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Purification and Characterization of Cellulase from the Edible Snail

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Abstract

The cellulase from internal organs of edible snails was purified by fractionation with ammonium sulfate, DEAE-Sephadex chromatography and gel filtration on Sephacryl S-200 and Superose 12 HR 10/30. The specific activity of the purified cellulase was 85.1 units/mg protein with 24.3 purification fold from crude extract. Molecular weight of the enzyme was estimated to be approximately 74,000 dalton by gel filtration chromatography and SDS-PAGE eletrophoresis. The isoelectric point of the enzyme was determined to be pH 4.6. The optimum temperature and pH of the enzyme were 50°C and pH 6.0, respectively. The enzyme was stable at 30~50°C and pH 6.0~10.0. It was activated by Mn^{2+}, but inhibited by Li^{2+}, Zn^{2+}, Ag^{2+} and Hg^{2+}.

Key words: cellulase, edible snail, purification

INTRODUCTION

The edible snail is a Gastropoda which has a spiral rind, as it is called "*wawoo*". It is a polyphagous shellfish living at high temperatures and high humidity and has an optimum temperature of 25 to 32°C and optimum humidity of 70 to 90% for its living conditions (1). Burgundy snail (*Helix pomatia*), Small gray snail (*Helix aspersa*) and Giant snail (*Achatina fulica*) have been used as edible snails but Giant snail, which has been mainly raised in snail farms of Korea, is the main source of edible snails. Snail was not only used as foods, but also medicines. According to an old Korean medicinal record, snails had been used for a hypotensive, for a diuretic, for medical treatment of laryngitis and for boils, fever and dermatitis in surgery disease (2). Snails have been distributed world wide and are used as a source of high nutritional food for human livers. Snail is frequently utilized as a tonic in France, Spain, China, Japan and Taiwan, because of its sulfate chondroitin.

Cellulase is an enzyme complex, consisting of 1,4-β-D-glucanglucanohydrolase (endoglucanase, C_x, EC 3.2.1.4), 1,4-β-D-glucancellobiohydrolase (exoglucanase, C_1, EC 3.2.1.91) and β-glucosidase (cellobiase, EC 3.2.1.21). Cellulase is classified into carboxymethylcellulase (CMCase), Avicelase and ρ-nitrophenyl-glucopyranosidase (PNPGase) by specific activity of enzyme (3). In general, the enzymatic mechanism of cellulase to fibrin is complex because three enzymes of endoglucanase, exoglucanase and β-glucosidase act sequentially. In the first reaction, endoglucanase reacts to β-1,4-glucosidic linkage randomly in the amorphous of fibrous cell, and produces low molecular weight the cellulose chains which had a non-reducing terminal (4-6). Then, exoglucanase acts on cellulose chains and disassembles them to the units of cellobiose and glucose. The final enzyme of β-glucosidase disassembles cellobiose and short chain oligosaccharides to glucose. As mentioned from the above sentences, cellulase has scientific importance because of its specific functions of the enzyme complex. As well, it has industrial value because it produces the decomposed products of glucose, xylose and oligosaccharide from cellulose (7).

Sources for production of cellulase were widely investigated from the natural products, for example, microorganisms, mollusks and higher animals. The digestive fluid of snails has been known to have the valuable carbohydrases including cellulase (8,9). Though there is some research about the cellulase from snails, most of the research is about the enzyme from *Helix pomatia* (8-11). The objectives of this study were to purify the crude enzyme from the edible snail, and to characterize the activity of the purified cellulase activity on such environmental factors as pH, temperature and metal ions.

MATERIALS AND METHODS

Materials

The edible snails (*Achatina fulica*) used for this study were obtained from raw materials of an agricultural farm in Dong-myon, Chil-kok, Kyung-buk province, Korea. They

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were washed and the shells were removed. Internals except muscles were stored at -35°C.

Enzyme extraction, isolation and purification

The internals of edible snails (100 g) were homogenized with 500 mL of 10 mM phosphate buffer (pH 6.5) and the homogenate was leaved at 4°C for 3 hours. The crude extract was obtained by centrifuging (SUPRA 21K, Hanil, Korea) at 8,000 rpm for 60 min. For the further purification of the enzyme, the crude extract was fractionated by salting out with ammonium sulfate (20 to 80%) and centrifuged at 8000 rpm for 1 hour. The precipitate was dissolved in 10 mM phosphate buffer (pH 6.5) and desalted with the same buffer for 24 hrs.

Five milliliters of the desalted and concentrated extract were applied to DEAE Sephadex A-50 column (3.0×25 cm) pre-equilibrated with 20 mM phosphate buffer (pH 6.5). Elution was carried out with a 320 mL linear gradient containing from 0 to 1.0 M in the same buffer at a flow rate of 0.4 mL/min and the eluted 8 mL was collected separately.

Five milliliters of the fraction obtained by ion chromatography were applied to Sephacryl S-200 (3.0×50 cm) column with a flow rate of 0.2 mL/min. Four milliliter were collected in each test tube. The fraction obtained by Sephacryl S-200 column was concentrated and applied to Superose 12 HR 10/30 column (1.0×30 cm) and purified by FPLC (AKTA, Phamacia, Sweden) at a flow rate of 0.5 mL/min. Five milliliters were collected to each be test tube. Enzyme actvities of each fraction were monitored by 280 nm. The eluted proteins isolated in each steps were concentrated with Amicon Diaflo system of Diaflo PM10 ultrafiltration membrane (MW cutoff; 10,000) by N_2 gas.

Enzyme activity

Cellulase activity was assayed by Wood and Bhat method (12). The mixture of 0.1 mL crude enzyme extract and 1% carboxylmethyl cellulose (ultralow viscosity, Aldrich, USA) in 0.1 M acetate buffer (pH 5.0) was reacted for 30 min at 50°C. And 0.5 mL 3,5-nitrosalicylic acid (DNS) solution was added to the reacted sample and the sample was heated in the water bath for 5 min. Then it was cooled rapidly. After 4 mL distilled water was added, the absorbance of the samples was measured at 540 nm. One unit of cellulase activity was defined as the amount of μL moles of glucose produced at 50°C for 30 min.

Determination of protein concentration

During the course of enzyme purification, the eluted protein from the column was measured by measuring absorbance at 280 nm and each concentration was measured by Lowry method (13) with bovine serum albumin (BSA) as a standard protein.

Determination of molecular weight

Molecular weight was determined by Sephacryl S-200 gel filtration chromatography of Whitaker (14) method. Sephacryl S-200 column (3.0×50 cm) was equilibrated with 20 mM sodium phosphate buffer and its void volume (Vo) was determined by blue dextran. Each elution volume (Ve) was determined by standard proteins injected to the column, and Ve/Vo about weight of standard protein was calculated to determine each molecular weight by a standard curve.

Electrophoresis was used by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Stacking gel and separating gel were each 5% and 10% polyacrylamide gels, respectively. The sample was heated with 2% sodium dodesylsulfate (SDS) and 5% 2-mercaptoethanol at 100°C for 5 min. Electrophoresis was performed at 20 mA for 90 min staining by 0.25% coomassie brilliant blue R-250 and destaining by the mixture of methanol : acetic acid : distilled water (4.5 : 1 : 4.5).

Determination of isoelectric point

The isoelectric point of enzyme was determined by isoelectric focucing (IEF). The enzyme was purified by 5% polyacrylamide gel from pH 3.0 to pH 10.0 The purified enzyme and pI standard proteins were carried out electrophoresis to determine isoelectric point with staining coomassie brilliant blue.

Effect of pH on cellulase activity

The effect of pH on enzyme activity was investigated between pH 2.0 and pH 11.0. The used buffers were KCl-HCl buffer (pH 2.0), sodium citrate buffer (pH 3.0~5.0), sodium phosphate buffer (pH 5.0~8.0), carbonate buffer (pH 9.0~11.0). Stability of pH for enzyme activity was measured by testing the remaining acivity of the enzyme in 100 mM buffer mixturefor 24 hrs at 4°C in the range of pH 2.0 and pH 11.0.

Effect of temperature on cellulase activity

The optimum temperature of cellulase activity was determined by measuring its activity of the purified enzyme for 30 min at 30°C to 80°C. Enzyme stability of the purified enzyme solution on reaction temperature was determined by measuring the remaining activity for 30 min at 30°C to 80°C.

Effect of metal ions on cellulase activity

The effect of metal ions on cellulase activity was studied in various metal ions of KCl, NaCl, $LiSO_4$, $CaCl_2$, $FeCl_2$, $HgCl_2$, $MgCl_2$, MnO_4, $ZnCl_2$, $AgNO_3$, $Pb(CH_3COO)_2$, $CuSO_4$ and $BaCl_2$. The metal ions were added up to 1 mM in final concentration and the remaining actvities were measured for 30 min.

RESULTS AND DISCUSSION

Purification of cellulase

The result of purification of crude extract of edible snail by DEAE Sephadex A-50 column is shown in Fig. 1. Among two peaks separated in the fraction No. 45~90, the larger peak was further purified in this study. So the fraction was collected and freeze-dried. The dried sample was injected to Sephacryl S-200 and its result is shown in Fig. 2. Two active peaks among fraction no. 36~56 were appeared. The fraction no. 36~42 which had more stronger activity, was collected and ultrafiltrated by Amicon concentrator. The ultrafiltrate was separated by Superose 12 HR 10/30 column. At its result, three peaks were separated as shown in Fig. 3. The first peak separated in the No. 26~39 had cellulase activity. The fractions of the first peak were known to have a band by electrophoresis.

Enzyme activities on each purification step of cellulase from snail internals are shown in Table 1. Total activity, protein concentration and non-activity of crude extract were 14,844.6 units, 4,277.4 mg, and 3.5 units/mg protein, respectively. After Superose 12 HR 10/30 gel filtration of the final purification step, its total activity, protein concentration and non-activity were 1,744.6 units, 20.5 mg, 85.1 units/mg protein, respectively. The final enzyme was purified 24.3 times from crude extracts.

Molecular weight of cellulase

The molecular weight of the purified cellulase was measured by Sephacryl S-200 column comparing with protein standards. The molecular weight of the cellulase was about 74,000 dalton as shown in Fig. 4. Also, the result of molecular weight of the cellulase by 10% SDS-PAGE was about 74,000 dalton as shown in Fig. 5. The result showed that the cellulase purified from snail internals was monomer consisting of one polypeptide. Three kinds of cellulases from *Bacillus* sp. KSM-522 were reported to have the molecular weights of 78,000, 61,000, 61,000 dalton (4), respectively. Those were similar to the result of this study. But the molecular weight of cellulase from digestive fluid of *Achatina fulica* snail was reported to be approximate 23,000 dalton (15). That was different from this re-

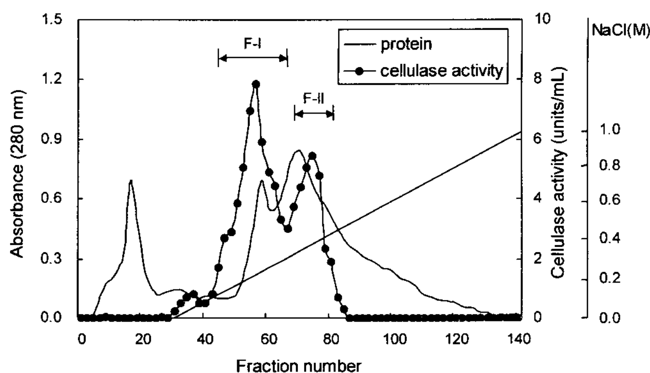

Fig. 1. Chromatograms of crude enzyme from edible snail on DEAE Sephadex A-50. Column size: 3.0×25 cm, Flow rate: 0.4 mL/min.

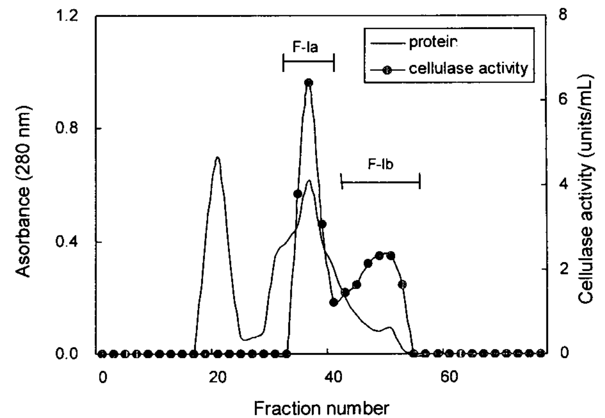

Fig. 2. Chromatograms of cellulase (F-I) from DEAE Sephadex A-50 on Sephacryl S-200. Column size: 3.0×50 cm, Flow rate: 0.3 mL/min.

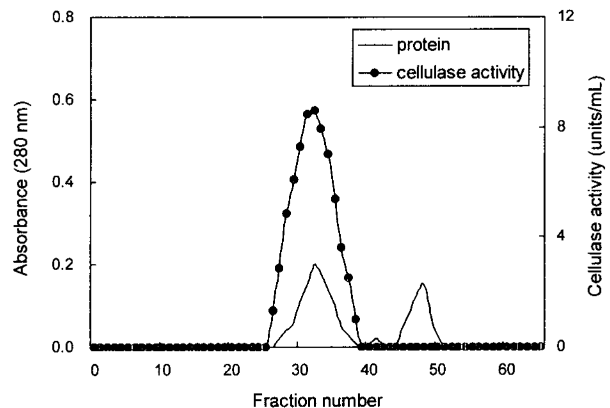

Fig. 3. Chromatograms of cellulase (F-Ia) from Sephacryl S-200 on Superose 12 HR 10/30. Column size: 1×30 cm, Flow rate: 0.5 mL/min.

Table 1. Purification steps and purity of cellulase from the edible snail

Purification steps	Total activity (units)	Protein content (mg)	Specific activity (units/mg protein)	Recovery (%)	Purification (fold)
Crude extract	14,844.6	4,277.4	3.47	100.0	1.0
$(NH_4)_2SO_4$	12,870.0	1,988.1	6.47	86.7	1.9
DEAE Sephadex A-50	6,534.6	314.7	20.78	44.0	6.0
Sephacryl S-200	2,564.2	54.3	47.22	17.3	13.6
Superose 12 HR 10/30	1,744.6	20.5	85.10	11.8	24.3

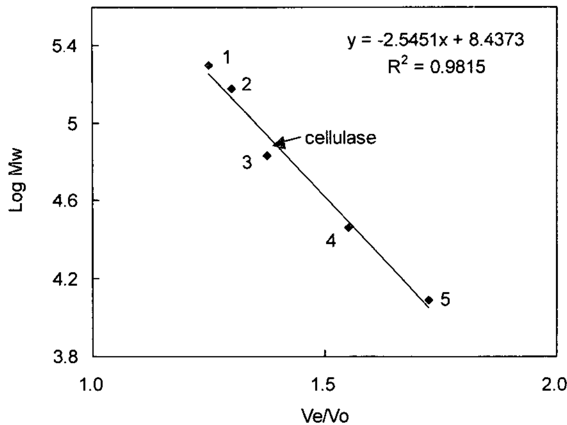

Fig. 4. Determination of molecular weight of cellulase by Sephacryl S-200 gel filtration. Vo: void volume, Ve: elution volume of each protein, 1: β-amylase (Mw. 200,000), 2: alcohol dehydrogenase, (Mw. 150,000), 3: albumin (Mw. 66,000), 4: carbonic anhydrase, (Mw. 29,000), 5: cytochrome c (Mw. 12,400).

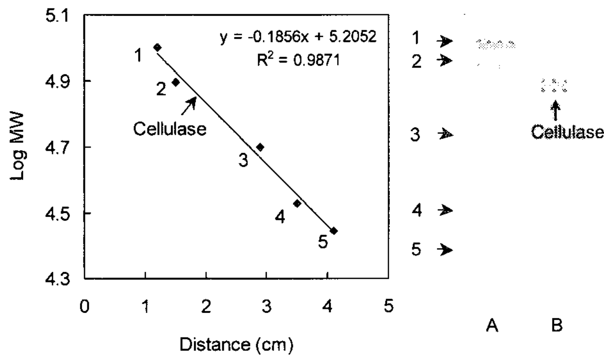

Fig. 5. Determination of molecular weight of cellulase by SDS-PAGE electrophoresis. 1. phosphorylase b (Mw. 101,000), 2. bovine serum albumin (Mw. 79,000), 3. ovalbumin (Mw. 50,000), 4. carbonic anhydrase (Mw. 34,700), 5. soybean trypsin inhibitor (Mw. 28,400).

sult. And the molecular weights of three kinds of endoglucanases purified from *Trichoderma viride* and the molecular weight of a kind of exoglucanase were 51,000, 59,000, 41,000, 62,000 dalton, respectively (16). That result explained that the molecular weights of the above-mentioned enzymes were lower than that of the cellulase purified from this study.

Isoelectric point of cellulase

In Fig. 6, the isoelectric point of the cellulase was determined to be pH 4.6 by measuring the relative distances of standard proteins. The isoelectric point of the cellulase isolated from green hard-shelled mussel (17) was reported to be pH 7.6, and the isoelectric points of the cellulases from microorganism were reported to be pH 3.8 to 7.0 of a very wide range (18-21). Moreover, the isoelectric point of cellulase of big snail internals was reported to be pH 5.3 and this result was different from the result

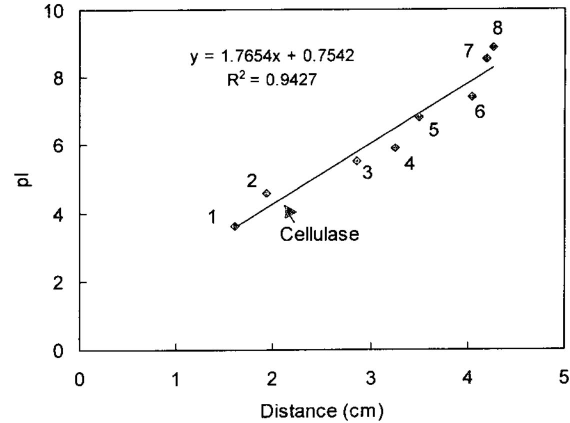

Fig. 6. Determination of pI of cellulase by isoelectric focusing. 1: amyloglucosidase (pI 3.6), 2: soybean trypsin inhibitor (pI 4.6), 3: β-lactoglobulin A (pI 5.1), 4: carbonic anhydarse II (pI 5.9), 5: carbonic anhydrase I (pI 6.6), 6: myoglobin (pI 7.2), 7: *Lens culinaris* lectin (pI 8.8), 8: trypsinogen (pI 9.3).

of this study.

Effect of pH

Effect of pH on enzyme activity is presented in Fig. 7 (A). The optimum pH of the cellulase of snail internals was pH 6.0. The result was similar to the optimum pH of *Dolabella auricularia* (22) and *Bacillus stearothermophilus* (21) which were pH 6.3 and 6.5, respectively.

Effect of pH on enzyme stability is shown in Fig. 7 (B). The highest activity of the cellulase was shown at pH 8.0 and its activity was maintained more than 80% from pH 6.0 to pH 10.0, but the activity was rapidly reduced below pH 5.0. Do and Kim (23) reported that cellulase activity was stable at pH 4.0 to pH 7.0 when the cellulase from *Ganoderma lucidum* was treated from pH 3.0 to pH 13.0 at 40°C for 1 hour. Kawai et al. (24) reported that cellulase partially purified from Neutrophilic *Bacillus* strain had high activities from pH 7.0 to pH 10.0 and it was stable from pH 5.0 to pH 12.0. The cellulase in this study had a more narrow pH range of stability than other reports. The optimum pH and stable pH for the cellulase activity were different from other results.

Effect of reaction temperature on cellulase activity

Enzyme activity of the cellulase on reaction temperature is shown in Fig. 8 (A). The activity of cellulase was highest at the reaction temp of 40°C and 50°C. The activities were decreased to 70% and 47% at 30°C and 60°C, respectively.

The optimum temperature of enzyme activity in the cellulase of green hard-shelled mussel was 50°C, and the enzyme maintained higher activity than 80% at 30°C to 50°C (17). The optimum temperature of cellulase from *Irpex lacteus* was 50°C, and it remained higher activity than 70% at 40°C to 60°C (25). This was similar to that

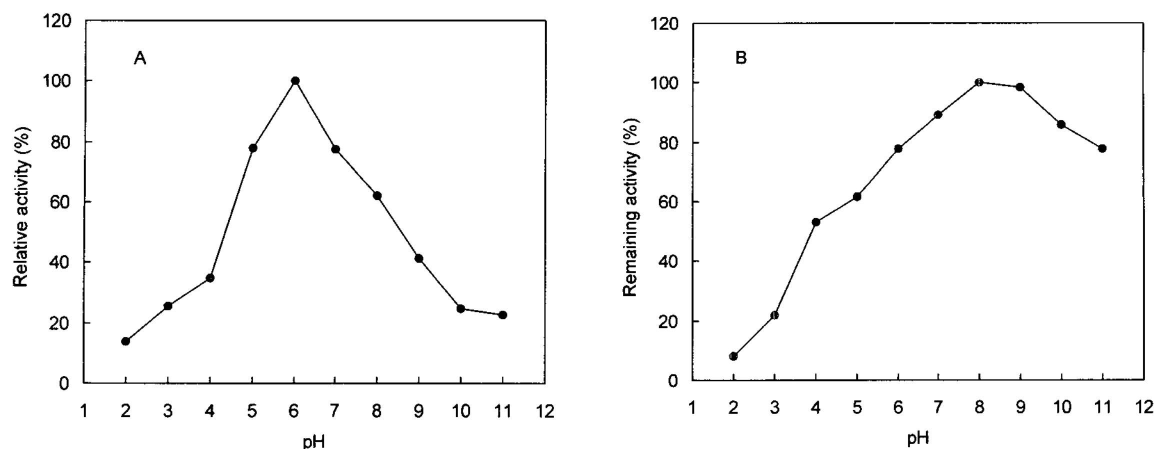

Fig. 7. Effects of pH on the activity (A) and stability (B) of cellulase from the ediblesnail.

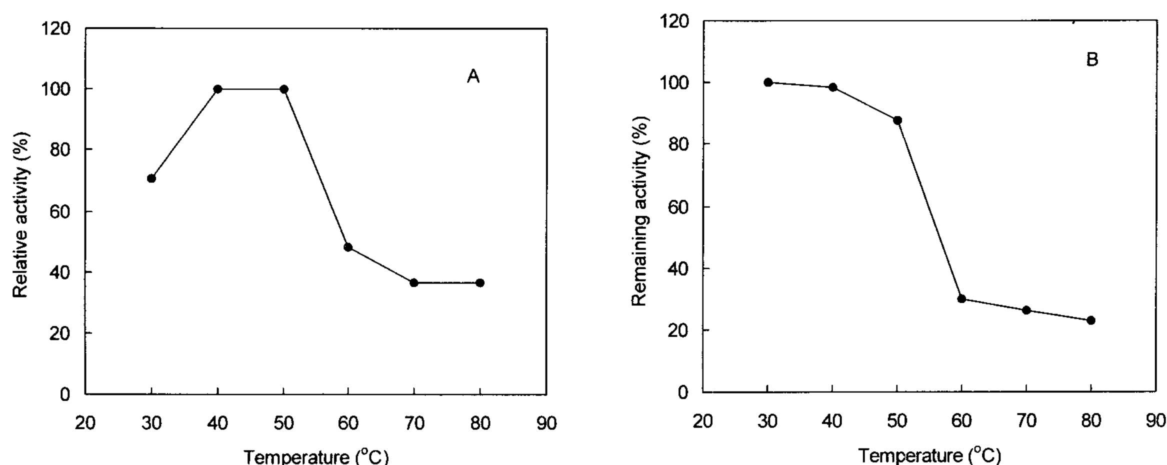

Fig. 8. Effects of temperature on the activity (A) and stability (B) of cellulase from the edible snail.

of this study. The proper temperatures of six kinds of endoglucanase and three kinds of exoglucanase from *Trichoderma viride* were 50°C to 60°C (26). It was reported, the other side, that two kinds of cellulases from *Thermotoga maritima* were heat-resisting enzymes showing the maximum activity at 95°C (27).

The remaining enzyme activities on heat stability after 30 min at each temperatures between 30°C and 40°C are shown in Fig. 8 (B). The remaining enzyme activities were 100% and 96% at 30°C and 40°C, respectively and its activity was rapidly decreased to 18% at 60°C.

Effect of metal ion on cellulase activity

The effect of enzyme activity on metal ions is shown in Table 2. The metal ions of $BaCl_2$ and $MnSO_4$ a little increased the cellulase activity or they little increased it. But in the case of $CaCl_2$, Pb $(CH_3COO)_2$ and $CuSO_4$, relative activities were 93, 93 and 94%, respectively, showing a little inhibition. But the other metal ions affected high inhibition. The relative activities of the cellulase on $LiSO_4$, $ZnCl_2$ and $AgNO_3$ were 61%, 64% and 51%, respectively. Especially, $HgCl_2$ strongly inhibited the enzyme activity, showing 18% remaining activity.

Table 2. Effects of metal ions on the activity of cellulase from the edible snail

Metal ions	Relative activity (%)
None	100
KCl	70
NaCl	70
Li_2SO_4	61
$CaCl_2$	93
$FeCl_2$	62
$HgCl_2$	18
$MgCl_2$	71
$MnSO_4$	108
$ZnCl_2$	64
$AgNO_3$	51
Pb $(CH_3COO)_2$	93
$CuSO_4$	94
$BaCl_2$	100

The cellulase purified from *Dolabella auricularia* had the remaining enzyme activities of 93.6 and 91.8%, on Ca^{2+} and Mg^{2+}, respectively, and the activities were completely inhibited by Cu^{2+} and Hg^{2+} (22). This result is different from the result of the cellulase of snail. The cellulase purified from digestive fluid of *Helix Pomatia* was stabilized and activated by Ca^{2+}, so the cellulase was proved to be a calcium reliable enzyme (10).

Generally, cellulases isolated from plants or microorganisms were known to be stabilized and activated by Ca^{2+}. However, the cellulase of snail internals in this study was not affected by Ca^{2+}, so the enzyme was determined to be a non-reliable enzyme by Ca^{2+}.

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