微生物学报 *Acta Microbiologica Sinica*
49(2):1613 – 1620;4 December 2009
ISSN 0001 – 6209;CN 11 – 1995/Q
http://journals.im.ac.cn/actamicrocn

Expression,purification and molecular characterization of elastase from *Aeromonas hydrophila* strain J-1

Xilong Meng[1,2],Yongjie Liu[1]
[1]College of Veterinary Medicine,Na
[2]College of Agriculture, Liaocheng

Abstract: [Objective] The enzym ... monas *hydrophila*. To know better about the molecular characterizatio ... activity. [Methods] We cloned a structural gene *ahyB* encoding for ... BL21 by using pET-32a as vector. We purified the recombinant enzy ... activity of the purified protein by incubation in a buffer containing 6 ... addition,we also purified the native enzyme from the culture supernata ... ion exchange chromatography and sephaceryl chromatography. We co ... yme showed a pH optimum at 8.5, but recombinant enzyme at 10.0. ... stable for heat. Both the elastase preparations showed some identical ... A,the cation chelator,and OPA,a Zn^{2+}/Fe^{2+} protease inhibitor. How ... inst the inhibitors. And the metal cation Zn^{2+} and Fe^{2+} strongly inh ... he similar enzymatic characteristics as the native enzyme from *A*. *hyd* ... talloendopeptidase. This is the first report on the recombinant expressio ... ase.

Keywords: *Aeromonas hydrophila* ...
CLC number: Q936 Document ...

Aeromonas hydrophila, ... produces toxins and a variet ... enzymes enhancing its ... extracellular proteases have ... including a 38 kDa thermostable metalloprotease [1-2], a 68 kDa temperature-labile serine protease [3-5], a 19 kDa zinc-proteinase [6], and a 22 kDa serine proteinase [7]. The contribution of the proteases to the pathogenesis was due to invasiveness and establishment of infection by overcoming initial host defenses and by providing nutrients for cell proliferation [8-9].

... ty, elastase is capable of ... s, including elastin, a major ... issues that is resistant to ... Elastase is therefore regarded as one of major virulence factors of some pathogenic bacteria. Many *A*. *hydrophila* strains secrete elastolytic activity into culture medium. Encoded by the *ahyB* gene, elastase is synthesized as a preproprotein with a 19-amino-acid signal peptide, a 164-amino-acid N-terminal propeptide, and a 405-amino-acid intermediate that is further processed into a mature protease and a C-terminal

Supported by the Program for New Century Excellent Talents in University (NCET-07-0440),the Jiangsu Provincial Natural Science Foundation of China (BK2007155) and the International Foundation for Science (A/4108-1)
*Corresponding author. E-mail:liuyongjie@njau.edu.cn
Received:19 July 2009/Revised:14 September 2009

propeptide in *A. hydrophila* AG2 [10].

The studies on the elastase of *A. hydrophila* mostly focused on its roles in the pathogenicity of this bacterium and the processing mechanism of protease, but biochemical characterization about this enzyme has not been sufficiently evaluated. In this study, we tried to express the elastase gene of *A. hydrophila* J-1 in *E. coli* BL21, purify the recombinant protein and investigate its property and activity. Meanwhile we also purified the native elastase from the culture supernatant

analyzed its property.

1 MATERIAL AND MET

1.1 Bacterial strains, pla conditions

A. hydrophila strain J-1
dead fish with haemorrhagic
China in 1989. The strain w
the species *A. hydrophila* by
amplification of 16s rDNA ge
as the recipient for initial clo
BL21 was used as expression
pET-32a was used in expres
pMD18-T as cloning vector.
Bertani (LB) broth or on a
hydrophila J-1 was cultured ir
5 g/L Sucrose, 2.5 g/L NaCl,

1.2 DNA manipulations

Bacterial genomic DNA w
the manuals for the genomic I
kits (QIAGEN). Plasmid D
QIAprep spin miniprep kit.
DNA Purification Kit were supplied by Takara.

1.3 Cloning of the elastase gene of *A. hydrophila* J-1

Oligonucleotides ahyB-F (5′-GGGGAATTCGGCAACGTC-AAGACTGGCAAGT-3′) and ahyB-R (5′-TTACTCGAGAGCCGCAGGCTCCCTGATCG-3′) corresponding to mature protease gene fragment were used to amplify *ahyB* gene from *A. hydrophila* J-1 by PCR. PCR conditions were as follows: initial denaturation at 94℃ for 2 min, followed by 35 cycles of denaturation at 92℃ for 1 min, annealing at 47.8℃ for 30 s and extension at 72℃ for 1 min. An extension step of 5 min at 72℃ was carried out following the last cycle in order to ensure full-length synthesis of the fragment. The PCR product was ligated to pMD18-T vector and transformed into *E. coli* DH5α. The internal fragment was recovered by *Eco*R I and *Xho* I restriction digestion (*Eco*R I and *Xho* I sites in the primers are underlined), and finally ligated into *Eco*R I and *Xho* I digested pET-32a which contains an ampicillin-resistant (Ap^r) gene and transformed into *E. coli* BL21. The recombinant transformants were selected using ampicillin (100 μg/mL on LB agar plates. The colonies containing the

identified and confirmed by

fication of the recombinant

ombinant clone of ahyB were
containing ampicillin (100
ed for 3 h by 1 mmol/L
ide (IPTG). After the
or 15 min, the precipitate was
iffer (0.5 mol/L NaCl,
l/L imidazole, pH 7.9) and
or 5 s with 5 s rests in an
TY92-II, Xinzhi Scientific
o, China). Most of elastases
body. The inclusion body
ed in binding buffer, filtered
d purified as described in the
Purification Kit (Novagen).
vity, the purified enzyme was
buffer containing 6 mol/L
nt removal of denaturant by
of protein was determined by
. The purified protein was
analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 5% stacking gel and 12.5% separating gel. The samples were heated at 100℃ for 5 min prior to electrophoresis. Twenty micrograms (20 μg) of proteins were loaded per lane and the protein bands were visualized by staining with Coomassie brilliant blue. The enzyme was stored at −20℃.

1.5 Purification of the elastase from culture supernatant of *A. hydrophila* J-1

Cells of *A. hydrophila* J-1 were grown in 2000 mL of TS medium at 28℃ for 48 h with shaking. After the cells were removed by centrifugation at 2500×g 5 min at 4℃,

the resulting supernatant was used as the crude enzyme sample.

The culture supernatant was concentrated to 87 mL by ultrafiltration using a 10 kDa nominal molecular weight cut-off membrane in Millipore (Pellicon) and then fractionated with ammonium sulfate. The 30 to 60% ammonium sulfate-insoluble precipitate was dissolved in 50 mmol/L Tris-HCl buffer (pH 8.0) and dialyzed against the same buffer at 4℃ for 24 h. The dialyzate was loaded onto a DEAE-cellulose DE-5
(1.0 cm × 20 cm) equilibrat
Tris-HCl, pH 8.5). The co
0.5 mol/L linear gradient of
buffer B (50 mmol/L Tris-HC
a flow rate of 0.1 mL/min.
and analyzed for elastalytic a
were pooled and loaded on a
Pharmacia Biotech, USA)
25 cm)[2-13]. The active e
peak with buffer A and used
the purified enzyme.

1.6 Properties of proteas

1.6.1 Thermostability of
purified elastase to heat w
elastase (0.2 mL) taken in a
30 min and 70℃ for 10 min
while the sample without heat

1.6.2 Metal ion requiremen
elastase activity was determi
purified enzyme with 0.3 mm
room temperature for 15min.
mixture without metal ions.
determined according to standard assay condition.

1.6.3 Effect of pH on elastase activity: The optimum pH for the elastolytic activity was examined by using buffers with a variety of pH: 0.05 mol/L citric acid at pH 4.0 to 6.0, 0.05 mol/L Tris-HCl at pH 7.0 to 9.0, and 0.05 mol/L glycine-NaOH at pH 10.0 to 11.0[1]. The mixtures containing purified elastase, buffer with different pH separately and substrate in an Eppendorf tube were incubated at 37℃ with shaking for 1 h. After incubating, the elastolytic activity was measured.

1.6.4 Effects of the inhibitors on elastase activity: The effects of the following inhibitors were determined: 10 mmol/L or 20 mmol/L ethylene diamine tetraacetate (EDTA), 10 mmol/L phenylmethylsulfonyl fluoride (PMSF), 10 mmol/L or 20 mmol/L 1,10-phenanthroline (OPA). The mixtures containing 50 μL of purified elastase and 50 μL of each of the selected inhibitors separately in an Eppendorf tube were preincubated at room temperature for 30 min, and then activity was measured as described above. Residual protease activity was expressed as a percentage of a control preincubation without

elastase activity: To evaluate
with identical concentrationg
astase activity, an attempt was
is-HCl, borate, glycine-NaOH
buffer (pH8.0) was separately
ure (containing elastase and
ivity was assayed after 1 h
ing.

say

was examined in a single-
fied enzymes in the wells on
plemented with 0.3% (wt/
es. The Petri dishes were
in the humidified chamber.
reveal activity. Quantitative
elastin Congo red assays.
L) of filtered enzyme (0.45-
dded to a reaction mixture
o red in 0.25 mL of distilled
L Tris-HCl buffer (pH 7.5).
ubated at 37℃ for 1 h with
the reaction was stopped by
adding 500 μL of 0.7 mol/L phosphate buffer (pH 6.0). After centrifugation at 13000 × g, the supernatant was detected. One elastolytic unit was defined as the amount of enzyme hydrolysing 0.25 mg elastin-Congo red in A_{495} for 1 h of incubation at 37℃[1]. Standard curve was drawn according to the method of Sarchar[4]. Each assay was performed in triplicate.

2 RESULTS

2.1 Molecular cloning and nucleotide sequence analysis of *A. hydrophila* ahyB gene

The *ahyB* gene was amplified from *A. hydrophila* J-1

genome by PCR using ahyB-F and ahyB-R as primers. Using pET-32a as an expression vector, the *ahyB* gene was constructed in *E. coli* BL21. Ap^r transformants were selected on LB agar plates supplemented with ampicillin (100 μg/mL) and elastin. Approximately thirty colonies with Ap^r and elatolytic positive were obtained.

The nucleotide sequence of the cloned gene revealed 787 bp with the capacity to encode a polypeptide of 262 amino acids and with a predicted molecular size of 28,820. The cloned sequence
protease sequences without s
nucleotide sequence of *ahyB*
GenBank nucleotide sequen
No. GQ494015.

The nucleotide sequence
DNAstar, furthermore, the cl
databases at NCBI showed 92
A. hydrophila strain AG2, 9
gene of *A. caviae* T-64 and
of *Pseudomonas aeruginosa* I

2.2 Purification of expres

The *ahyB* gene was cl
expressed in *E. coli* BL2
bacterial cells were induced b
formed with inclusion body.
was further purified by His
The elastolytic activity of
recovered by incubation in
guanidine HCl and subseque

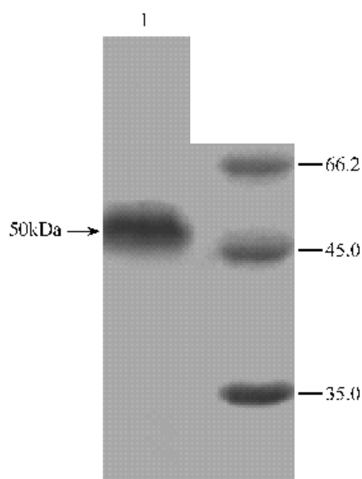


Fig. 1 SDS-PAGE analysis of recombinant elastase expressed in *E. coli* BL21. Lane M, molecular mass standards (Da); Lane 1, purified elastase.

dilution. Twenty millilitres (20 mL) of expressed protein with the protein concentration of 10 mg/mLwas obtained and the elastolytic activity was up to 50 U/mL. The purified enzyme was moved as a single band on SDS gels, indicating its homogeneity. The molecular weight of fusion protease was approximately 50 kDa as estimated by SDS-PAGE gel electrophoresis (Fig. 1), which agreed well with the calculated molecular mass (His fragment 22 kDa + interest protein 28 kDa) of the recombinant enzyme.

e elastase from culture
hila J-1

rom the extracellular medium
emoving the bacteria by low-
ation using 10 kDa nominal
nbrane, 30 to 60% ammnium
change chromatography (Fig.
aphy (Fig. 3). The results of
l in Table 1. The specific
ne preparation was up to
4-fold increase compared to
cular weight of this protease
estimated by SDS-PAGE gel

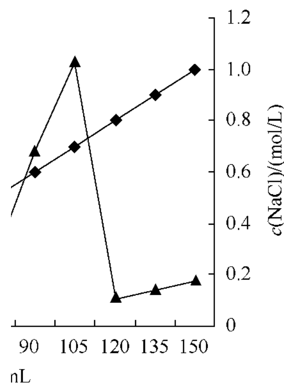


Fig. 2 Ion-exchange Chromatography of AhJ-1 elastase.

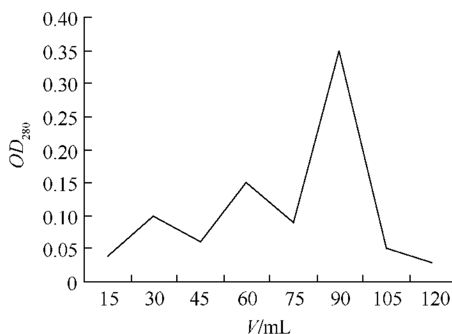


Fig. 3 Sephaceryl Chromatography of AhJ-1 elastase.

electrophoresis using protein markers of known molecular weight (Fig. 4).

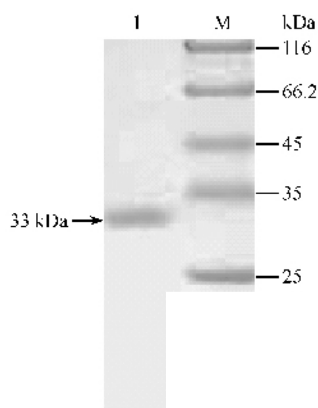


Fig. 4 SDS-PAGE analysis of purif
of *Aeromonas hydrophila* J-1. Lane M
Lane 1, purified elastase.

2.4 Properties of protease

Both elastase preparations, native enzyme from *A. hydrophila* J-1 and recombinant enzyme from *E. coli* BL21 show elastolytic activity when detected on 1.0% agarose supplemented with 0.3% elastin (Fig. 5).

To examine the roles of Zn, Ca, Fe and other metal ions in elastase production, each ion was added alone prior to inoculation of 18 h. The elastase obtained from *A. hydrophila* J-1 was strongly influenced by the presence of
metal cation Mg^{2+}, Zn^{2+},
ibited the enzyme activity,
l the elastase activity that was
n the control. Ca^{2+}, Na^{+},
distinct effects on elastase

Purification Stage	ivity	Purification fold
Crude extract		1.0
Utrafiltration		1.3
Ammonium precipitate		1.45
Ion-exchange Chromatography		2.9
Sephaceryl Chromatography		4.0

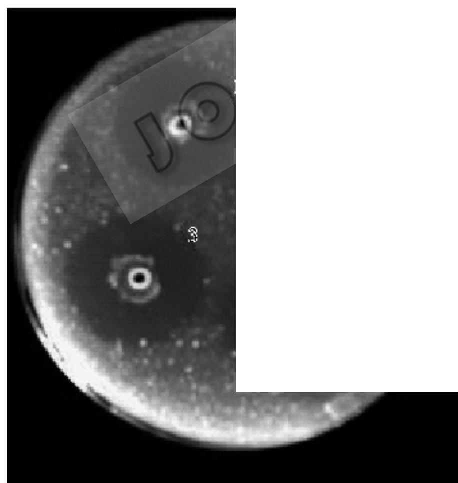


Fig. 5 Elastolytic activity detected on 1.0% (wt/vol) agarose supplemented with 0.3% (wt/vol) elastin in Petri dishes. The Petri dishes were incubated at 37℃ for 24 h in the humidified chamber. 1, 2: purified elastase from culture supernatant of *Aeromonas hydrophila* J-1; 3, 4: recombinant elastase expressed in *E. coli* BL21.

Both elastase preparations showed some identical properties concerning inhibitors. EDTA, the cation chelator, and OPA, the Zn^{2+}/Fe^{2+} protease inhibitor,
astase to a larger extent at
L and 20 mmol/L, but the
protease inhibitor PMSF
bit the enzyme, and even
as above 10% more than the
he whole, the recombinant
e than native enzyme against

ons showed some identical properties concerning different buffers. The enzyme activity was the highest in Tris-HCl buffer, then glycine-NaOH and borate buffer, and the activity was the lowest in phosphate buffer. The ezyme activity was the 1.7-fold in Tris-HCl buffer than in phosphate buffer.

Compared with native enzyme, the recombinant enzyme was more stable for heat, and remained 75% of its original activity at 56℃ for 30 min and 48% at 70℃ for 10 min. The native enzyme remained 65% and 40% of its original activity at the two different temperatures, respectively.

Table 2 Effect of metal ions on elastolytic activity

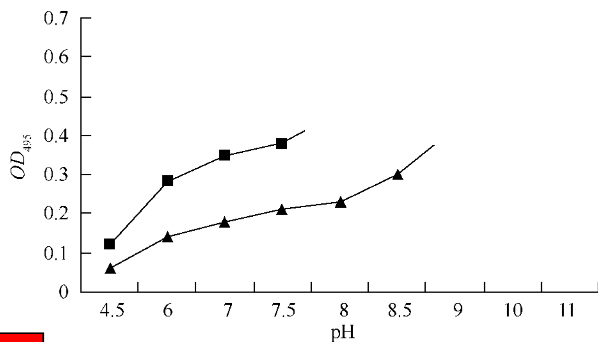
Metal ion	Residual activity/%	
	Native enzyme	Recombinant enzyme
No treatment	100.0 ± 0.5	100.0 ± 0.4
$MgCl_2$	55.4 ± 1.1	57.6 ± 1.0
NaCl	101.2 ± 0.38	98.0 ± 0.13*
$ZnCl_2$	47.1 ± 0.9	50.3 ± 0.7*
$CaCl_2$	96.4 ± 1.2	100.4 ± 0.9
$FeCl_2$	55.3 ± 1.2	58.0 ± 1.2*
$MnCl_2$	100.3 ± 1.4	103.2 ± 1.2*
$CuCl_2$	94.4 ± 0.7	[illegible]
$CoCl_2$	84.5 ± 1.3	
$BaCl_2$	109.4 ± 1.	
KCl	110.3 ± 1.	

Note: * and ** mean significant di recombinant enzyme within the same respectively. The same as below.

Table 3 Effects of some inhil

Inhibitor	Concentration /(mmol/L)	Nativ
EDTA	10	48.
	20	12.
PMSF	10	112.
OPA	10	20.
	20	6.

The effect of pH on the en: carrying out assays at differe pH, there was a distinct enha two elastase preparations. enzyme was 8.5 and 10. (Fig. 6).



. 6 Effect of pH on protease activity. Elastase activity was measured for 1 h at 37℃ at the pH values indicated. (■) Activity of native elastase from culture supernatant of *A. hydrophila* J-1 (▲) Activity of recombinant elastase expressed in *E. coli* BL21.

3 DISCUSSION

Much of the virulence of *A. hydrophila* has been attributed to its ability to secrete toxic or degradative enzymes. Elastase is responsible for the majoring of the proteolytic activity found in the supernatant of most strains of *A. hydrophila*. It had been reported that some bacterial elastase genes had been efficiently expressed in *E. coli*, when strong promoter and efficient vector were used [5-16]. In this study we reported the efficient expression of *A. hydrophila* J-1 elastase gene without signal peptide sequence in *E. coli* BL21 using pET-32a

n protein indicated elastase

nzyme from *A. hydrophila* J-

f 33 kDa was purified. There

lar weight of mature elastase

l strains of *A. hydrophila*.

eported concerning a 38 kDa

ophila AG2 [0] and a 31 kDa

hydrophila EO63 [1]. There

difference in the recognition

e protease processing may be

ondition.

ortant factors that regulate

. The pH influences the

es) and hence governs their

ctivities of both enzyme

nt decrease in acid buffer.

H optimum at 8.5, but

. However, they both belong

served between both enzyme

eristics such as pH optima,

neless, it is interesting to note that their behavior toward various inhibitors is almost the same. Only a slight difference could be found in the inhibition level, and recombinant elastase has a higher tolerance than native enzyme.

Both elastase preparations showed some identical properties concerning inhibitors, metal ions, buffers, etc, but the recombinant elastase had a higher tolerance toward some inhibitors and temperature than native enzyme. The probable mechanism appeared to be an outcome of the fusion fragment of the recombinant enzyme which connects C-terminal of interest protease to prelong primary structure

and influences higher structure of protease [7]. The analysis showed that His fusion amino residues might be helpful to improve the enzymatic thermostability of fusion protease.

To assign elastase into one of the four known classes of protease, serine, aspartic, thiol, or metalloendopeptidase [8], we examined the inhibition properties of the enzyme. The EDTA treated elastase show significant inhibition, indicating that the
for activity and stability.
significantly inhibited by C
inhibitor, but not by a serine
conclude that elastase is
metalloendopeptidase. The
analysis of the *A. hydrophil*
using the PROSITE compute
Bioinformatics), which revea
positions 128-VAAHEVSHGF
further supported by the inhib
iron ions, a property characte
dependant peptidases [9].

In conclusion, recombina
E. coli and purified to ho
product showed the same en
native enzyme from *A. hyd*
system might be suitable f
analysis of elastase. Howeve
recovered as insoluble ma
bodies. Production of soluble
for structure function anal
problem concerns the expression of soluble proteins with the activity of elastase. Fortunately, two new fusion partners have been identified to address these solubility problems. One of the tags was derived from a bacteriophage T7 protein kinase and the other one from a small *E. coli* chaperone, Skp [10].

The present work is the first report on recombinant expression and subsequent molecular chracterization analysis of *A. hydrophila* elastase. This study provided a basis for further investigation of enzyme reaction mechanism and its potential application.

REFERENCES

[1] Leung, KY, Stevenson, RM. Characteristics and distribution of extracellular proteases from *Aeromonas hydrophila*. *Journal of General Microbiology*, 1988, 134: 151 – 160.

[2] Rivero O, Anguita J, Paningua C, et al. Molecular cloning and characterization of an extracellular protease gene from *Aeromonas hydrophila*. *Journal of Bacteriology*, 1990, 172: 3905 – 3908.

lateos D, et al. Cloning and
acellular temperature-labile serine
leromonas hydrophila. *FEMS*
81: 1 – 8.

ma Y. Cloning, sequencing and
e encoding the extracellular
onas caviae. *Microbiology and*
– 347.

Secretion of haemolysins and
eparation and characterization of
talloprotease. *Journal of Applied*
– 1001.

czorek M, et al. Purification and
Zinc-proteinase from culture of
e Journal of Biological Chemistry,

, Nieto TP. Purification and
racellular metalloprotease, serine
Aeromonas hydrophila strain B_{32}:
robial Pathogenesis, 1992, 13: 17

Secreted enzymes of *Aeromonas*.
, 1997, 152: 1 – 10.

, J. A secreted metalloprotease
a exhibits prothrombin activator
activity. *Blood Coagul Fibrinolysis*, 2004, 15: 169 – 178.

[10] Cason A, Yugueos J. A majar secreted elastase is essential for pathogenicity of fish. *Infection and Immunity*, 2000, 68: 3233 – 3241.

[11] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Annals of Biochemistry*, 1976, 72: 248 – 254.

[12] Nakasone N, Tpma C, Song T, et al. Purification and characterization of a novel metalloprotase isolated from *Aeromonas caviae*. *FEMS Microbiology Letters*, 2004, 237: 127 – 132.

[3] Lan XQ, Zhang X, Hu JH, et al. Cloning, expression and characterization of a chitinase from the chitinolytic bacterium *Aeromonas hydrophila* strain SUWA-9. *Bioscience, Biotechnology and Biochemistry*, 2006, 70: 2437 – 2442.

[4] Sachar LA, Kathleen K, Winter D. Photometric method for elastase activity. *Proceeding of Society for Experimental Biology and Medcine*, 1955, 90: 323 – 326.

[5] Nirasawa S, Nakajima Y, ZHang Zh, et al. Molecular cloning and expression in *Escherichia coli* of the extracellular endoprotease of *Aerom* ... aminopeptidase processing ... *Acta-Protein Structure anc* ... 1433: 335 – 342.

[6] Clark DJ, Hawrylik SJ, ... characterization of a ... *Micrococcus luteus*. *Protein Expression and Purification*, 2000, 18: 46 – 55.

[7] Qi W, Hongning W, Likou Z. Expression of phytase phyC from *Bacillus subtilis* in *Escherichia coil* and its effect on enzymatic thermostability. *Chinese High Technology Letters*, 2004, 5: 23 – 27.

[8] Barrett AJ. Classification of peptidases. *Methods in Enzymology*, 1994, 244: 1 – 15.

[9] Auld DS. Removal and replacement of metal ions in ... *s in Enzymology*, 1995, 248: 228

... anced soluble protein expression ... tags. *Protein Expression and* ... – 129.

嗜水气单胞菌 ... 特性分析

孟喜龙[1,2], 刘永杰[1 *] ...

([1]南京农业大学动物医学院, ...

([2]聊城大学农学院, 聊城 252...

摘要:【目的】表达、纯化嗜 ... 进行分析。【方法】以 pET-32a 为表达载体将弹性蛋 ... 表达, 表达重组酶用 His TaqNi^{2+} 亲和层析柱纯化并 ... 子交换层析和分子筛层析对嗜水气单胞菌培养上清 ... 培养上清液中获得的弹性蛋白酶原酶的最适 pH 为 ... 酶。两种形式酶的性质有一些相似性, 其酶活性都能 ... 剂的耐受性要高于原酶。金属离子 Zn^{2+}、Fe^{2+} 能抑制 ... /Fe^{2+} 型金属蛋白酶, 且具有相似的酶学性质。本研 ... 了基础。

关键词:嗜水气单胞菌 J-1 ...

中图分类号:Q936 文献标识码:A 文章编号:0001-6209(2009)12-1620-08

(本文责编:张晓丽)

基金项目:新世纪优秀人才支持计划(NCET-07-0440);江苏自然科学基金(BK2007155);国际科学基金项目(B/4108-1)

* 通信作者。E-mail:liuyongjie@njau.edu.cn

作者简介:孟喜龙(1979 –),女,河南濮阳人,硕士,研究方向兽医微生物。E-mail:mengxilong@lcu.edu.cn

收稿日期:2009-07-19;修回日期:2009-09-14