Elastolytic mechanism of a novel M23 metalloprotease pseudoalterin from deep-sea *Pseudoalteromonas* sp. CF6-2: cleaving not only the glycyl bonds in the hydrophobic regions, but also the peptide bonds in the hydrophilic regions involved in crosslinking

Hui-Lin Zhao[1], Xiu-Lan Chen[1], Bin-Bin Xie[1], Ming-Yang Zhou[1], Xiang Gao[1], Xi-Ying Zhang[1], Bai-Cheng Zhou[1], Anthony S. Weiss[2], Yu-Zhong Zhang[1]

[1] State Key Laboratory of Microbial Technology, Marine Biotechnology Research Center, Shandong University, Jinan 250100, P. R. China
[2] School of Molecular Bioscience, The University of Sydney, NSW 2006, Australia

Running title: Elastolytic mechanism of a novel M23 metalloprotease

Address correspondence to: Yu-Zhong Zhang, State Key Laboratory of Microbial Technology, Marine Biotechnology Research Center, Shandong University, Jinan 250100, P. R. China. Tel: +86-531-88364326; Fax: +86-531-88564326. E-mail: zhangyz@sdu.edu.cn

Supplementary Data

Part I. Purification and characterization of pseudoalterin

Table S1. Purification of the protease secreted by *Pseudoalteromonas* sp. CF6-2

Procedure	Volume (ml)	Protein concentration (mg/ml)	Total protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Purification (fold)	Yield (%)
Culture supernatant	100	0.1123	11.23	1504	133.93	1	100
DEAE sepharose fast flow	20	0.126	2.52	506.6	201.03	1.50	33.68

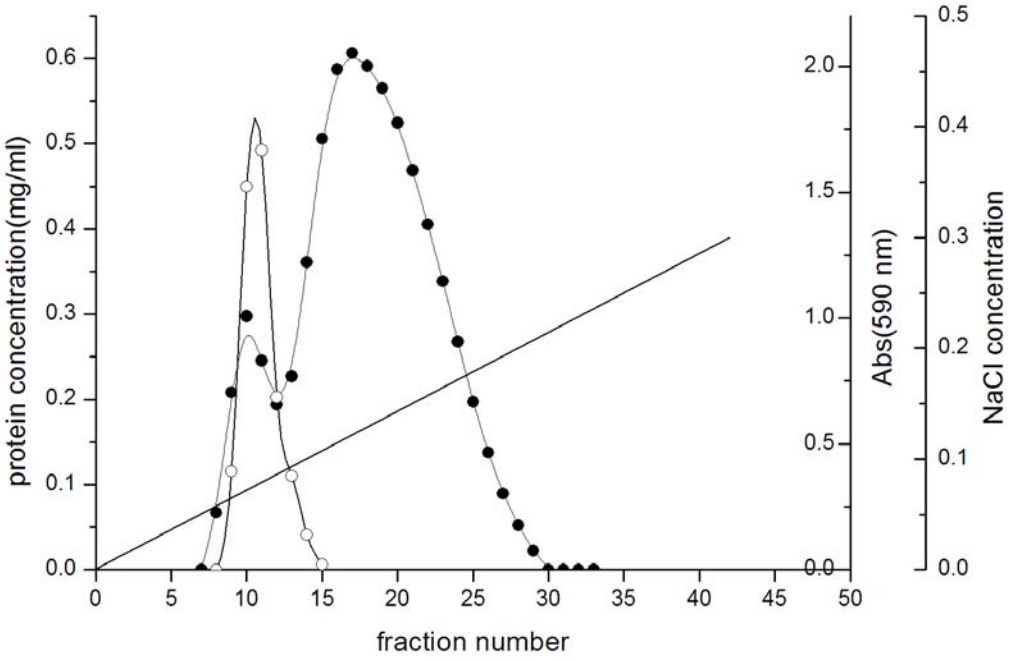


Fig. S1. Purification of pseudoalterin by ion-exchange chromatography on a DEAE-Sepharose Fast Flow column. ●, protein concentration; ○, relative protease activity; ─, NaCl concentration.

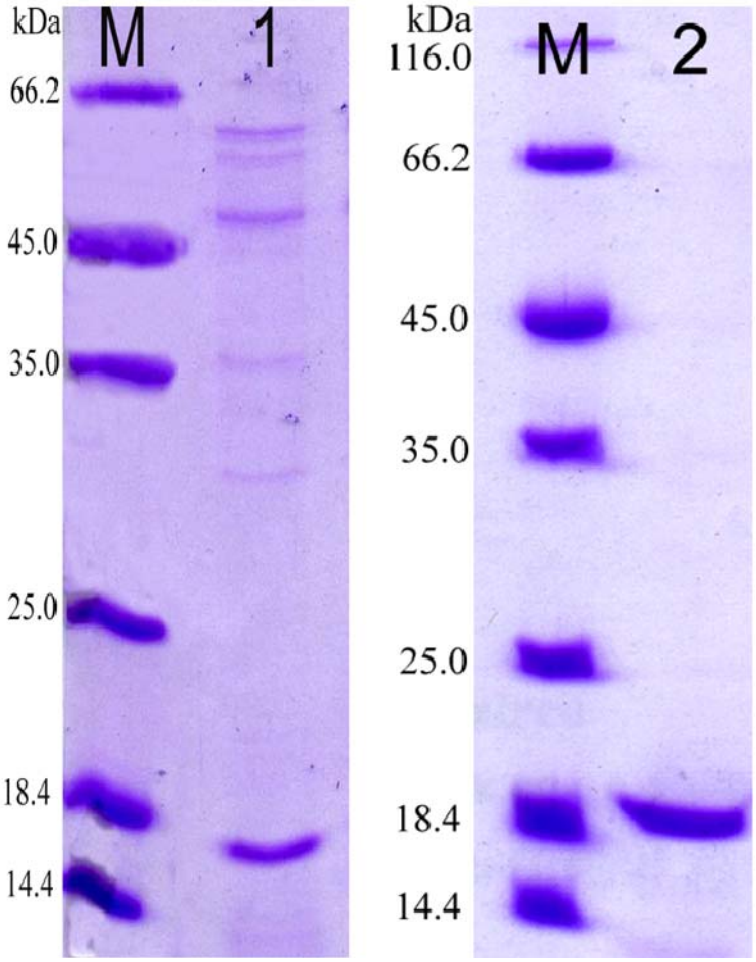
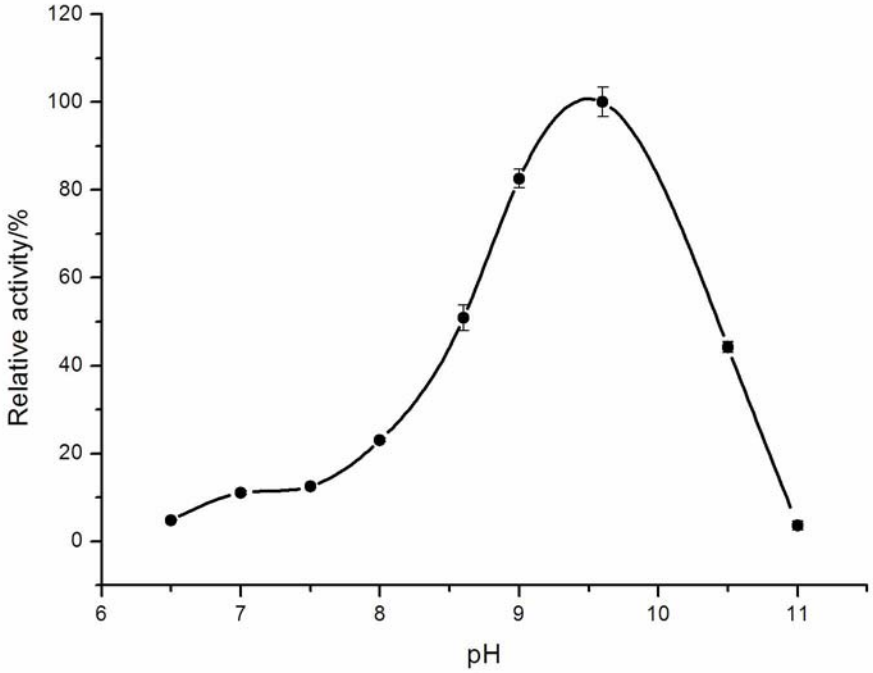
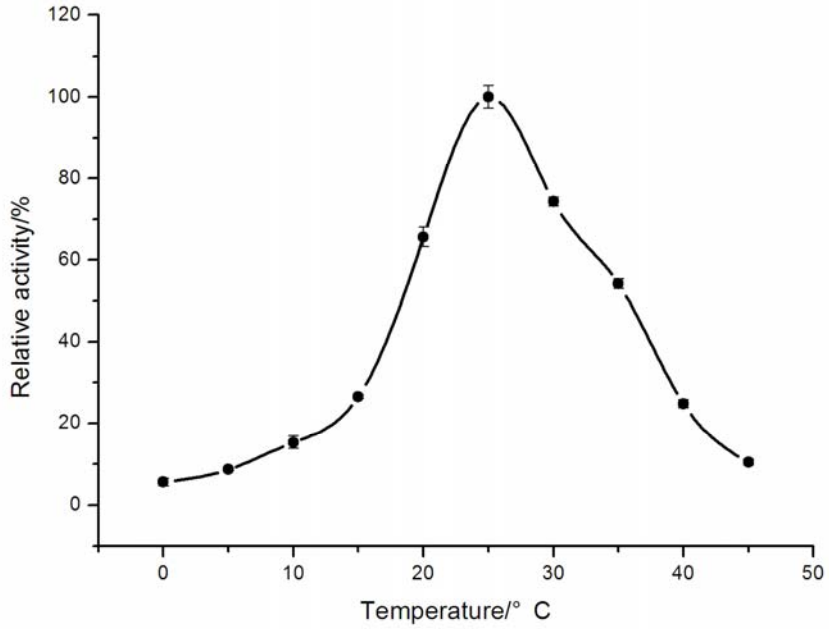


Fig. S2. Analysis of the purity of pseudoalterin by SDS-PAGE.1, proteins in *Ps.* sp. CF6-2 culture supernatant; 2, pseudoalterin purified by ion exchange chromatography; M: marker.


120
100
80
60
40
20
0
Relative activity/%
6
7
8
9
10
11
pH

A


120
100
80
60
40
20
0
Relative activity/%
0
10
20
30
40
50
Temperature/° C

B

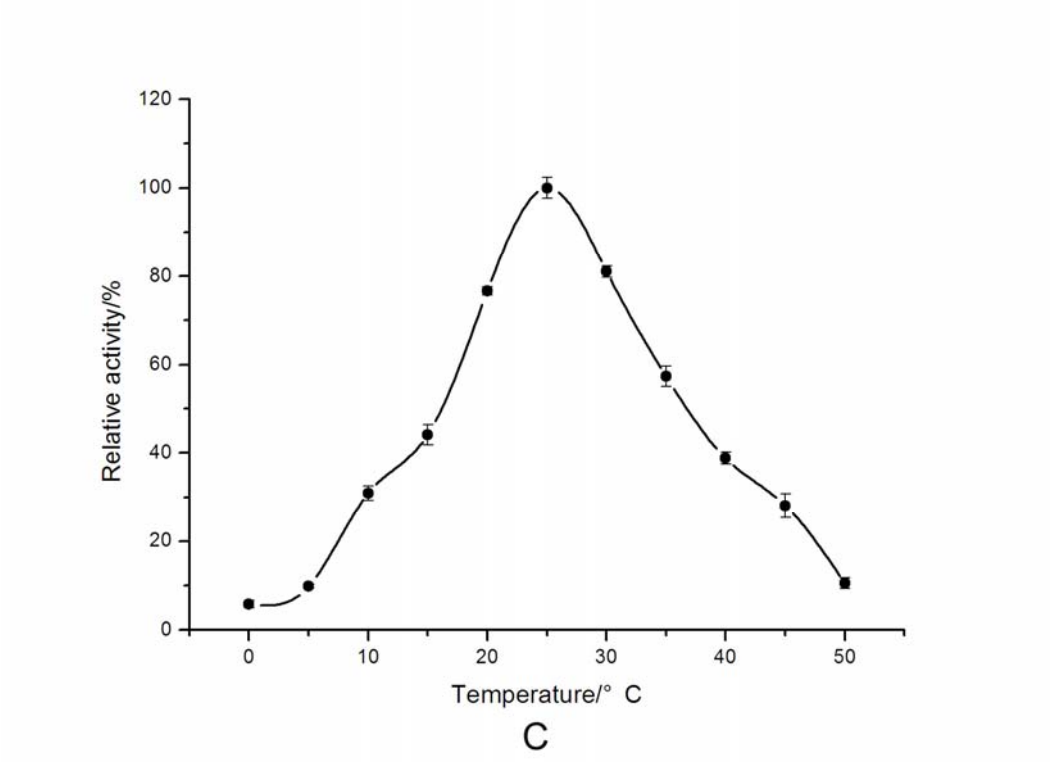


Fig. S3. Effect of temperature and pH on the activity of pseudoalterin. A, Effect of pH on pseudoalterin activity. The optimum pH was determined by measuring the activities of pseudoalterin with elastinorcein at 30°C in Na_2HPO_4-NaH_2PO_4 buffer at pHs ranging from 6.5 to 8, barbital sodium-HCl buffer at pHs ranging from 7.5 to 9.6 and $NaHCO_3$-NaOH buffer ranging from 9.6 to 11.0. B, Effect of temperature on pseudoalterin activity. C, Effect of temperature on pseudoalterin activity in artificial sea water. The optimum temperature was determined by measuring the activities of pseudoalterin with elastinorcein in 50 mM Tris buffer (pH 9.0) or in artificial sea water at temperatures ranging from 0 to 45°C. The data shown are the mean of three repeats with standard deviations ≤ 5%.

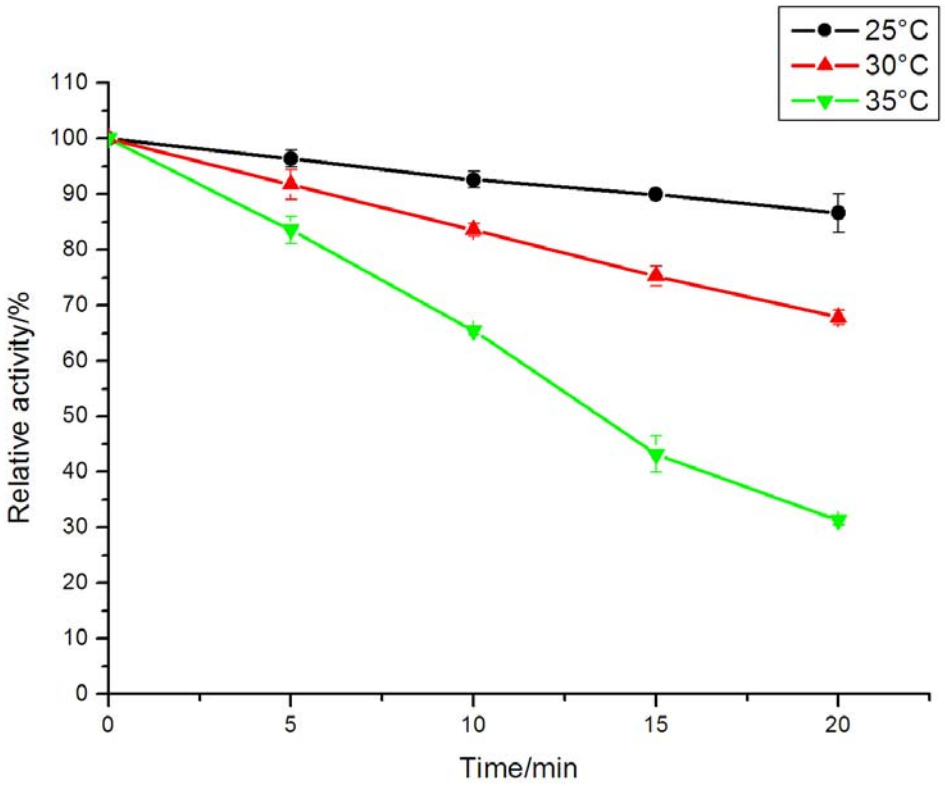


Fig. S4. Effect of temperature on the thermostability of pseudoalterin. The concentration of the incubated pseudoalterin is 0.1 mg/mL. The data shown are the means of three repeats with standard deviations≤ 5%.

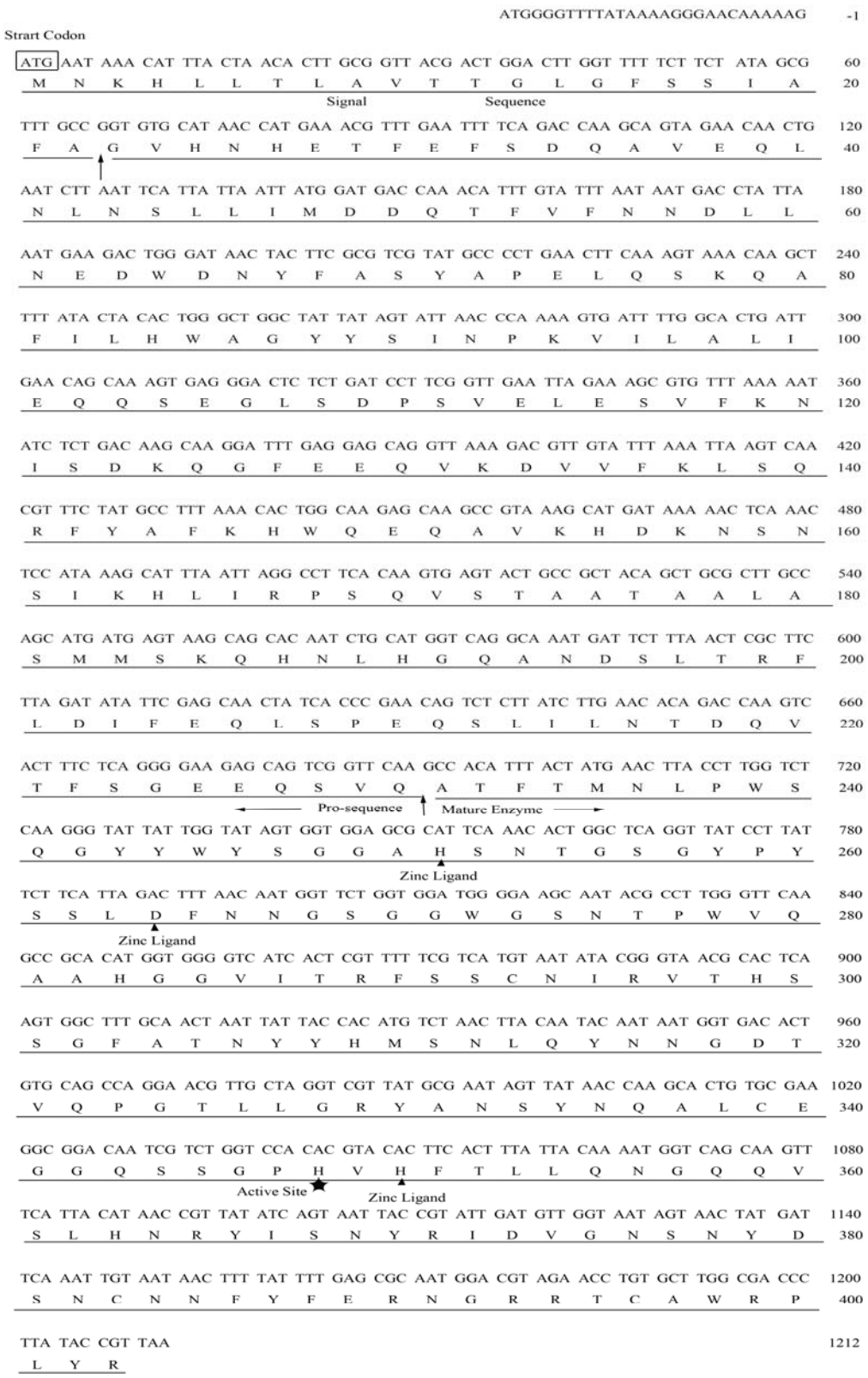


Fig.S5. The nucleotide and deduced amino acid sequences of pseudoalterin.

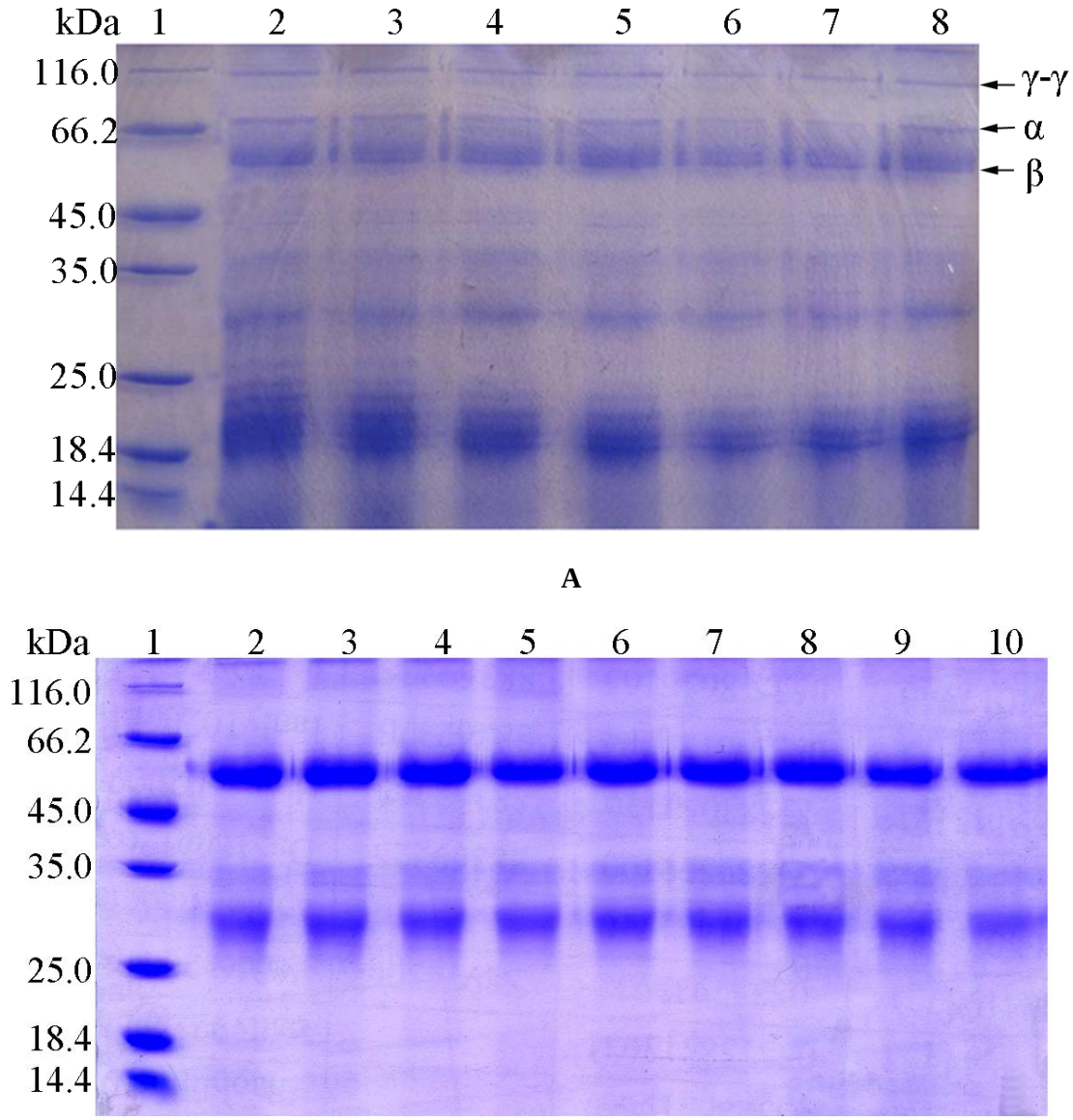


Fig.S6. SDS-PAGE analysis of the hydrolytic activities of pseudoalterin on fibrin and gamma-globulin. A, the hydrolysis of fibrin; 1, marker; 2, 3, 4, 5, 6, 7, 8 are samples taken at 0, 20, 40, 60, 80, 100, 120 min; B, the hydrolysis of gamma-globulin; 1, marker; 2, 3, 4, 5, 6, 7, 8, 9, 10 are samples taken at 0, 10, 20, 30, 40, 50, 60, 70, 80 min.

Part II. Analysis of the cleavage pattern of oxidized insulin B chain by pseudoalterin.

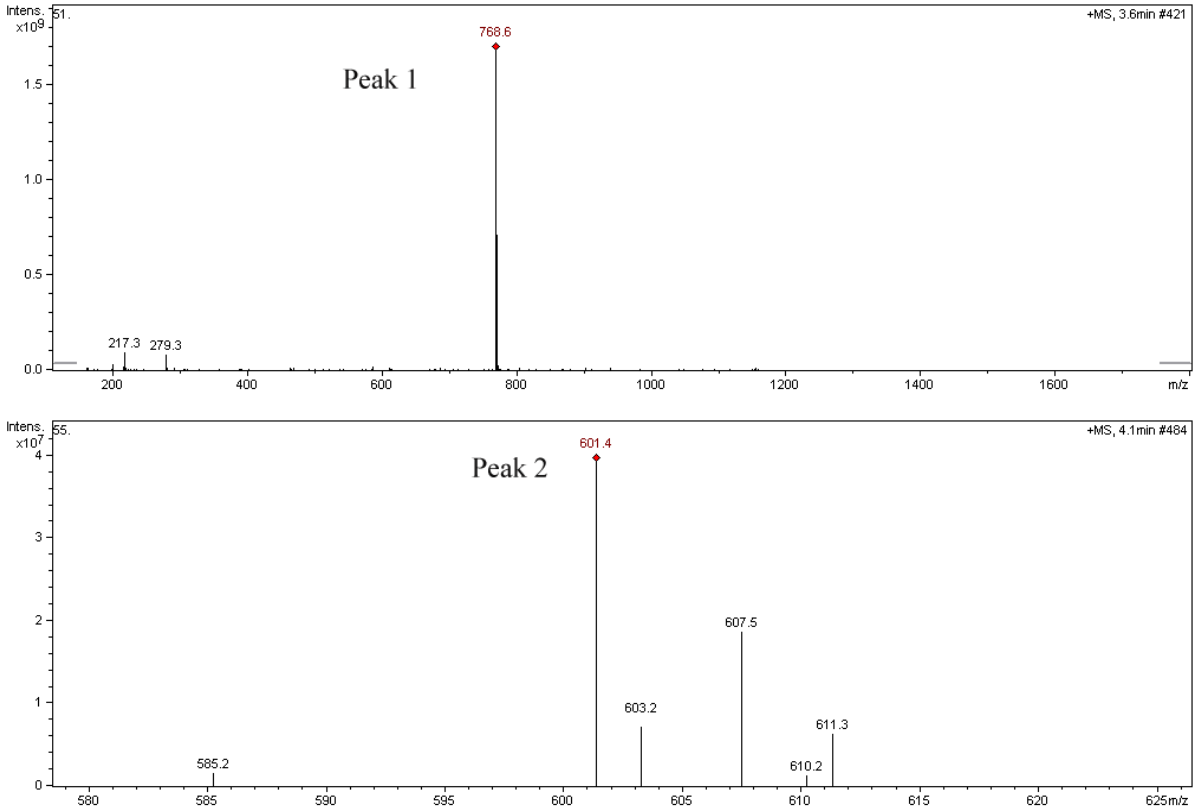


Fig. S7. Partial LC-MS analysis of the molecular weights of the peptides released from the oxidized insulin B chain by pseudoalterin.

Table S2. The determined molecular masses and sequences of the 16 peptides released from oxidized insulin B chain by pseudoalterin.

Peak number	m/z Measured (Da)	MH^+ Matched (Da)	Peptide[a]	RT (min)	Position
1	768.65	768.43	(G)SHLVEAL(Y)	3.59	9-15
2	601.64	1201.60	(C)GERGFFYTPK(A)	4.15	20-29
3	543.87	1086.57	(E)RGFFYTPKA	4.17	22-30
4	636.82	1272.64	(C)GERGFFYTPKA	4.72	20-30
5	564.92	1128.84	(E)ALYLVCGERG(F)	4.88	14-23
6	726.41	1451.82	(L)VCGERGFFYTPK(A)	5.22	18-29
7	546.36	1091.72	(L)YLVCGERGF(F)	5.26	16-24
8	761.96	1522.92	(L)VCGERGFFYTPKA	5.30	18-30
9	783.00	1565.00	(Y)LVCGERGFFYTPK(A)	5.63	17-29
10	818.48	1635.96	(Y)LVCGERGFFYTPKA	5.66	17-30
11	647.89	1294.78	(G)SHLVEALYLVC(G)	6.52	9-19
12	992.06	1983.12	(E)ALYLVCGERGFFYTPKA	6.67	14-30
13	921.09	1841.18	(G)SHLVEALYLVCGERGF(F)	6.69	9-24
14	1274.67	2548.34	(G)SHLVEALYLVCGERGFFYTPKA	6.77	9-30
15	826.49	2477.47	(G)SHLVEALYLVCGERGFFYTPK(A)	6.78	9-29
16	1166.00	3495.00	FVDQHLCGSHLVEALYLVCGERGFFYTPKA	8.39	1-30

a The sequence of each peptide in black was determined by liquid chromatography-mass spectrometry and MASCOT MS/MS Ion Research tools. The left bracketed residue of each peptide indicates the P1-site residue of the left cleavage site. The right bracketed residue of each peptide indicates the P1'-site residue of the right cleavage site. The underline cysteine residues indicate that they are oxidized cysteine residues.

Part III. Analysis of the cleavage sites of pseudoalterin on bovine elastin fibers

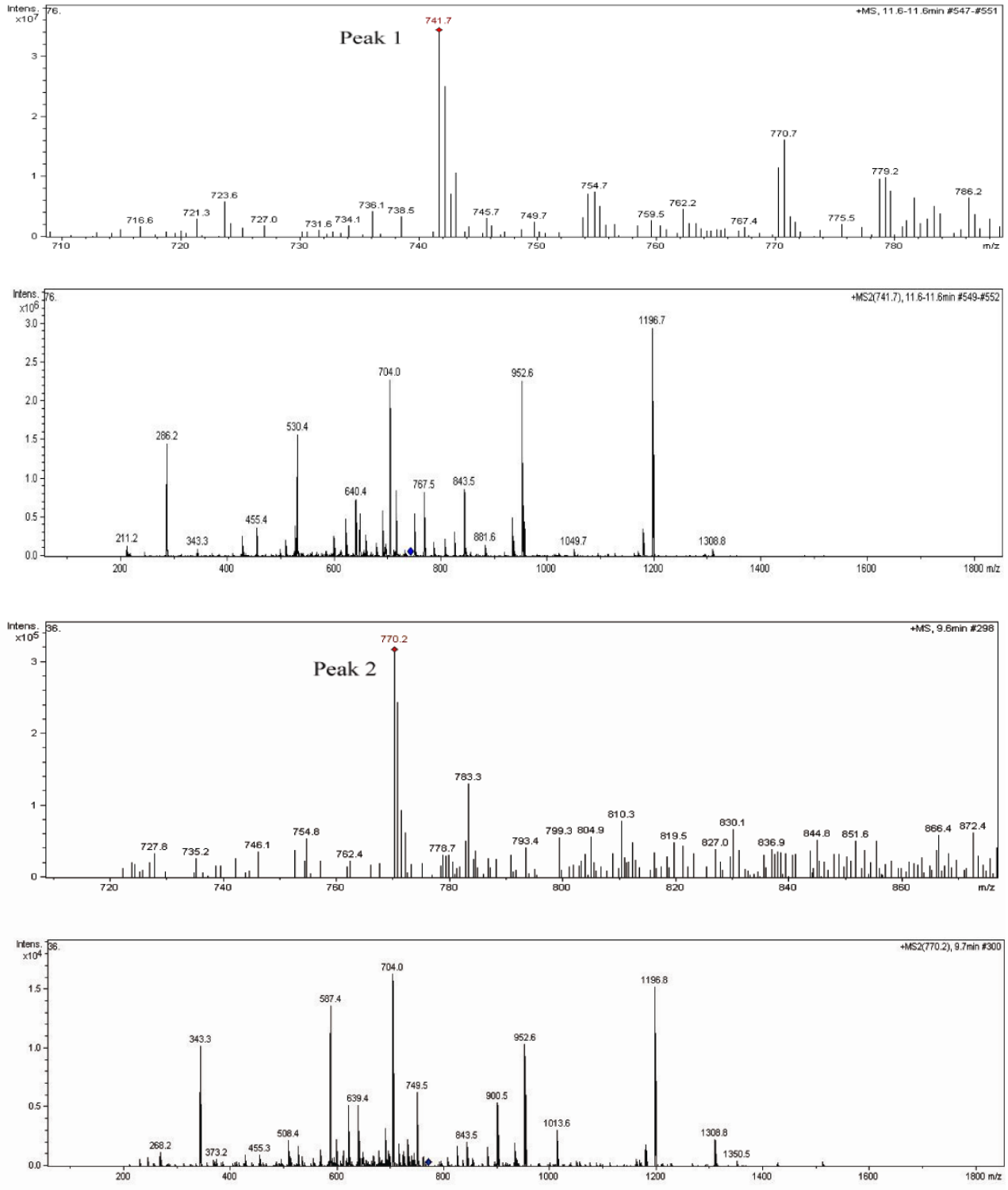


Fig. S8. Partial LC-MS analysis of the molecular weights of the peptides released from bovine elastin fibers by pseudoalterin. For each peptide, the first spectra image is MS analysis and the second spectra image is the MS peak analyzed by secondary MS.

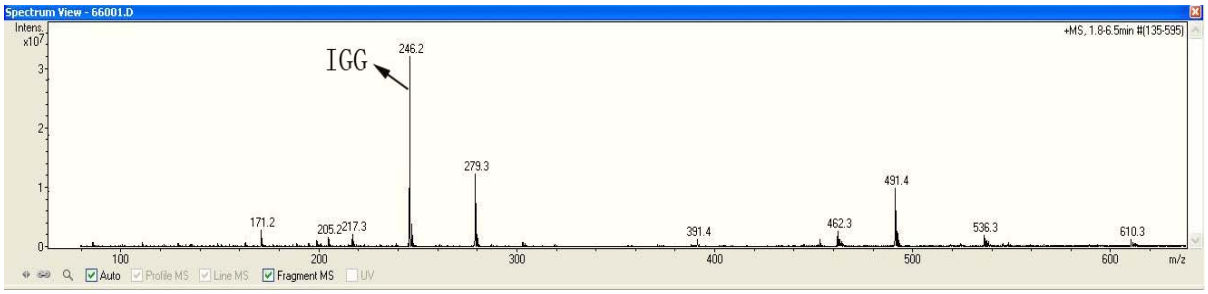
Table S3. The determined molecular masses and sequences of the 27 peptides released from bovine elastin fibers by pseudoalterin.

Peak number	m/z Measured (Da)	MH^+ Matched (Da)	Peptide[a]	RT (min)	Position
1	741.67	1481.811	(G)AAGLGGVLGAGQPFPIG(G)	11.60	697-713
2	770.22	1538.833	(G)AAGLGGVLGAGQPFPIGG(G)	9.68	697-714
3	770.09	1538.854	(G)GLGVSTGAVVPQLGAGVG(A)	8.40	125-142
4	933.68	1865.991	(G)GVPGAVPGGVPGGVFFPGAGLG(G)	11.22	28-49
5	1005.93	2010.175	(G)VAPGVGVVPGVGVVPGVGVAPGIG(L)	9.98	505-528
6	556.99	1112.610	(G)GVLGAGQPFPIG(G)	9.68	696-707
7	734.65	1467.817	(G)GVGGLGVSTGAVVPQLG(A)	8.46	122-138
8	782.69	1563.886	(G)GVGGLGVSTGAVVPQLGAGVG(A)	11.47	122-142
9	1331.44	2660.550	(G)VAPGVGVVPGVGVVPGVGVAPGIGLGPGGVIG(A)	11.67	505-536
10	642.07	1282.715	(G)LGGVLGAGQPFPIG(G)	9.19	694-707
11	1011.75	2021.139	(G)LGVGGIGGVGGLGVSTGAVVPQLG(A)	11.51	115-138
12	848.19	1694.944	(G)GIGGVGGLGVSTGAVVPQLG(A)	9.17	119-138
13	999.27	1996.159	(G)LGPGVGVAPGVGVVPGVGVVPGVG(V)	11.87	499-522
14	585.50	1169.631	(G)GVLGAGQPFPIGG(G)	8.02	696-708
15	778.62	1555.827	(G)AVPGGVPGGVFFPGAGLG(G)	10.09	32-49
16	1571.02	3140.819	(G)LGPGVGVAPGVGVVPGVGVVPGVGVAPGIGLGPGGVIG(A)	14.06	499-536
17	628.05	1254.705	(G)GLGVSTGAVVPQLG(A)	7.58	125-138
18	484.49	967.557	(G)GLGAVPGAVGLG(G)	9.07	665-676
19	422.49	843.436	(G)AGQPFPIGG(G)	5.78	700-708
20	990.19	1979.092	(G)GIGGVGGLGVSTGAVVPQLGAGVG(A)	10.10	119-142
21	676.06	1350.775	(G)GLGVGGLGAVPGAVGLG(G)	9.06	660-676
22	599.56	1197.684	(G)LGVSTGAVVPQLG(A)	7.85	126-138
23	413.38	825.483	(G)AGVLPGVGVG(G)	6.66	281-290
24	441.91	882.204	(G)GAGVLPGVGVG(G)	6.46	280-290
25	1137.82	2275.256	(G)AGVPGPGAVPGTLAAAKAAKFGPGGVG(A)	11.09	590-616
26	719.60	1437.821	(G)ARFPGIGVLPGVPTG(A)	8.83	169-183
27	893.2	1786.908	(S)AGKAGYPTGTGVGPQAAAAAA(K)	19.8	250-270

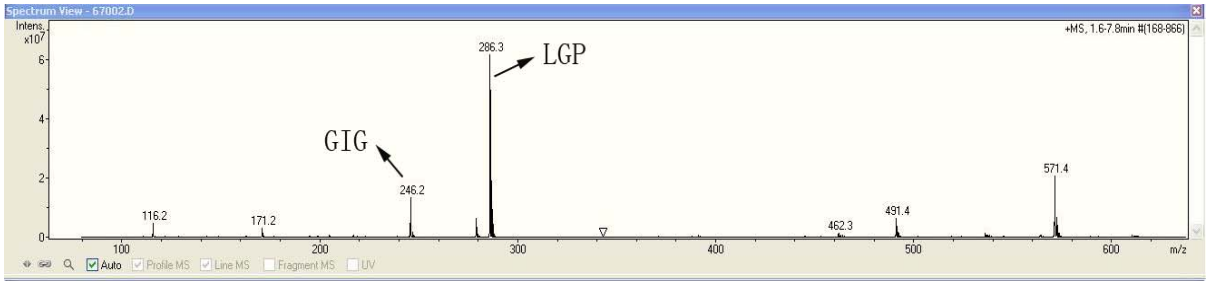
a The sequence of each peptide in black was determined by liquid chromatography-mass spectrometry and MASCOT MS/MS Ion Research tools. The left bracketed residue of each peptide indicates the P1-site residue of the left cleavage site. The right bracketed residue of each peptide indicates the P1'-site residue of the right cleavage site.

Table S4. Specificity matrix of pseudoalterin to bovine elastin fibers based on 32 cleavage sites.

Amino acid	P4	P3	P2	P1	P1’	P2’	P3’	P4’
Gly	11	17	3	31	14	12	13	15
Ala	6	4	5	1	11	5	2	2
Ser	1	0	0	0	0	0	1	1
Gln	0	3	0	0	0	0	1	0
Pro	6	2	2	0	0	1	6	4
Val	3	4	9	0	2	7	7	5
Leu	3	0	6	0	4	5	1	2
Lys	0	1	0	0	1	0	0	2
Arg	0	0	0	0	0	1	0	0
Phe	2	0	2	0	0	0	1	0
Thr	0	0	1	0	0	0	0	0
Ile	0	1	4	0	0	1	0	1


Spectrum View - 66001.D
+MS, 1.8-6.5min #(135-595)
IGG
246.2
279.3
491.4
171.2
205.2
217.3
391.4
462.3
536.3
610.3
m/z

A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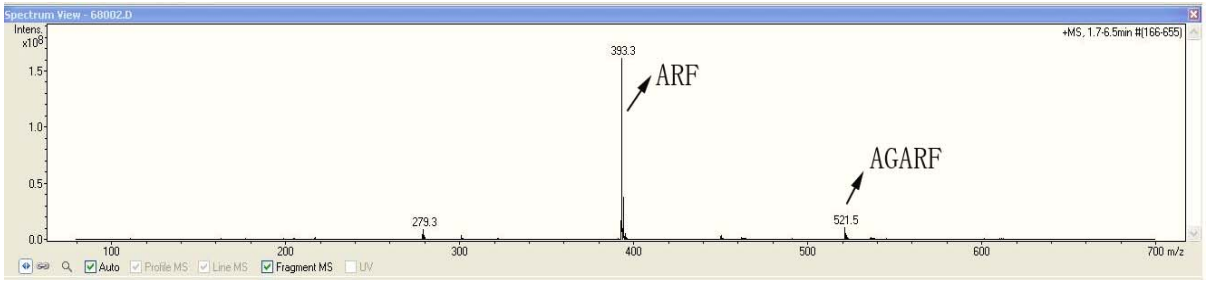
+MS, 1.6-7.8min #(168-866)
286.3
LGP
GIG
246.2
571.4
116.2
171.2
462.3
491.4
m/z

B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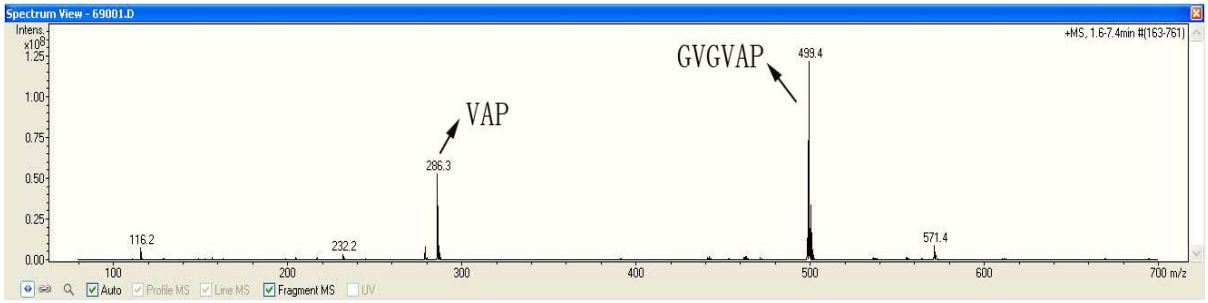
+MS, 1.7-6.5min #(166-655)
393.3
ARF
AGARF
279.3
521.5
m/z

C


Spectrum View - 69001.D
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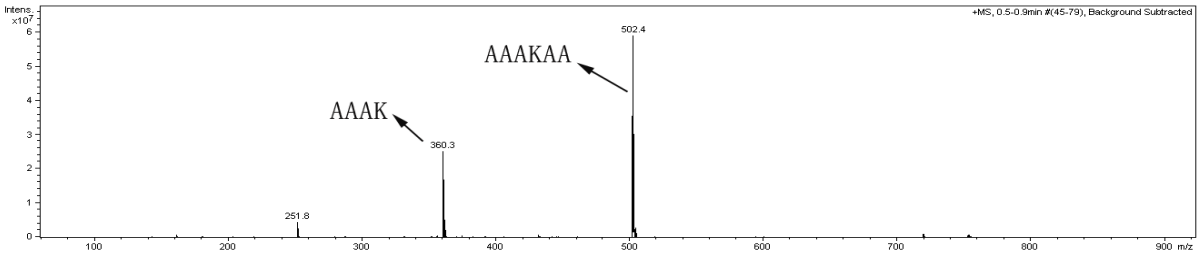
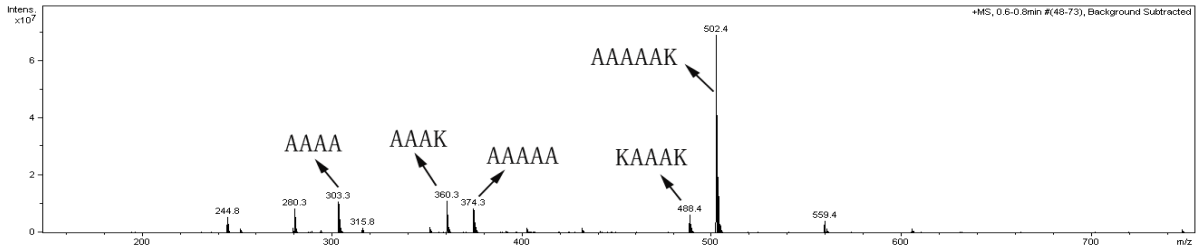
499.4
VAP
286.3
116.2
232.2
571.4
m/z

D



E



F

Fig. S9. Analysis of the degradation of the synthetical peptides hydrolysates with pseudoalterin. The molecular masses and sequences of all the peptides were analyzed by LC/MS and FindPept Tool at expasy website, respectively. A, IGGGAGG; B, GIGLGP; C, PGAGARF; D, GVGVAP; E, AAAKAA; F, AAAAAKAAAK.

Table S5. M23 family proteases[a] predicted from marine sediment and hydrothermal vent bacteria

Strains	Genome No.	Location	Accession No. of proteases in M23
Polaromonas sp. JS666 (1)	CP000316	Sed[b]	ABE46000.1, ABE42783.1
Roseobacter denitrificans OCh 114 (2)	CP000362	Australia sed	ABG30058.1
Nautilia profundicola Am-H (3)	CP001279	Ven[c]	ACM93731.1, ACM93477.1
Nitratiruptor sp. SB155-2 (4)	AP009178	Ven in Iheya	BAF69457.1
Sulfurovum sp. NBC37-1 (5)	AP009179	Ven in Iheya	BAF72920.1, BAF72695.1
Thermotoga maritima MSB8 (6)	AE000512	Sed near Vulcano	AAD36886.1
Persephonella marina EX-H1 (7)	CP001230	Ven in Pacific	ACO04799.1, ACO03251.1
Candidatus Ruthia magnifica Cm (8)	CP000488	Ven	ABL02478.1, ABL01827.1
Thiomicrospira crunogena XCL-2 (9)	CP000109	Ven	ABB41394.1, ABB40948.1
Alcanivorax borkumensis SK2 (10)	AM286690	Sed in North Sea	CAL17579.1, CAL16054.1, CAL16619.1, CAL17648.1
Desulfobacterium autotrophicum HRM2 (11)	CP001087	Mediterranean sea sed	ACN14260.1
Hahella chejuensis KCTC 2396 (12)	CP000155	Cheju Island sed	ABC28216.1, ABC28556.1, ABC28713.1, ABC29831.1, ABC31053.1, ABC31896.1, ABC32524.1, ABC32886.1, ABC32948.1, ABC33568.1, ABC29581.1
Shewanella sediminis HAW-EB3 (13)	CP000821	Sed in Halifax Harbor	ABV39073.1, ABV36423.1, ABV35714.1
Shewanella halifaxensis HAW-EB4 (14)	CP000931	Sed in Emerald Basin	ABZ77255.1, ABZ75798.1, ABZ74618.1
Shewanella sp. W3-18-1 (15)	CP000503	Sed in Pacific Ocean	ABM25854.1, ABM26841.1, ABM24560.1, ABM24105.1, ABM25390.1
Shewanella piezotolerans WP3 (16)	CP000472	Sed in Pacific	ACJ29884.1, ACJ29020.1, ACJ28150.1
Salinispora arenicola CNS-205 (17)	CP000850	Tropical sed	ABW00492.1, ABV99987.1, ABV99986.1, ABV98343.1, ABV97281.1, ABV97111.1, ABV96162.1
Idiomarina loihiensis L2-TR (18)	AE017340	Lo'ihi Seamount ven	AAV81179.1, AAV83434.1, AAV82097.1, AAV81287.1, AAV81075.1
Shewanella loihica PV-4 (19)	CP000606	Naha ven	ABO24321.1, ABO23341.1, ABO23082.1
Oceanobacillus iheyensis strain HTE831 (20)	BA000028	Iheya deep sea mud	Not found
Geobacillus kaustophilus strain HTA426 (21)	BA000043	Mariana Trench sed	Not found

a The proteases were all secretary proteins predicted with SignalP 3.0 (possibility >0.5).

b Sed, sediment.

c Ven, hydrothermal vent.

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