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# Purification and characterization of exo-polygalacturonase from *Zygoascus hellenicus* V25 and its potential application in fruit juice clarification

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**Abstract** The purification and characterization of the extracellular polygalacturonase from *Zygoascus hellenicus* V25 submerged culture using orange peel waste were investigated. This polygalacturonase, with a molecular weight of 75.28 kDa, was purified to 16.89 purification fold with a recovery of 18.46% and specific activity of 2469.77 U/mg protein by ammonium sulfate precipitation, DEAE cellulose chromatography, and Sephadex G-100 gel filtration. The enzyme exhibited maximum activity at 60°C and pH 5.0 and was stable over a wide range of pH levels (3.0-11.0). Moreover, enzyme activity was enhanced by $Cu^{2+}$ and cysteine, whereas it was strongly inhibited by $Hg^{2+}$. The extent of enzymatic hydrolysis was negatively correlated with the degree of pectin esterification. $K_m$ and $V_{max}$ values of the polygalacturonase were 5.44 mg/mL and 61.73 μmol/(min·mg), respectively. The polygalacturonase was applied in the juice clarification of four fruits, and results showed that the percentage transmittance at 660 nm increased by 3.51, 4.36, 8.04, and 12.2%.

**Keywords:** *Zygoascus hellenicus* V25, exo-polygalacturonase, purification, characterization, juice clarification

## Introduction

Pectin is a complex structural polysaccharide that is composed of a linear chain of partially methyl-esterified galacturonic acid subunits linked by α-1,4-glycosidic linkages (1). Pectinases are a complex of enzymes that degrade pectin substances. Based on their mechanisms in degrading pectin substances, pectinases can be classified into protopectinases, polygalacturonases (EC 3.2.1.15), lyases (EC 4.2.2.10), and pectinesterases (EC 3.1.1.11) (2,3). Pectinases are mainly used in the beverage industry for the extraction and clarification of juice and wine to remove pectic substances, which are responsible for the viscosity, turbidity, and consistency (4). In the current fruit juice industry, gelatin tannins, refrigeration, bentonite addition, and natural clarification cannot effectively reduce juice cloudiness caused by pectin substances, but pectinase can degrade pectin to improve the effect of juice clarification and might increase juice yield and decrease juice viscosity (5-7). Moreover, enzymatic clarification is advantageous in terms of high catalytic efficiency, low activation energy, and high degree of specificity.

In food-processing industries, a polygalacturonase is widely used in the extraction and clarification of fruit juices (5,8). Polygalacturonase treatment decreases the size of granule particles in fruit juices and removes their web-like aspect (9). The reduction in the size of these particles and the subsequent decrease in viscosity can partially explain how depectinization improves flux (10). Polygalacturonases are widely distributed in microbial kingdoms such as fungi, bacteria, and yeast.

To the best of our knowledge, no report has been published concerning the extracellular polygalacturonase from *Zygoascus hellenicus* V25. Therefore, the characterization of the purified polygalacturonase from *Zygoascus hellenicus* V25 is important in distinguishing among enzyme components, optimal conditions for enzyme activity, and the metabolism of enzyme synthesis so that the enzyme can be better utilized in the food industry (11).

In the present study, as a pectinase producer, strain V25 was screened and identified as *Zygoascus hellenicus*. Herein, we report the purification and characterization of the pectinase from *Zygoascus hellenicus* V25 submerged culture using orange peel waste, which can stimulate the approval of the enzyme in biotechnological applications. In addition, the application of the purified enzyme in fruit juice clarification was studied.

## Materials and Methods

**Chemicals** Galacturonic acid-citrus pectin with three different methyl-esterified degrees (30, 72, and 85%) of carboxyl groups-polygalacturonic acid, xylan, bovine serum albumin, and trigalacturonic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). All chemicals and solvents used were of analytical grade.

**Microorganism** As a pectinase producer, strain V25 was screened and isolated from soil samples of citrus fruits using pectin as the sole carbon source. According to the ratio of color ring diameter and colony diameter, flat color change on the bromophenol blue tablet and the pectinase activity of fermentation, the strain was determined. After being cultivated and maintained in a yeast extract/peptone/dextrose medium (YEPD), the strain was inoculated to the YEPD liquid medium and incubated at 30°C for 14 h; then, the cell suspension was transferred to the fermentation medium (orange peel powder 31.2 g/L, pepton 1 g/L, beef extract 3 g/L, $CaCl_2$ 0.5 g/L, $K_2HPO_4{\cdot}3H_2O$ 0.094 g/L, $KH_2PO_4$ 0.5 g/L) and incubated at 30°C for 48 h. The fermented medium was centrifuged at 4,500x *g* for 20 min, and the supernatant (crude enzyme) was collected for further enzyme purification.

**Enzyme assay and protein determination** Polygalacturonase activity was determined by measuring the amount of galacturonic acid using the Miller method (12). The reaction solution, composed of 1.8 mL of 1% (10 g/L) citrus pectin and 0.2 mL of the enzyme in 2 mL of a 50 mM citrate phosphate buffer (pH 5.0), was incubated at 50°C for 30 min in a water bath. After incubation, 3 mL of DNS was added to the mixture. The mixture was then boiled for 10 min to terminate the reaction. Reduced sugars released were quantified using an ELISA reader (BioTek, Winooski, VT, USA) by measuring the absorbance at 540 nm. Heat-deactivated enzyme was used as a control. One unit of enzyme activity was defined as the amount of the enzyme that releases 1 μmol of galacturonic acid per minute at 50°C under pH 5.0. Protein concentration was measured using the Lowry method (13). The results are expressed as the mean value of three readings with an estimated error of ±10%.

**Enzyme purification** Attempts were first made to concentrate the crude enzyme *via* ammonium sulfate precipitation to determine a suitable saturation degree by measuring the activity in all fractions of 10-90% saturation through a gradual addition of powdered ammonium sulfate under gentle stirring. During the next formal purification, the crude enzyme was brought to 60-90% saturation. After overnight preservation at 4°C, the mixture was centrifuged at 10,000 rpm for 20 min and the precipitate was dissolved in a 50 mM sodium phosphate buffer (pH 7.0) for further purification. The dissolved enzyme solution was desalinated by the 50 mM sodium phosphate buffer (pH 7.0) at a flow rate of 1 mL/min using a Sephadex G-25 column (0.9×30 cm) after the column was pre-equilibrated with the same buffer. Fractions with high polygalacturonase activity, determined using the Miller method, were loaded onto a DEAE cellulose column (3×20 cm), and then, the column was eluted with a linear gradient of a NaCl (0-40 mM) buffer including 50 mM sodium phosphate (pH 5.4) at a flow rate of 2 mL/min. These fractions were collected and concentrated at 6,000 rpm for 30 min in an ultrafiltration tube (Millipore, Billerica, MA, USA). Finally, the concentrated enzyme solution was processed using a Sephadex G-100 column (0.9×30 cm), and the column was eluted with a 50 mM sodium phosphate buffer (pH 7.0) at a flow rate of 0.1 mL/min. Fractions with high polygalacturonase activity were collected and stored at 20°C for further experiments.

**Gel electrophoresis** The purity and molecular weight of the purified polygalacturonase were checked *via* SDS-PAGE by Laemmli (14). Protein bands were stained with Coomassie brilliant blue G-250. The molecular weight of the purified polygalacturonase was determined by comparing its heterogeneity with those of marker proteins (14.4-116.0 kDa).

**Enzyme characterization**

***Effects of temperature on enzyme activity and stability*:** The effect of temperature on polygalacturonase activity was determined by performing an enzymatic hydrolysis reaction for 30 min at various incubation temperatures (30-80°C). To evaluate the thermal stability of the purified polygalacturonase, it was incubated without a pectin substrate for 4 h at various incubation temperatures (30-70°C), and residual polygalacturonase activity was determined at a 1-h interval.

***Effects of pH on enzyme activity and stability*:** The effect of pH on polygalacturonase activity was investigated by performing an enzymatic hydrolysis reaction at various pH levels (3.0-11.0) for 30 min. The stability of the purified polygalacturonase at these pH levels was studied by incubating the polygalacturonase at 50°C for 4 h, and residual polygalacturonase activity was determined at a 1-h interval. The types of reactant buffers were set as follows: citrate/disodium hydrogen phosphate (50 mM, pH 3.0-5.0), sodium phosphate (50 mM, pH 6.0-7.0), Tris-HCl (50 mM, pH 8.0), glycine/sodium hydroxide (50 mM, pH 9.0-10.0), and sodium hydroxide/disodium hydrogen phosphate (50 mM, pH 11.0).

***Effect of metal ions on enzyme activity*:** The effect of various metal ions on polygalacturonase activity was determined after 5 or 10 mM of $Cu^{2+}$, $Ba^{2+}$, $Zn^{2+}$, $Hg^{2+}$, $Mg^{2+}$, $Fe^{2+}$, $Na^{+}$, $K^{+}$, $Co^{2+}$, $Al^{3+}$, or $Pb^{2+}$ was incubated with the polygalacturonase at 30°C for 1 h. No metal ion was used in the control sample solution.

***Effects of inhibitors and surfactants on enzyme activity*:** The effects of inhibitors and surfactants on the activity of the purified polygalacturonase were evaluated after incubating the enzyme with 5 or 10 mM of urea, cysteine, β-mercaptoethanol, PMSF, GSH, EDTA, EDC, Tween 80, or Tween 20.

***Substrate specificity*:** The substrate specificity of the purified polygalacturonase was investigated by incubating the polygalacturonase

with different substrates (citrus pectin with three methyl-esterified degree -30, 72, and 85%- of carboxyl groups; polygalacturonic acid; xylan; and carboxymethylcellulose) in a 50 mM disodium hydrogen phosphate/citrate buffer.

***Kinetic parameters*:** $K_m$ and $V_{max}$ values of the purified polygalacturonase were determined with different substrate concentrations following the Lineweaver-Burk double-reciprocal plot (15). Kinetic constants were calculated using citrus pectin (with a 30% degree of esterification) as a substrate.

***Thin-layer chromatography*:** The degradation products of the enzymatic hydrolysis of the purified polygalacturonase were analyzed by thin-layer chromatography for various incubation periods, in which heat-deactivated samples containing reaction mixtures were spotted on silica gel sheets (Branch of Qingdao Haiyang Chemical Co., Qingdao, China), and n-butanol:acetic acid:water (7:5:10, v/v/v) was used as the mobile phase (16). Thereafter, the dried plate was sprayed with a chromogenic reagent (0.4-g diphenylamine, 0.4 mL aniline, 2 mL phosphoric acid, 20 mL propanone), and then, it was kept at 105°C for 10 min.

***Application of purified enzyme in fruit juice clarification*:** Four kinds of fruits, i.e., tangerine, orange, grapefruit, and apple, were purchased from a local market, and their juices were extracted using a lab mixer grinder. The extracted pulp was filtered using muslin cloth to obtain the fruit juice, which was centrifuged at 4,500 rpm for 10 min, and the resulting supernatant was collected for further application test (17). The pH levels of orange, tangerine, grapefruit, and apple juices were 3.94, 3.47, 3.58, and 3.82, respectively. The purified polygalacturonase was added to the fruit juice (9.58 U/mL fruit juice; 4.98%, v/v), which was then incubated at 50°C for 24 h. The blank contained a heat-deactivated enzyme. The reaction was terminated by heating the reaction mixture up to 90°C for 2 min and then centrifuged at 4,500 rpm for 10 min. The supernatant was analyzed for juice clarity by measuring the percentage transmittance at 660 nm (18). Water was used as a control (percentage transmittance at 660 nm=100%).

## Results and Discussion

**Microorganism** One isolate, V25, showed the maximum pectinase activity of 29.02±0.06 U/mL. Strain V25 was identified and deposited by the China Center for Type Culture Collection (CCTCC, Wuhan, China) with a culture collection number of CCTCC M 2015183. Fragments of 18S rRNA were sequenced and subjected to phylogenetic analysis to further identify strain V25. Nucleotide-nucleotide BLAST in NCBI results showed that strain V25 has more than 99% sequence similarity with the first hit of *Zygoascus hellenicus*. Based on the 18S rRNA gene sequence, a phylogenetic tree was made in MEGA 5.2, and it showed 99.09% sequence similarity to *Zygoascus hellenicus* (AB018152.2). According to the results reported above, it is reasonable to identify strain V25 as *Zygoascus hellenicus*.

**Purification of polygalacturonase from *Zygoascus hellenicus* V25**
The dissolved precipitates of 90% saturation showed the highest polygalacturonase activity compared with the other saturations of ammonium sulfate. The desalination profile of the enzyme from Sephadex G-25 gel filtration is presented in Fig. 1A. Fractions with the first protein peak were observed to contain the highest polygalacturonase activity, whereas fractions with the third protein peak were observed to contain most of the yellow pigment derived from orange peel. The elution profile from anion exchange chromatography is shown in Fig. 1B. Fractions with the third protein peak were observed to contain high polygalacturonase activity, whereas for fractions with the first protein peak, most contaminant proteins were eluted from the target protein. No protein peak occurred when the NaCl concentration in the anion exchange column reached 40 mM, indicating that all proteins were rushed out. The elution profile from Sephadex G-100 gel filtration is shown in Fig. 1C. The higher polygalacturonase activity at the second protein peak fractions was detected whereas it had little polygalacturonase activity at the first half of second protein peak, which could be attributed to the very small difference of relative molecular mass between the proteins. All purification steps are summarized in Table 1, which shows 16.89 purification fold with a recovery of 18.46% and specific activity of 2469.77 U/mg proteins.

The homogeneity of the purified polygalacturonase was checked *via* SDS-PAGE. Only a single protein band was observed after Sephadex G-100 gel filtration, indicating that the purified polygalacturonase was almost pure. The molecular weight of the purified polygalacturonase, determined by plotting the log of the molecular weights of markers *versus* their relative mobilities, is approximately 75.28 kDa, which is similar to the results reported about polygalacturonases from *Paecilomyces variotii* (77.3 kDa) and *Aspergillus giganteus* (69.7 kDa) (8,16).

**Enzyme characterization**

***Effects of temperature on enzyme activity and stability*:** The effect of temperature on polygalacturonase activity is shown in Fig. 2A. The polygalacturonase exhibited the highest activity at 60°C, and it is in accordance with the behavior of fungal polygalacturonase which show frequently optimum temperatures between 30 and 60°C (8,11). The purified polygalacturonase was stable between 30 and 50°C, retaining more than 90% of its activity, but the thermal stability of the enzyme obviously declined at 60-70°C (Fig. 2B), which can be attributed to the denaturation of the tertiary structure of the purified enzyme under high temperatures (19,20).

***Effects of pH on enzyme activity and stability*:** The effect of pH on polygalacturonase activity is shown in Fig. 2C. The polygalacturonase was active at pH 3.0-7.0 and exhibited the highest activity at pH 5.0, indicating that the enzyme was competent in an acidic medium as most fungal polygalacturonases exhibit optimal activity at pH 4.0-5.0 (4,21). The purified enzyme was very stable for retaining more than

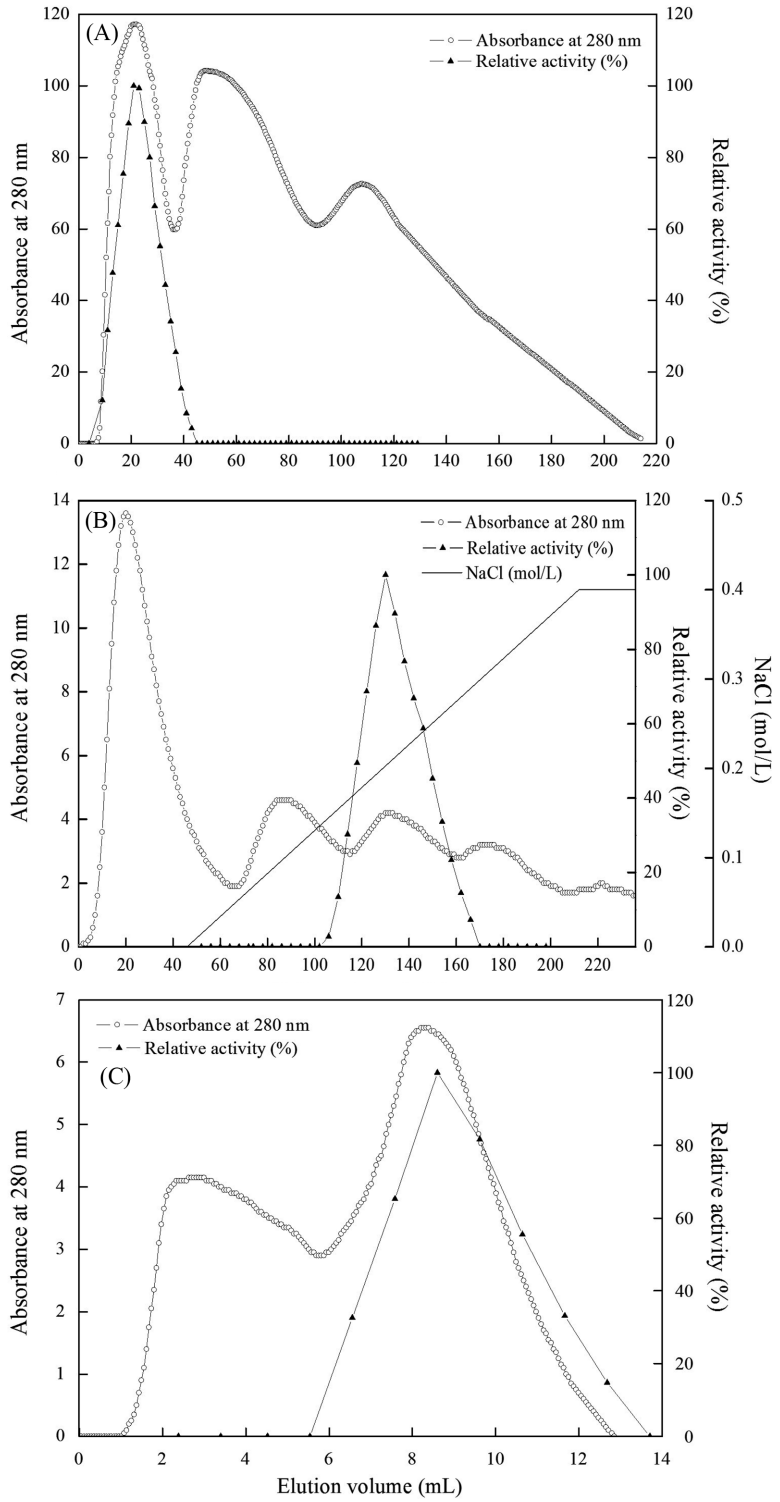


**Fig. 1.** (A) Sephadex G-25 chromatography elution *via* ammonium sulfate precipitation, (B) DEAE cellulose chromatography elution *via* Sephadex G-25 chromatography, and (C) Sephadex G-100 chromatography elution *via* DEAE cellulose chromatography.

85% of its activity at pH 3-11 in 4 h (Fig. 2D). Most polygalacturonases obtained from different microbial sources were stable in an acidic medium within pH 3.0-6.0, e.g., *Thermococcus aurantiacus* (22), *Aspergillus awamori* (23), and *Saccharomyces cerevisiae* (24), or stable only in a neutral range of 7.0-11.0, e.g., *Bacillus* sp. KSM-P410 (20) and *Fusarium oxysporum* f. sp. *lycopersici* (25). This wide range of pH stability implies that the polygalacturonase from *Zygoascus hellenicus* V25 can be versatile in various industrial processes. A comparison of the pH optimum curve with pH stability curve can contribute to the determination of reversible and irreversible effects

**Table 1.** Purification of polygalacturonase from *Zygoascus hellenicus* V25

| Purification steps | Total activity (U) | Total protein (mg) | Specific activity (U/mg) | Yield (%) | Purification fold |
|---|---|---|---|---|---|
| Crude filtrate | 8,773.80 | 60.02 | 146.19 | 100.00 | 1.00 |
| $(NH_4)_2SO_4$ | 6,894.15 | 16.77 | 411.01 | 78.58 | 2.81 |
| Sephadex G-25 | 6,364.54 | 9.94 | 640.60 | 72.54 | 4.38 |
| DEAE-Sephadex A-52 | 2,896.27 | 1.47 | 1,971.91 | 33.01 | 13.49 |
| Sephadex G-100 | 1,620.00 | 0.66 | 2,469.77 | 18.46 | 16.89 |

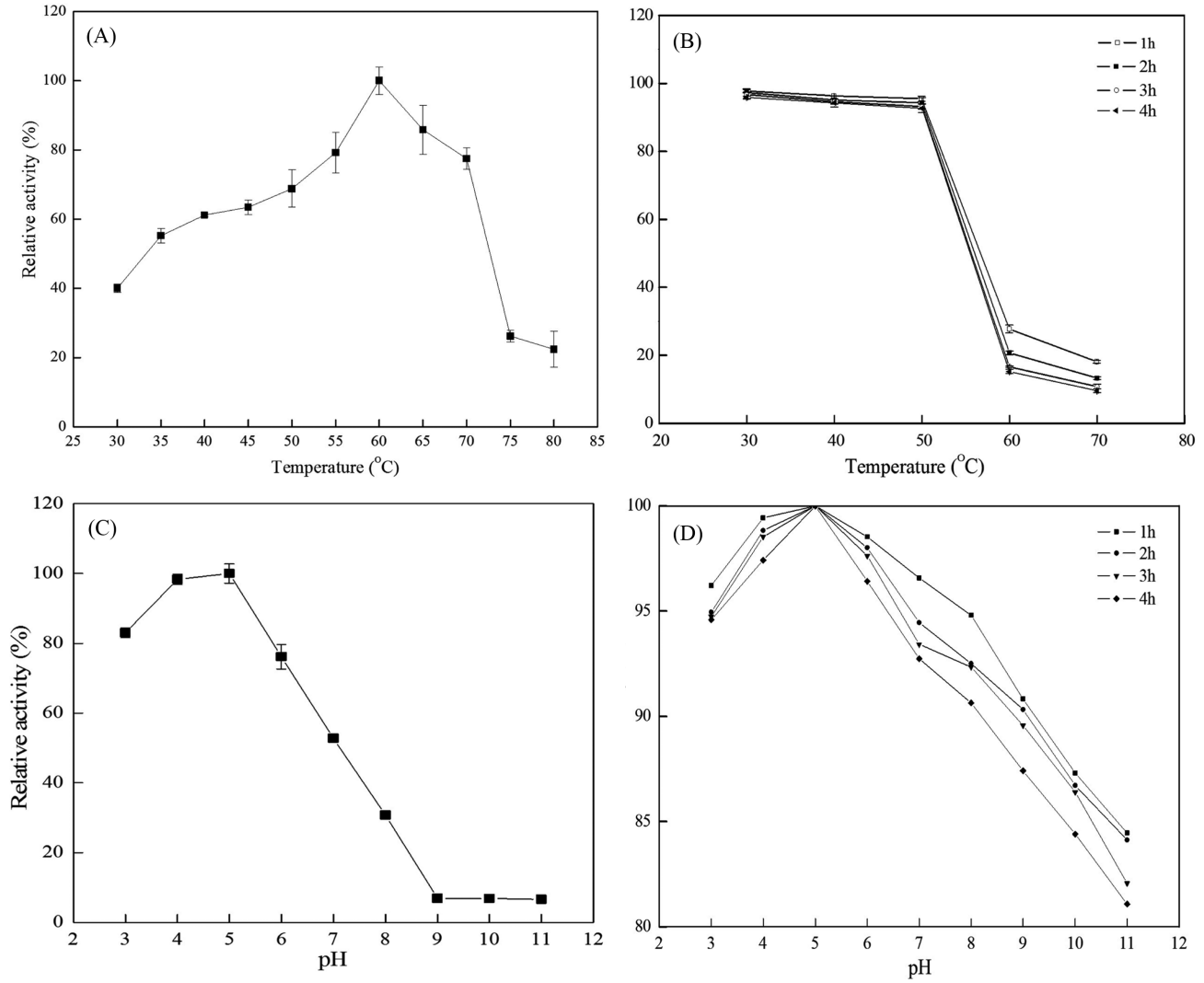


**Fig. 2.** Effect of temperature on activity (A) and thermal stability (B) of the polygalacturonase from *Zygoascus hellenicus* V25; effect of pH on activity (C) and stability (D) of the polygalacturonase from *Zygoascus hellenicus* V25.

of pH on enzyme activity (2).

***Effect of metal ions on enzyme activity*:** The effect of metal ions on polygalacturonase activity is shown in Table 2. The enzyme showed the maximum stimulation by $Cu^{2+}$. Enzyme activity was strongly stimulated by $Zn^{2+}$, $Mg^{2+}$, $Na^{+}$, or $K^{+}$. The stimulation might be attributed to "blocks" of carboxyl groups, which might trap enzyme molecules and thus prevent the progress of the enzymatic hydrolysis reaction (26). Nevertheless, enzyme activity was inhibited by $Ba^{2+}$, $Hg^{2+}$, $Fe^{2+}$, $Co^{2+}$, $Al^{3+}$, or $Pb^{2+}$ at high concentrations. The enzyme showed the maximum inhibition by $Hg^{2+}$, possibly owing to its effective combination with sulfydryl groups, suggesting that there should be some important cysteine residues inside or in the vicinity of an active site of the enzyme.

***Effects of inhibitors and surfactants on enzyme activity*:** The effects of inhibitors and surfactants on polygalacturonase activity are reported in Table 2. The enzyme exhibited the maximum stimulation in the presence of 5 mM cysteine, while it showed the maximum inhibition in the presence of 10 mM PMSF. Polygalacturonase

**Table 2.** Effect of substance on polygalacturonase activity from *Zygoascus hellenicus* V25

| Substance | Relative activity (%)* | |
|---|---|---|
| | 5 mM | 10 mM |
| $Cu^{2+}$ | 135.76±1.57 | 140.11±0.17 |
| $Ba^{2+}$ | 85.48±1.67 | 85.24±1.17 |
| $Zn^{2+}$ | 112.25±3.03 | 112.44±0.93 |
| $Hg^{2+}$ | 35.48±3.07 | 31.41±1.96 |
| $Mg^{2+}$ | 102.84±1.61 | 113.53±0.79 |
| $Fe^{2+}$ | 93.14±2.64 | 90.07±3.73 |
| $Na^{+}$ | 112.75±3.55 | 108.74±0.82 |
| $K^{+}$ | 115.42±0.64 | 117.79±0.63 |
| $Co^{2+}$ | 108.45±1.64 | 87.23±1.53 |
| $Al^{3+}$ | 99.99±0.15 | 84.27±2.29 |
| $Pb^{2+}$ | 107.26±1.11 | 87.53±2.88 |
| Urea | 110.38±0.99 | 100.64±0.26 |
| Cysteine | 121.40±3.86 | 109.53±0.34 |
| β-Mercaptoethanol | 116.31±1.97 | 107.42±0.45 |
| PMSF | 113.77±0.53 | 81.14±2.56 |
| GSH | 97.25±1.29 | 83.26±0.80 |
| EDTA | 107.84±3.41 | 112.08±0.62 |
| EDC | 90.89±1.06 | 82.84±0.40 |
| Tween 80 | 115.47±1.31 | 90.04±0.90 |
| Tween 20 | 109.11±1.34 | 94.28±0.72 |

*control: (100.00±2.26)%

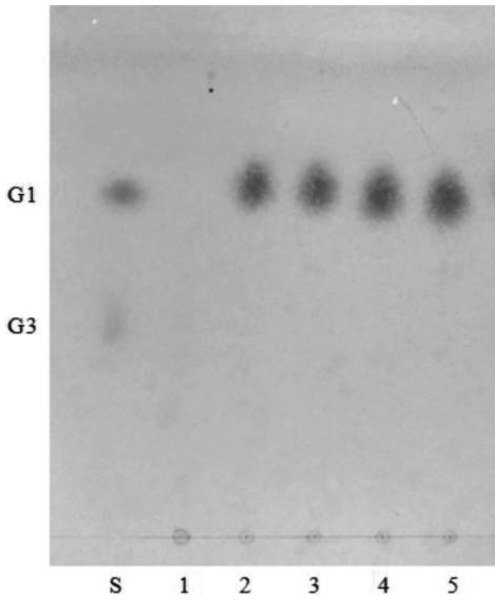


**Fig. 3.** Degradation products of citrus pectin by the purified polygalacturonase from *Zygoascus hellenicus* V25. Pectin substrate was incubated in the presence of the enzyme for 0 h (lane 1), 0.5 h (lane 2), 6 h (lane 3), 12 h (lane 4), and 24 h (lane 5). Standards: G1 monogalacturonic acid, G3 trigalacturonic acid.

activity was activated in the presence of 5 mM urea, cysteine, β-Mercaptoethanol, PMSF, Tween 80, and Tween 20, while the activity was inhibited in the presence of 10 mM PMSF, Tween 80, and Tween 20. β-Mercaptoethanol could counteract the oxidation effects of the S-S linkage between cysteine residues, indicating that cysteine should be involved in hydrogen bonding with the substrate-enzyme complex and the formation of a covalent glycosyl-substrate intermediate. PMSF, which can contribute to the sulfonylation of serine residues, was employed here to detect the presence of serine residues inside or in the vicinity of an active site of the enzyme. The addition of EDTA had a strong stimulation on enzyme activity, indicating that there should be some free cations in the maintenance of the enzyme's three-dimensional structure (8).

***Substrate specificity*:** The substrate specificity of the purified polygalacturonase was determined by performing an enzymatic hydrolysis reaction with a variety of substrates. The relative activity of the purified enzyme reacting with citrus pectin (DE 30%) was the highest, which was taken as 100±2.4%. The relative activities of the purified enzyme reacting with citrus pectin (DE 72%) and citrus pectin (DE 85%) were 58.19±3.1 and 7.18±0.5%, respectively. The extent of the hydrolysis reaction was negatively correlated with the degree of esterification, suggesting that the purified enzyme has a high affinity for a low-esterified pectin substrate. The degree of methyl esterification appears to be a determinant for the biological activity of enzymatic hydrolysis (27). The relative activity of the purified enzyme reacting with other polysaccharides as substrates, including xylan and CMC, was not found, suggesting that the purified polygalacturonase was a kind of a substrate-specific enzyme.

***Kinetic parameters*:** The kinetic parameters of the purified polygalacturonase for enzymatic hydrolysis with citrus pectin (DE 30%) at 50°C under pH 5.0 were obtained using the Lineweaver-Burk plot. It was found that the $K_m$ and $V_{max}$ values of the polygalacturonase were 5.44 mg/mL and 61.73 μmol/(min·mg) protein, respectively. The $K_m$ value is similar to those of polygalaturonases from *Neurospora crassa* (5.0 mg/mL) (16) and *Paenibacillus amylolytics* (4.6 mg/mL) (28).

***Action mode of polygalacturonase from Zygoascus hellenicus V25*:** The action mode of polygalacturonase from *Zygoascus hellenicus* V25 was merely a preliminary study, in which the degradation products of citrus pectin (DE 30%) were analyzed by thin-layer chromatography (Fig. 3). Soluble products released after enzymatic hydrolysis were monogalacturonic acid and its derivatives by esterification with various fatty acids or L-ascorbic acid by yeast conversion (29). As no galacturonan oligosaccharide was found among the products, it is reasonable to postulate that the purified enzyme was exo-polygalacturonase (EC 3.2.1.67), which cleaved pectin by catalyzing hydrolytic cleavage at a non-reducing end of the substrate to generate monogalacturonic acid (9).

***Fruit juice clarification by purified enzyme*:** The purified polygalacturonase was found to have the stimulatory effect in the juice clarification of four fruits. As shown in Fig. 4, percentage transmittances at 660 nm in the juice clarification of four fruits have increased by 3.51, 4.36, 8.04, and 12.2%. The *p* values were 0.036, 0.059, 0.079, and 0.007 by SPSS statistical analysis, showing that the purified polygalacturonase had a noticeable effect on the juice clarification of tangerine and

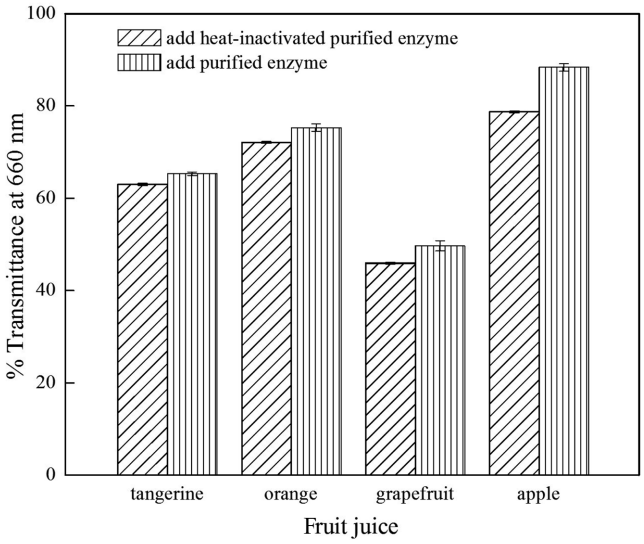


**Fig. 4.** Effect of the purified polygalacturonase on juice clarification.

apple. Apple juice clarification was the most noticeable, where the percentage transmittance at 660 nm increased from 78.7 to 88.31%. Fruit juice clarification might be due to the fact that the polygalacturonase can degrade insoluble pectin molecules into soluble galacturonic acid; as a result, the senses of color and taste of fruit juice products were also improved. The formation of pectin-protein flocs facilitated production of a clear clarification with the removal of colloidal part of the juice might also contribute to fruit juice clarification (30). In addition, studies on the synergism of the polygalacturonase and other enzymes such as pectin lyase, pectinesterase, and amylase in the fruit juice industry are undergoing in our lab to increase juice yield and the pressing efficiency of fruits for juice extraction.

**Disclosure** The authors declare no conflict of interest.

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