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ARTICLE

Isolation, identification and characterization of a novel elastase from *Chryseobacterium indologenes*

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Abstract Elastase is a type of protease that specifically degrades elastin. It has broad application prospects in medicine, food industry, and daily-use chemical industry. In this study, we isolated a bacterial strain WZE87 with high elastin-hydrolysis activity, which was identified as *Chryseobacterium indologenes* based on morphology, physiological and biochemical characteristics, and 16S rDNA sequence analysis. The elastase produced by this strain was purified by three steps: ammonium sulfate precipitation, Q-Sepharose fast-flow anion-exchange chromatography, and Sephadex G-75 gel-filtration chromatography. The purified elastase was 2376.5 U/mg in activity (a 8.3-fold increase in specific activity), and the recovery was 5.8%. Its molecular mass was estimated to be 26 kDa by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. This enzyme was stable in the pH range of 5.0–10.5 at 37 °C. The optimal temperature and pH were 37 °C and 7.5, respectively. The activity of this elastase was found to decrease when the temperature was higher than 50 °C. The activity was also inhibited by Zn^{2+}, Fe^{2+}, Fe^{3+}, and Mn^{2+} ions. The specific hydrolytic ability of this enzyme was similar to that of papain on substrates like gelatin, casein, soybean-isolated protein and bovine hemoglobin. However, this elastase preferentially hydrolyzed elastin in a protein mixture because of its specific adsorption. Considering its promising properties, this protease may be considered a potential candidate for applications in related industries.

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Keywords *Chryseobacterium indologenes* · Elastase · Enzyme property · Protein hydrolysis specificity · Purification

Introduction

Elastin is an insoluble protein that exists in the extracellular matrix and connective tissues. It is crosslinked with nonpolar amino acid residues, such as Ala, Leu and Ile, and is combined with microfibrils to form elastic fibers in vivo [1, 2]. Elastase is an endopeptidase that hydrolyzes insoluble elastin [3, 4]. Because of its hydrolytic bias for peptide bonds at the carboxyl end of neutral aliphatic amino acids, elastase shows a high specificity for elastin. It hydrolyzes insoluble elastin much more efficiently than other proteases and has been widely applied in many fields, such as curing hyperlipidaemia and arteriosclerosis in medicine [5].

In China, the people's favorite parts of livestock, apart from pork and beef, are the tendon, skin, and stomach tissues. However, the rich elastin in these tissues strongly limits the taste and texture of these foods. Accordingly, Chen et al. [6] reported the tenderization of pork by elastase by testing the meat shear force. Sachar et al. [7] reported the tenderization and sensory evaluations of beef by elastase by improving meat mouthfeel and dish flavor. Hence, elastase has become a promising meat tenderizer.

Since the reports by Balo and Banga that protease from the pancreas could hydrolyze elastin [8, 9], elastase has been widely isolated from bovine, moose, North Atlantic salmon, and ovine pancreas [10–12]. However, the high cost of elastase sourced from animals has impeded its application. Microbial elastase serves as a preferred option not only because of the low cost and the extensive

distribution of elastase in microorganisms, but also the ease to genetically manipulate enzyme properties for various requirements [3]. Since the first isolation and purification of elastase from *Flavobacterium elastolyticum* by Ines et al. [13], elastase from *Flavobacterium* sp. R-102-87, *Bacillus alcalophilus* Ya-B, *Vibrio cholerae*, and *Micrococcus luteus* was reported which are amenable to industry production [7, 12, 14, 15].

In this study, we describe the purification and characterization of the elastase produced by the strain *Chryseobacterium indologenes* WZE87. Particularly, we analyzed and evaluated its hydrolysis specificity toward different protein substrates. This research provides a valuable reference for the further application of elastase in meat tenderization and animal protein process in future.

Materials and methods

Strain identification

An elastase-producing bacterium, designated strain WZE87, was isolated from the mud of a meat market in Ya'an city. It was grown in Luria-Bertani (LB) broth (1.0% w/v peptone, 0.5% w/v yeast extract, 0.5% w/v NaCl, 1.5%–2.0% w/v agar powder, pH 7.0) for 24 h at 37 °C. Bacteria with the highest HC value (the ratio of hydrolysis halo to colony diameter) were then isolated from the growth medium (culture conditions: 60 h at 37 °C in bovine tendon powder (BTP) medium consisting of 1.0% w/v BTP, 1.5%–2.0% agar powder, pH 7.0–7.5). The physiological and biochemical properties were identified using a VITEK2 automated bacterial identification system (Biomerieux Biotech Co., Ltd., France), and then 16S ribosomal DNA (rDNA) was amplified. Primers are as follows:

upstream primer 27F(5′-AGAGTTTGATCCTGGCTCAG-3′)
downstream 1492R(5′-TACGG(C/T)TACCTTGTTACGACTT-3′).

Elastase activity assay

Elastase activity was measured using a modification of a previous method [5]. Orcein elastin (20 mg) in Tris-HCl buffer (6 mL of 200 mM, pH 8.0) was mixed with standard elastase solution (0.1 mL of 1500 U/mL) and incubated for 1 h at 37 °C. Tris-HCl buffer (200 mM, pH 8.0, no enzyme and substrate) was used as the control group. A standard curve was generated from the reaction where elastase hydrolyzed orcein elastin. One unit of elastase activity was defined as the amount of enzyme required to hydrolyze 1 mg elastin per minute at 37 °C.

Elastase purification

Strains were grown in seed medium (0.5% glucose, 1.0% peptone, 0.5% yeast extract, 0.5% NaCl, pH 7.0) for 18 h at 30 °C and then inoculated in fermentation medium (0.5% glucose, 1.0% peptone 0.4% w/v cornflour, 0.4% BTP, 0.1% w/v $K_2HPO_4{\cdot}3H_2O$, 0.05% w/v $MgSO_4{\cdot}7H_2O$, pH 7.0) for 26 h at 30 °C. After centrifugation of the culture, the supernatant was retained as the crude enzyme. The crude enzyme samples were precipitated separately with 25, 30, 40, 50, 60, 70, 75, and 80% ammonium sulfate saturation to ascertain the optimal precipitation saturation by measuring the elastase activity of the supernatant. Then the enzyme sample was precipitated with the optimal precipitation saturation and dissolved in Buffer A (200 mM Tris-HCl, pH 7.5) and concentrated by PEG-20,000. The concentrated protein (2 mL) was subjected to a Q-Sepharose fast-flow chromatography column (1.5 × 20 cm) after pre-equilibration with distilled water and Buffer A, which was eluted with a linear gradient of 1 mM NaCl, at a flow rate of 3 mL/min. Afterward, the active peak (1 mL) was pooled and applied to a Sephadex G-75 column (1.5 × 50 cm) previously equilibrated with Buffer A. Specific protein was eluted with Buffer A at a flow rate of 0.5 mL/min. Finally, aim fractions with active elastase activity were collected, concentrated, and refrigerated at 4 °C for further analysis. The protein concentration was determined by the method of Marion et al. [16]. Gradient concentrations of bovine serum albumin (BSA) were used to make a standard curve. All the purification steps were conducted at temperatures not exceeding 4 °C.

Elastase molecular mass determination

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed with 12% polyacrylamide gel [15]. The standard molecular weight markers were purchased from Takara Biotechnology Co., Ltd. (Da Lian, Liaoning province, China). The specific fraction (10 μL) was added into each lane of the gel. The protein bands were stained with Coomassie Brilliant Blue R-250.

Enzyme characterization

The optimal reaction temperature was determined by measuring the elastase activity in a water bath at different temperatures (20, 25, 30, 37, 45, 50, 55, 60, 65, 70 and 80 °C). For the thermostability analysis, elastase activity was measured in the range of 30–80 °C.

To determine the optimal reaction pH of the elastase, the enzyme was mixed with 200 mM buffer (sodium acetate, sodium phosphate, Tris-HCl, boric acid, or sodium carbonate) in a pH range of 3.5–10.5. To analyze the stability of the elastase at different pH, 0.1 mL of enzyme solution was added to 0.6 mL of 20 mM Tris-HCl buffer in a pH range of 3.5–12.5 and left for 24 h at room temperature. Enzyme activity was measured at the optimal condition in 200 mM Tris-HCl buffer.

The effect of metal ions on the enzyme activity was investigated by separately adding three monovalent ions (Na^+, K^+, and Li^+), five divalent ions (Zn^{2+}, Mn^{2+}, Ca^{2+}, Mg^{2+}, and Fe^{2+}) and Fe^{3+} at the final concentration of 5 mM of the reaction mixture. Meanwhile, the influence of chemical reagents on elastase activity was also tested by 5 mM ethylenediaminetetraacetic acid (EDTA), 5 mM sodium dodecyl sulfate (SDS), and 0.01 mM β-mercaptoethanol. The residual activities were determined as a percentage of the activity in the control sample without any extra additive, measured at optimum temperature and pH.

Elastin adsorption and selective hydrolysis of elastase

After 20 mg orcein elastin and 20 mg BTP were separately added to 1 mL of enzyme solution (150 U/mL), the mixtures were incubated in an ice-water bath for 20 min. Then the elastase enzyme activity (EEA) was measured using each of the supernatants. The adsorption rate (AR) was calculated by the formula as follows: $AR = (EEA_{Control-} - EEA_{Sample})/EEA_{Control} \times 100\%$. Furthermore, for the characterization prior to hydrolysis, the elastase (after 60/120-folds dilution) was added to the reaction systems as follows: (1) 10 mg orcein elastin; (2) 10 mg orcein elastin + 10 mg casein; (3) 10 mg orcein elastin + 10 mg gelatin; (4) 10 mg orcein elastin + 10 mg BSA.

Hydrolysis of different proteins by the purified elastase and by papain

Elastase and papain, which have the same hydrolysis activity toward casein, were used to hydrolyze 20 mg of each protein as follows (40 °C, 30 min): orcein elastin, casein, gelatin, BSA, bovine hemoglobin, isolated soybean protein, and feather meal. After adding 2 mL of 10% (400 mM) trichloroacetic acid followed by centrifugation (10 min at 9000 rpm), the absorbance at 680 nm (A_{680}) of the supernatant was measured by the Lowry method [17].

Statistical analysis

Data were analyzed using the unduplicated two-factor analysis of variance. A P value of 0.05 was considered to be significant, and a P value of 0.01 was considered to be extremely significant.

Results

Morphologic identification of the strain producing elastase

On the LB medium plate, the single colony of strain WZE87 appeared golden in color, wet and viscous on the surface, and round in shape after 24 h of incubation at 37 °C. Cells were characterized as gram-negative, in the absence of flagella or spores, and 3.1–3.7 × 9.0 μm in size under a light microscope. After 60 h of cultivation on the BTP medium, the BTP, which in the hydrolysis halo has the highest HC value (4.0), was completely hydrolyzed to be transparent. Additionally, its initial elastase activity of crude enzyme culture was 71.06 U/mL.

Physiological and biochemical identification of bacteria

Furthermore, the strain WZE87 was tentatively identified as *Chryseobacterium indologenes* from the physiological and biochemical characteristics (Table 1). In order to confirm the identity, a full 16S rDNA sequence with 1484 bp in length was amplified and determined on GenBank (Accession No. HQ848390). The blasting result indicated that the strain WZE87 showed 97–99% identity with *Chryseobacterium indologenes*. Therefore, we concluded that strain WZE87 is a strain of *Chryseobacterium indologenes*.

Purification of elastase

According to the supernatant activities of the enzyme sample after ammonium sulfate precipitation (Fig. 1), 30 and 60% ammonium sulfate saturation were chosen for gradient precipitation. Then, the final precipitate was dissolved in Buffer A, and the solution was dialyzed against Buffer A. Afterward, the dialysate was applied to a Q-Sepharose column. As shown in Fig. 2, elastase activity was detected at two active peaks. The stronger active fractions (Peak 1) were pooled and concentrated by PEG-20,000 at 4 °C. Finally, the enzyme solution was subjected to a Sephadex G-75 column. The proteins were eluted as one wide peak (Peak 1) and two sharp peaks (Peak 2 and Peak 3) (Fig. 3). Because the elastase activity was detected in Peak 2 only, its fractions were pooled, concentrated, freeze-dried, and stored at − 20 °C. The results of the elastase purification are summarized in Table 2. By a three-step procedure, this enzyme with 2376.5 U/mg

Table 1 Physiological and biochemical identification of *C. indologenes*

Index	Result	Index	Result
Ala-Phe-Pro-Arylamidase	+	Glu-Gly-Arg-Arylamidase	+
Adonitol	−	Saccharose/sucrose	−
L-Pyrrolydonyl-Arylamidase	+	D-Tagatose	−
L-Arabitol	−	D-Trehalose	−
D-Cellobiose	−	Citrate (sodium)	+
β-Galactosidase	−	Malonate	−
H_2S production	−	5-Keto-D-Gluconate	−
β-N-Acetyl-Glucosaminidase	+	L-Lactate alkalinization	−
Glutamyl arylamidase pNA	+	α-Glucosidase	+
D-Glucose	−	Succinate alkalinization	−
Gamma-Glutamyl-Transferase	+	*N*-Acetyl-Galactosaminidase	−
Fermentation/glucose	−	α-Galactosidase	−
β-Glucosidase	+	Phosphatase	+
D-Maltose	−	Glycine arylamidase	+
D-Mannitol	−	Ornithine decarboxylase	−
D-Mannose	−	Glu-Gly-Arg-Arylamidase	+
β-Xylosidase	−	Saccharose/sucrose	−
β-Alanine arylamidase pNA	−	D-Tagatose	−
L-Proline arylamidase	+	D-Trehalose	−
Lipase	+	Citrate (sodium)	+
Palatinose	−	L-Malate assimilation	−
Tyrosine arylamidase	+	Ellman	−
Urease	−	L-Lactate assimilation	−
D-Sorbitol	−		

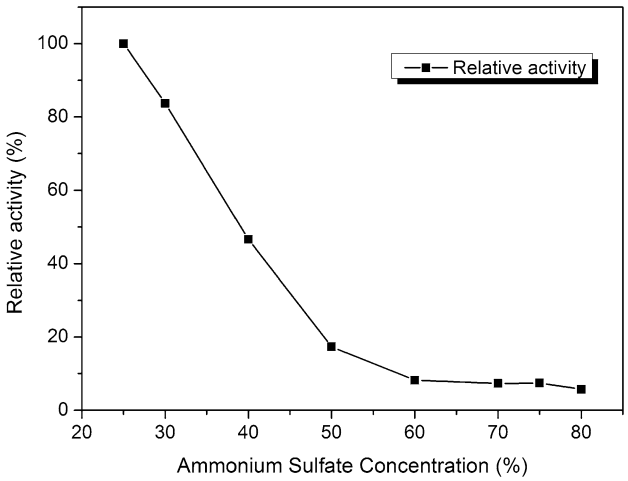

Fig. 1 Relative activity of the supernatant from ammonium sulfate precipitation

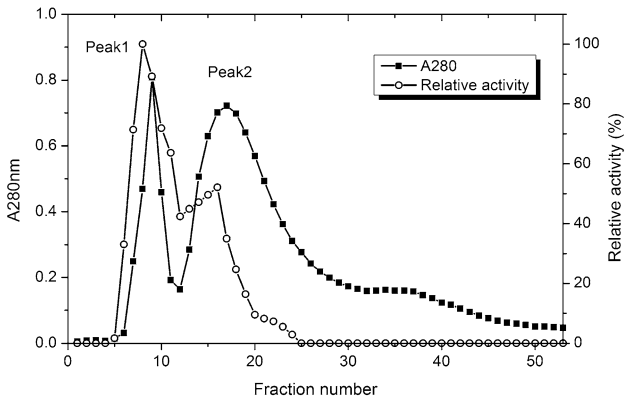

Fig. 2 Elution curve from Q-Sepharose column at A_{280}

relative activity was purified 8.3-fold with a recovery ratio of 5.8% from the crude extract. As seen from the SDS–PAGE analysis, elastase from *C. indologenes* WZE87 was purified to homogeneity with the relative molecular mass 26 kDa (Fig. 4).

Effect of temperature on enzyme activity and stability

The optimum activity of the enzyme was observed at 37 °C (Fig. 5A). When the elastase was treated at 50 °C or at lower temperatures for 30 min, the residual enzyme activity remained over 85% of the original enzyme activity. However, the enzyme activity dropped sharply by about 90% of the full enzyme activity when at 60 °C for 30 min,

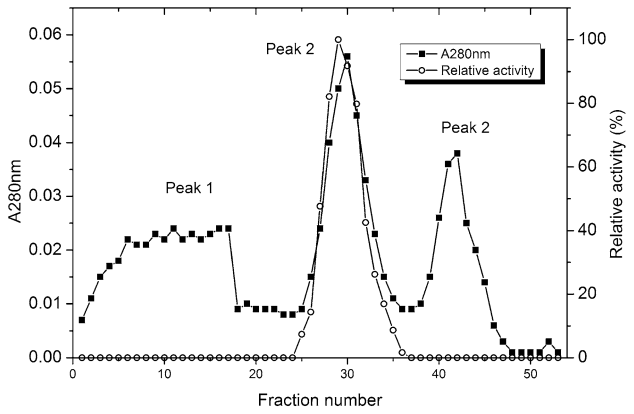

Fig. 3 Elution curve from Sephadex G-75 at A_{280}

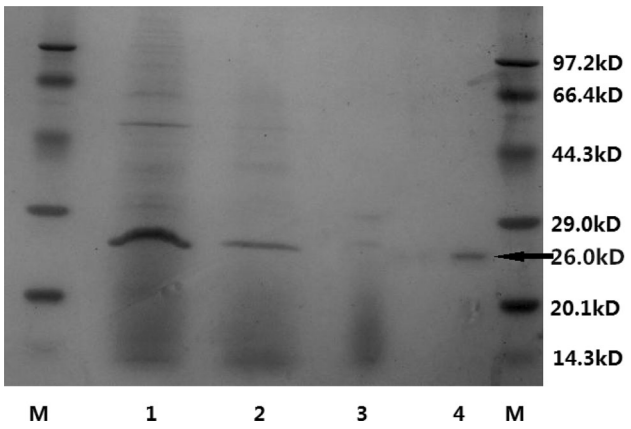

Fig. 4 SDS–PAGE of elastase from *C. indologenes* strain WZE87. (Lane M: low–molecular–weight protein marker; lane 1: crude enzyme solution; lane 2: concentrated sample from ammonium sulfate precipitation; lane 3: active peaks from Q-Sepharose column; lane 4: active peaks from Sephadex G-75 column)

and the enzyme activity was completely lost at temperatures over 70 °C (Fig. 5B).

Effect of pH on enzyme activity and stability

The optimal reaction pH for the elastase was obtained at pH 7.5 in Tris-HCl buffer and at pH 7.7 in boric acid buffer (Fig. 6A). The pH optimal stability was determined in a pH range of 5.0–10.6 (Fig. 6B). After 30 min of incubation at 37 °C, the peak of residual enzyme activity was observed at pH 6.4 with 84% of activity. According to the results, the residual enzyme activity remained at over 70% of activity in all tests of pH stability (72.5–84%), and there is no significant difference in different pH, except at the optimal pH.

Effect of metal ions and reagents on enzyme activity

Most of the metal ions exhibited a significant inhibition effect on the elastase (Table 3). Some of the metal ions such as Fe^{2+}, Fe^{3+}, and Zn^{2+} almost completely inhibited the enzyme activity, while the Mn^{2+} ion showed a sharp inhibition of the enzyme by 63%. Further, Ca^{2+}, K^{+}, Na^{+}, and Li^{+} appeared to inhibit the elastase slightly, but only Mg^{2+} increased its activity marginally. Additionally, the elastase activity was drastically inhibited about 31–56% by the reagents including SDS, EDTA, and β-mercaptoethanol.

Effect of substrate adsorption on selective hydrolysis

The adsorption rate of elastase toward insoluble substrates was likely to affect its hydrolysis ability. As seen in Table 4, the elastase showed a significant adsorption to elastin. 39% orcein elastin and 30% BTP were absorbed by the elastase. Meanwhile, both in high and low concentrations (60/120-folds dilution), casein and gelatin remarkably affected the selective hydrolysis of the elastase ($P < 0.05$) (Fig. 7A). However, BSA showed no significant effect on the selective hydrolysis of the elastase ($P > 0.05$).

Elastase versus papain of hydrolysis activity to animal protein

In order to evaluate the potential application of bacterial elastase in foodstuff industry, *C. indologenes* elastase and papain were used to degrade edible proteins (Fig. 7B). The result showed that the two enzymes have the similar hydrolytic characteristics toward the different proteins, and the relative activity was as follows: soybean-isolated protein > BSA > gelatin > bovine hemoglobin > feather meal. However, the relative activity of elastase to elastin could reach up to 69.7%, and it was not detected in the

Table 2 Purification of elastase from *C. indologenes* strain WZE87

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Crude extract	206.03	58,719.12	285	1	100
Ammonium sulfate precipitation	43.8	19,972.99	456	1.6	34.9
Q-Sepharose	6.85	13,470.53	1966.5	6.9	23
Sephadex G-75	1.44	3422.17	2376.5	8.3	5.8

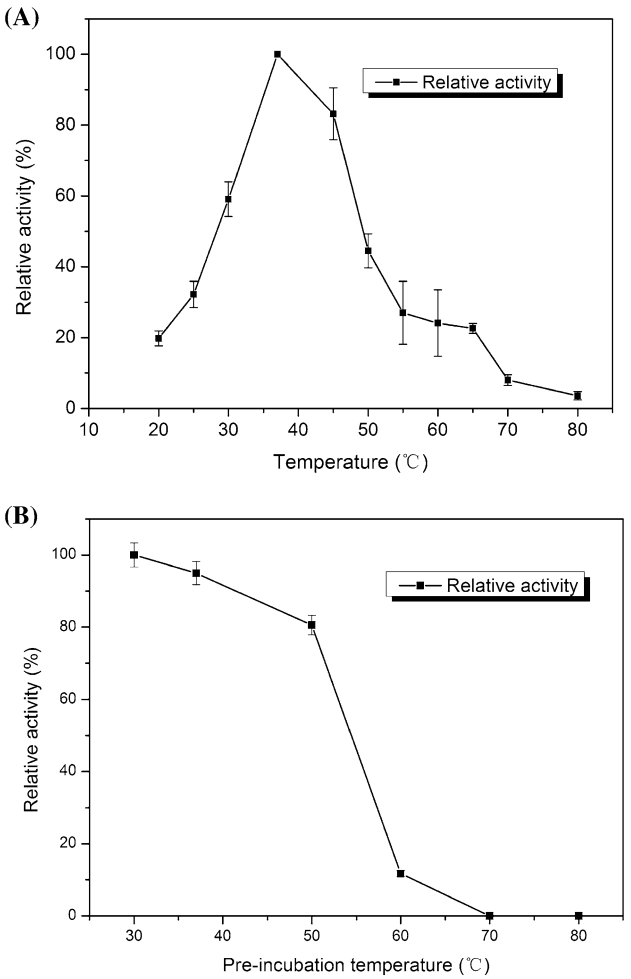

Fig. 5 Effects of temperature on *C. indologenes* elastase activity (**A**) and thermal stability (**B**)

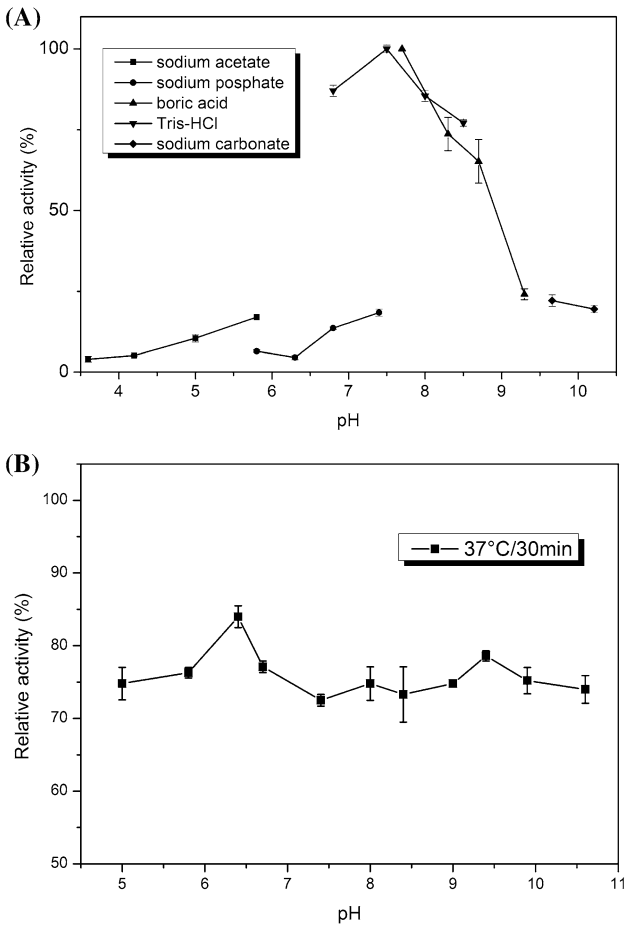

Fig. 6 (**A**) Effects of pH on elastase from *C. indologenes*. (**B**) pH stability of elastase from *C. indologenes*

papain–elastin group. Hence, the elastase in this research may have a similar but better hydrolytic characteristic than papain.

Discussion

Elastase was initially extracted from animal viscera, but this source cannot satisfy the requirements of industrial and daily use. Actually, microorganism isolation of elastase with high activity is an important and direct way to attain more elastase. Ying et al. isolated an elastase with 18 U/mL activity from *Pseudomonas* bacteria [1]. Fang et al. [18] attained an elastase with 74.25 U/mL activity from *Bacillus subtilis*. Chen et al. isolated an elastase with up to 100 U/mL initial enzyme activity from *Bacillus* sp. [19]. In this study, we isolated a new elastase-active strain, *Chryseobacterium indologenes*, and its initial enzyme activity was 71.06 U/mL. Compared with other elastases reported, elastase of this strain has a relatively high natural enzyme activity. Furthermore, the specific activity of the purified elastase was 2376.5 U/mg, which is higher than the reports

Table 3 Effects of chemicals and reagents on elastase from *C. indologenes*

Reagent	Concentration (mM/L)	Relative activity (%)
CK	0	100
EDTA	5	66.7
SDS	5	44.1
β-Mercaptoethanol	0.01	69.4
KCl	5	85.9
NaCl	5	77.5
LiCl	5	96.4
$ZnCl_2$	5/0.05	7.2/24.5
$MnCl_2$	5	36.9
$CaCl_2$	5	86.5
$MgCl_2$	5	102.7
$FeCl_2$	5	0
$FeCl_3$	5	0

Table 4 Elastin adsorption of elastase from *C. indologenes*

Index	Control group	Orcein elastin	BTP
Supernatant enzyme activity (U)	146	89	102
Adsorption rate	–	39%	30%

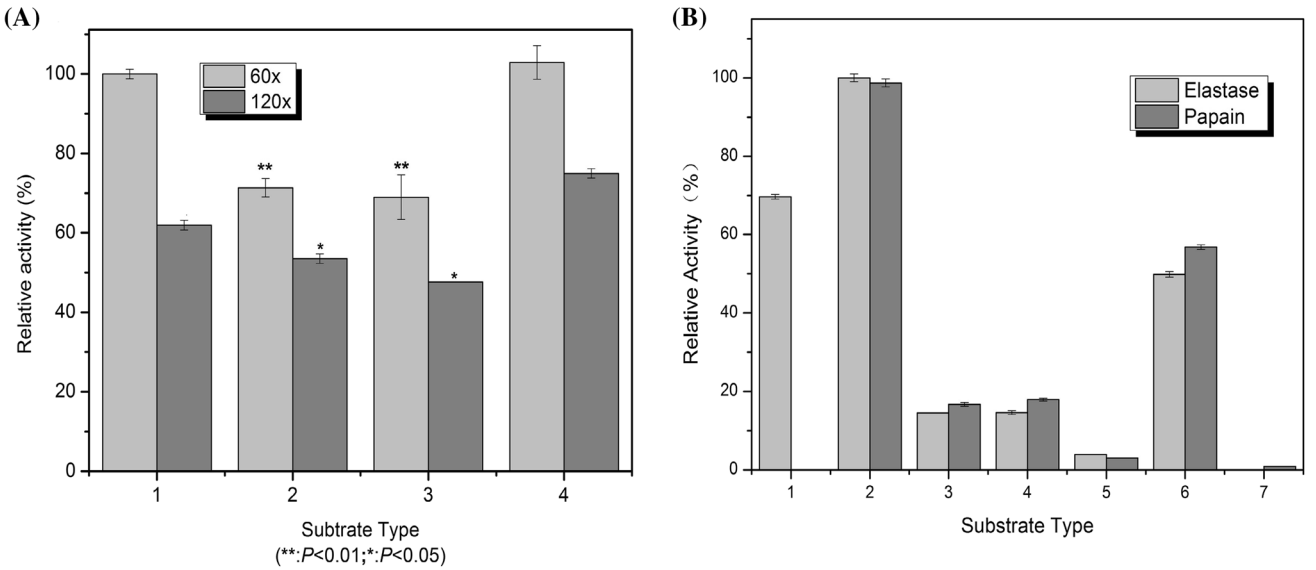

Fig. 7 (**A**) Elastin degradation by *C. indologenes* elastase alone or in a mixture with other proteins (1) Orcein elastin; (2) orcein elastin + casein; (3) orcein elastin + gelatin; (4) orcein elastin + BSA. (**B**) Effects of elastase and papain on different proteins' degradation as measured by the Lowry method. (1) Orcein–elastin; (2) casein; (3) gelatin; (4) BSA; (5) bovine hemoglobin; (6) soybean-isolated protein; (7) feather meal

of Chen et al. (292 U/mg) and Olfa et al. (81 U/mg) [20, 21], and similar to that reported by Han et al. [22] (2427 U/mg). Additionally, as a monomeric enzyme, SDS–PAGE analysis showed that the molecular weight of the elastase was 26 kDa, which was different from all reported microbial elastases [23].

Reaction temperature and pH are two crucial parameters of protease industrial production. The optimal reaction temperature and pH of reported microbial elastases were generally higher than 45 °C and 7.0 [14, 24–26], respectively. The elastase in our research was thermostable below 60 °C, but its optimal reaction temperature was 37 °C. So, it may be used in physianthropy and other fields as pancreatic elastase. Meanwhile, it has a wide range of pH stability (pH 5.0–10.5) with high residual enzyme activity (72.5–84%). Hence, this elastase could be applied to tenderize the elastin-rich tissues of food animals.

Further in the study, 5 mM Mg^{2+} slightly activated this enzyme, while 5 mM Li^{+}, Na^{+}, Ca^{2+}, K^{+}, EDTA and 0.01 mM β-mercaptoethanol inhibited it indistinctively. This differs from the results of Tsai et al. and Chen et al. who reported that Ca^{2+} could stabilize the activity of the enzyme and K^{+} could activate the elastase [19, 26]. Generally, SDS improved the elastase activity, but 5 mM SDS inhibited the activity of the elastase. Also, 5 mM Zn^{2+}, Fe^{2+} and Fe^{3+} significantly reduced the activity of this enzyme, which is consistent with the reports of Yan et al. and Liu et al. [17, 27]. To determine whether the previous concentration of Zn^{2+} which inactivated this elastase (residual activity 7.2%) was too high in the test, 0.05 mM Zn^{2+} was utilized in further analysis. However, it still inhibited the activity (residual activity 24.5%). Thus, it can be seen that elastase from *Chryseobacterium indologenes* is not a metalloproteinase [28]. However, EDTA, as a low-concentration metal–ion-chelating agent, inhibited the activity of this elastase significantly. This showed that a certain kind of metal ion was crucial in the stabilization of elastase activity, which needs to be further investigated. Additionally, the inhibitory activity of β-mecaptoethanol toward this elastase indicated the existence of a disulfide bond. Hence, the distinctive enzyme properties of the elastase in this research manifest that it was a new microbial elastase.

Elastase is a broad spectrum proteolytic enzyme which is capable of hydrolyzing insoluble protein. Chen et al. [6] reported that the tenderization effect of recombinant elastase was better than commercial papain on pork shear force and tenderization rate. Kanako et al. [24] purified and crystallized an elastase named KFP 419, which the relative activity of elastin/casein of it was higher than that of

poronase. In the current research, when coexisting with other substrates, elastin was selectively hydrolyzed by the elastase. According to the substrate specificity, this elastase can hydrolyze elastin while papain cannot hydrolyze elastin. This is significantly important for the tenderization of food animal tissues which are rich in elastin, such as bovine stomach and cowheels. Moreover, the elastase and papain showed no remarkable hydrolysis difference to the substrates ($P > 0.05$), although the elastase slightly hydrolyzed the feather meal.

In summary, a novel elastase was isolated and purified from *Chryseobacterium indologenes* in this study. Due to its specific hydrolysis toward elastin, the elastase has a foreseeable application in meat tenderization. Ultimately, the intensive investigation of this elastase should focus on hydrolysis optimization and food safety evaluation for its industrialization and commercialization.

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