contents of the secondary structures from the final purified amylase. The CD spectrum was shown in Figure 2. The theoretical values of -helix, -sheet and random coil were 25, 26 and 49% (http://www.embl.de/~andrade/k2d/), respectively. The errors involved in calculating the percentages of secondary structure were estimated at about ±5%. These

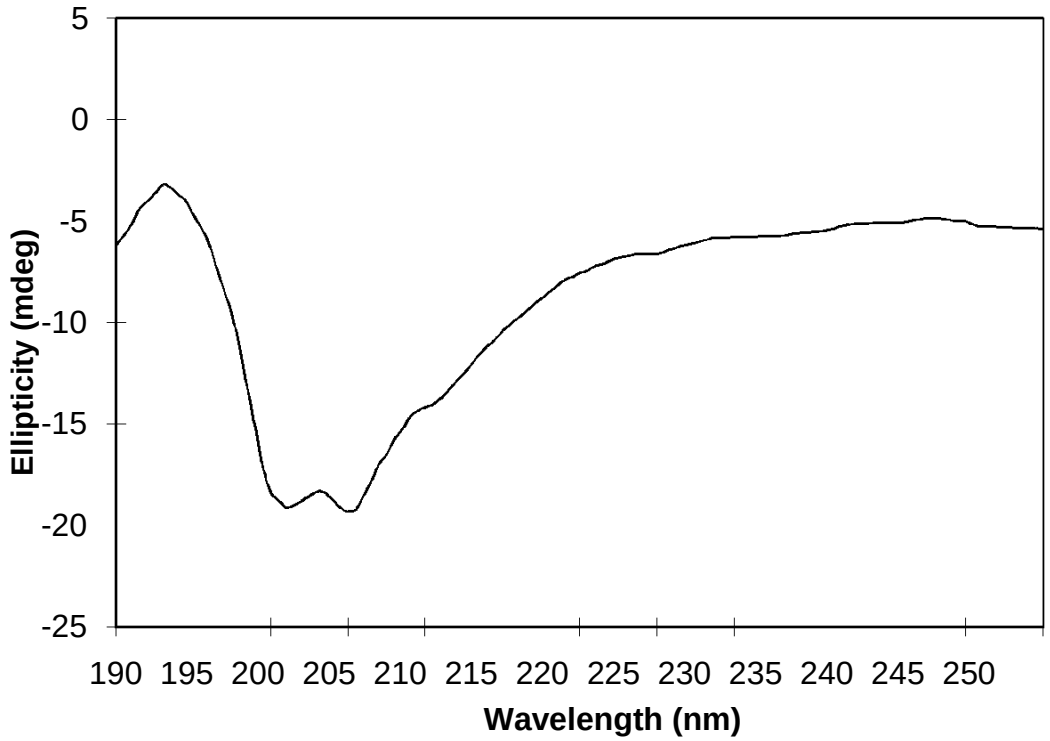

Figure 2. Circular dichroism spectrum of the purified amylase sample.

secondary structure results were very close to the literature values (-helix: 21.3%, -sheet: 22.9% and coil: 55.8%) (Chang et al., 1997), thereby indicating that the purified amylase folded with a reasonable secondary structure.

Kinetic parameters

Lineweaver-Burk plots in Figure 3 shows that apparent K_m and V_{max} values of the purified amylase for soluble starch were 2.08 ± 0.3 mg/ml and 1.26 ± 0.02 mg/ml/min, respectively.

Effect of pH and temperature on amylase activity

The activity of *Wangia* sp. C52 amylase was measured over a wide range of assay pH levels (4.0 to 8.0). As shown in Figure 4, *Wangia* sp. amylase exhibited optimal activity at pH 6.0, which was close to that of cold-active -amylase from *Eisenia foetide* (pH 5.5) (Ueda et al., 2008). The effect of temperature on the amylase activity revealed that the enzyme was optimally active at 30°C (Figure 5). This optimal temperature was much lower than those of cold-active -amylases from *Alteromonas haloplanctis* (43.7°C) (Feller et al., 1999) and *E. foetide* (50°C) (Ueda et al., 2008). When the temperature exceeded 30°C, the enzyme activity decreased rapidly.

Thermostability

The thermostability was investigated by pre-incubating the enzyme in 50 mM citrate buffer (pH 6.0) for 1 h. As Figure 5 shows, the enzyme was rapidly inactivated at temperatures above the optimum (30°C) . The results of optimal temperature and thermostability of *Wangia* sp. C52 amylase were in agreement with the character of the cold-active enzymes (Gerday et al., 2000).

Effects of chemicals on amylase activity

The effect of chemicals on amylase activity was tested (Table 2). Among the cations used, Ca^{2+} and Co^{2+} were found to have a stimulating effect on the enzyme activity, whereas Cu^{2+}, Hg^{2+}, Mn^{2+}, Zn^{2+}, Fe^{3+} and Al^{3+} were found to be inhibitors. In the presence of chelating agents, SDS, EDTA and EGTA inhibited the enzyme activity, indicating that the amylase was a metalloenzyme (Ramirez-Zavala et al., 2004). In addition, PMSF and DTT were found to have no effect on the enzyme activity, suggesting that Ser residues were not essential for the enzyme active sites

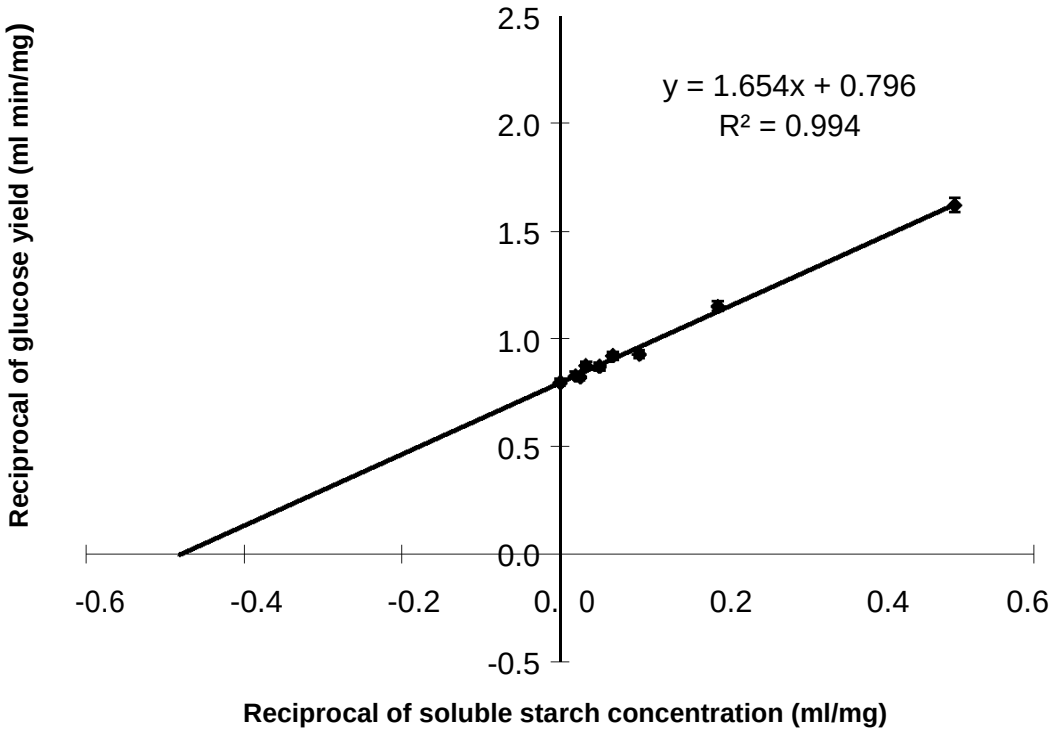

Figure 3. Lineweaver-Burk plot for K_m and V_{max} values of the purified amylase in the different concentrations of soluble starch.

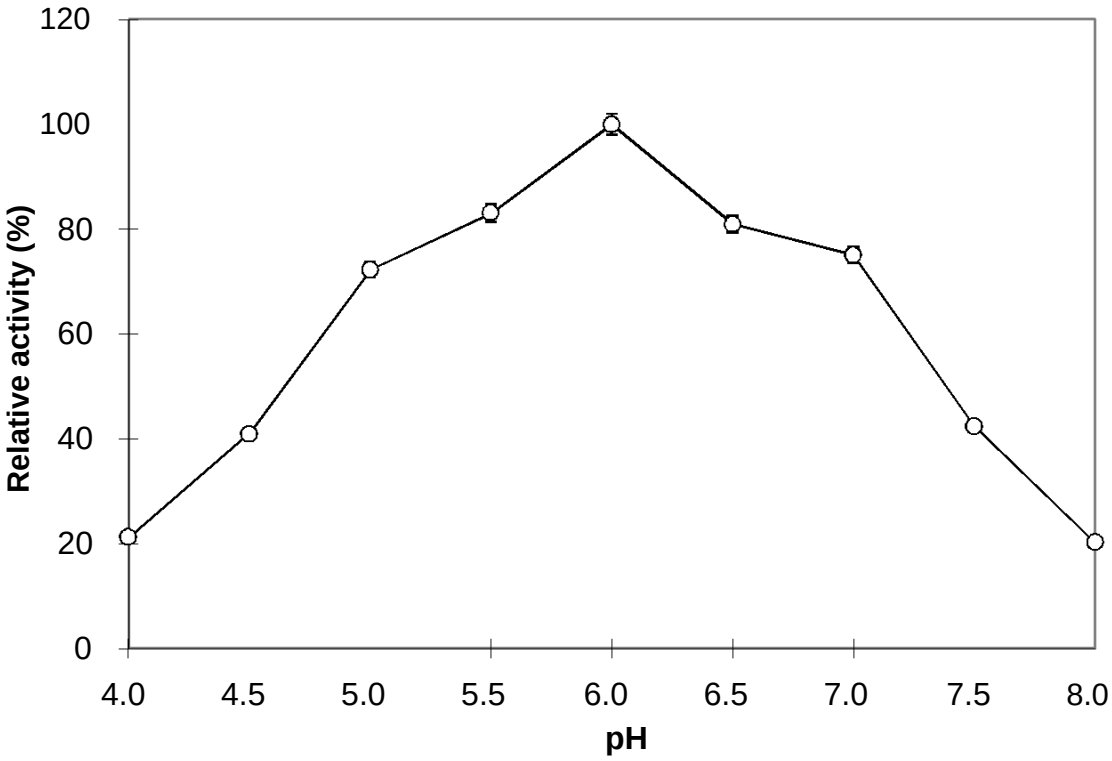

Figure 4. Effect of pH on the activity of *Wangia* sp. C52 amylase. For each pH, activity was assayed at 30°C and expressed as relative activity.

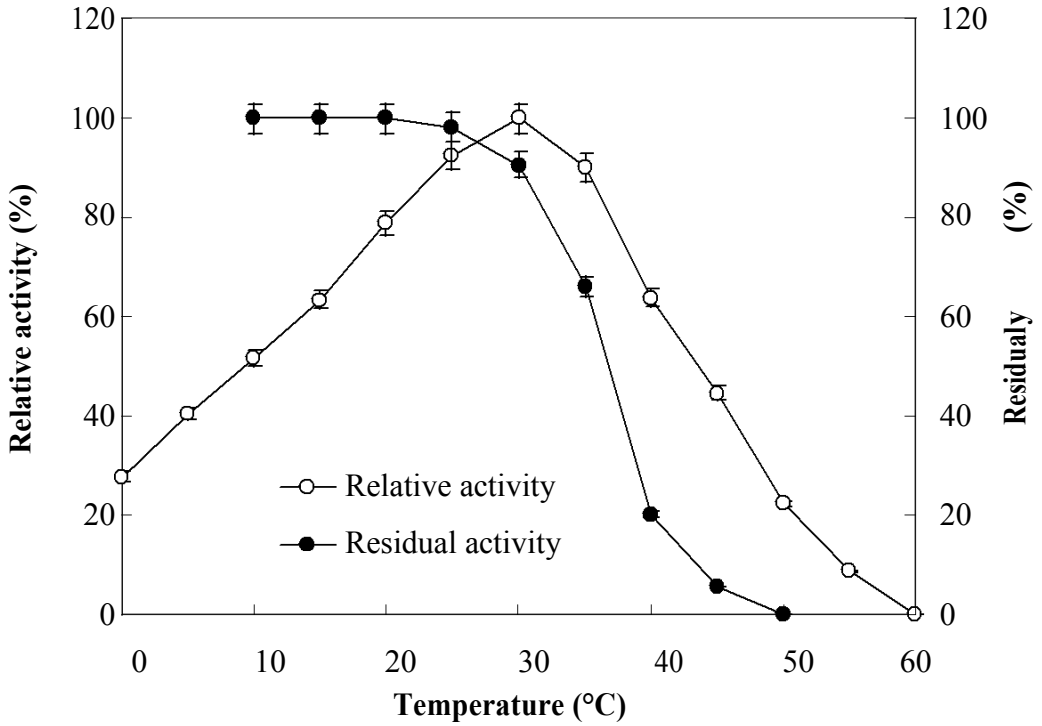

Figure 5. Effect of temperature on the activity and stability of *Wangia* sp. C52 amylase. Enzyme activity was assayed for each temperature after an incubation period of 30 min ( ) . For thermostability experiments ( ), the enzyme extract was pre-warmed at the indicated temperature for a 1 h period, and tthe remaining activity hen was determined.

Table 2. Effect of chemicals on the activity of *Wangia* sp. C52 amylase.

Reagent	Relative activity (%)	Reagent	Relative activity (%)
None	100	Zn^{2+}	86
K^{+}	101	Fe^{3+}	72
Cu^{2+}	48	Al^{3+}	57
Ca^{2+}	116	SDS	59
Hg^{2+}	29	EDTA	72
Mn^{2+}	82	EGTA	64
Co^{2+}	148	PMSF	98
Mg^{2+}	104	DTT	99

Enzyme activities were determined using the standard method of assay described in materials and methods after pre-incubation of the enzymes in 50 mM citrate buffer (pH 6.0) containing the chemicals at 4°C for 1 h. Concentration of metal ions and protease inhibitors is 5 mM.

(Urek and Pazarlioglu, 2004).

Conclusions

In the present work, a cold-active amylase was successfully purified from marine *Wangia* sp. C52 by ammonium sulfate precipitation and gel filtration chromatography. The purified enzyme was then characterized by various biotechnical methods to obtain the molecular properties. To our knowledge, this is the first study on purification and characterization of a cold-adapted enzyme from the genus of *Wangia* sp. Further work is in progress to study the physicochemical properties of the enzyme, which may have potential implications for both industrial use and fundamental

studies.

ACKNOWLEDGEMENT

The authors are grateful for the financial support of the Fundamental Research for the Central Universities.

REFERENCES

Aghajari N, Feller G, Gerday C, Haser R (1998b). Structures of the psychrophilic *Alteromonas haloplanctis* alpha-amylase give insights into cold adaptation at a molecular level. Structure, 6: 1503-1516.

Bernfeld P (1955). Amylases, and . Methods Enzymol., 1: 149-158. Chang QL, Chen JY, Zhang XF, Zhao NM (1997). Effect of the cosolvent type on the extraction of -amylase with reversed micelles: circular dichroism study. Enzyme Microb. Technol., 20: 87-92.

D'Amico S, Gerday C, Feller G (2003). Temperature adaptation of proteins: engineering esophilic -like activity and stability in a cold-adapted alpha-amylase. J. Mol. Biol., 332: 981-988.

Dang HY, Zhu H, Wang J, Li TG (2009). Extracellular hydrolytic enzyme screening of culturable heterotrophic bacteria from deep-sea sediments of the Southern Okinawa Trough. World J. Microbiol. Biotechnol., 25: 71-79.

Feller G, D'Amico D, Gerday C (1999). Thermodynamic stability of a cold-active -amylase from the Antarctic bacterium *Alteromonas haloplanctis*. Biochem., 38: 4613-4619.

Feller G, Lonhienne T, Deroanne C, Libioulle C, Van Beeumen J, Gerday C (1992). Purification, characterization, and nucleotide sequence of the thermolabile alpha-amylase from the Antarctic psychrotroph *Alteromonas haloplanctis* A23. J. Biol. Chem., 267: 5217-5221.

Gerday C, Aittaleb M, Bentahir M, Chessa JP, Claverie P, Collins T, D'Amico S, Dumont J, Garsoux G, Georlette D, Hoyoux A, Lonhienne T, Meuwis MA, Feller G (2000). Cold-adapted enzymes, from fundamentals to biotechnology. Trends Biotechnol., 18: 103-107.

Mannisto MK, Haggblom MM (2006). Characterization of psychrotolerant heterotrophic bacteria from Finnish Lapland. Syst. Appl. Microbiol., 29: 229-243.

Miller GL (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem., 31: 426-428.

Nichols D, Bowman J, Sanderson K, Nichols CM, Lewis T, McMeekin T, Nichols PD (1999). Developments with antarctic microorganisms: culture collections, bioactivity screening, taxonomy, PUFA production and cold-adapted enzymes. Curr. Opin. Biotechnol., 10: 240-246.

Polymenakou PN, Lampadariou N, Mandalakis M, Tselepides A (2009). Phylogenetic diversity of sediment bacteria from the southern Cretan margin, Eastern Mediterranean Sea. Syst. Appl. Microbiol., 32: 17-26.

Ramirez-Zavala B, Mercado-Flores Y, Hernadez-Rodriguez C, Villa-Tanaca L (2004). Purification and charaterization of lysine aminopeptidase from *Kluyveromyces marxiamus*. FEMS Microbiol. Lett., 235: 369-375.

Sedmak JJ, Grossberg SE (1977). A rapid, sensitive and vertile assay for protein using Coomassia Brilliant Blue G250. Anal. Biochem., 79: 544-552.

Ueda M, Asano T, Nakazawa M, Miyatake K, Inouye K (2008). Purification and characterization of novel raw-starch-digesting and cold-adapted -amylases from *Eisenia foetida*. Comp. Biochem. Physiol. B, 150: 125-130.

Urek RO, Pazarlioglu NK (2004). Purification of partial characterization of manganese peroxidase from immobilized *Phanerochaeto chrysosporium*. Process. Biochem., 39: 2061-2068.

Zhang JW, Zeng RY (2008). Purification and characterization of a cold-adapted alpha-amylase produced by *Nocardiopsis* sp. 7326 isolated from Prydz Bay, Antarctic. Mar. Biotechnol., 10: 75-82.