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# Isolation and characterization of novel cadmium-inducible peptides from the barnacle, *Megabalanus volcano*

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**Abstract:** Novel cadmium-inducible peptides were purified from the barnacle, *Megabalanus volcano*. They lacked cysteine and contained high amounts of acidic residues. Their molecular weights were much smaller than those of other cadmium-inducible/binding proteins. These peptides, megabalanein A (CdIP1) and megabalanein B (CdIP2) may be the smallest metal-inducible peptides that have been found in vertebrates and invertebrates. 

## INTRODUCTION

Marine vertebrates and invertebrates in coastal zones are considered to be exposed to many metals in sea water. On exposure to metals, they synthesize metal-inducible peptides/proteins, such as metallothionein (MT)[1] or other metal-binding/inducible proteins,[2-7] for detoxification purposes. With MT, cysteines are known to bind metals including cadmium, zinc, copper and other soft metals.

Previously we reported isolation of a low-molecular-mass cadmium-inducible peptide, megabalanein A (CdIP1), from the barnacle, *Megabalanus volcano*.[8] Megabalanein A contains no cysteines, but it is rich in acidic amino acids (glutamic acid and aspartic acid) which are considered to provide binding sites to metals. In this paper, we describe the details of megabalanein A and a newly isolated barnacle cadmium-inducible peptide, megabalanein B (CdIP2). The amino acid sequence of megabalanein B consists of megabalanein A plus four amino acids added to the amino-terminal end of megabalanein A, and is thought to have a function similar to that of megabalanein A because it is also rich in acidic amino acids.

## RESULTS

*Isolation of cadmium-inducible peptides from M. volcano*

Cadmium-inducible peptides were isolated from the internal organs of *M. volcano* using three chromatographic steps. The supernatant of the homogenate of the internal organs of cadmium-exposed barnacles was first fractioned by gel filtration chromatography on a Superdex 75 column (Fig. 1). Fractions

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of 2 ml were analyzed for cadmium with an inductively-coupled plasma (ICP) spectrometer, and fractions corresponding to the low-molecular-mass cadmium-binding region (apparent molecular mass = 10 kDa; fractions 37-44) were pooled and diluted with distilled water to a concentration of 3 mM Tris. The diluted fractions were chromatographed on an anion-exchange Mono Q column, which was developed with a linear gradient of Tris (Fig. 2). Fractions of 1 ml were analyzed for cadmium and cadmium-binding fractions 19-26 were pooled and separated by reverse-phase HPLC. Four major peaks were resolved by this method (Fig. 3 (a)). Peaks with retention times of 18.1 and 26.4 were individually pooled and concentrated by evaporation under reduced pressure to give pure peptides.

For control purposes, the same purification from unexposed barnacles was performed. The homogenate of the internal organs was similarly separated by gel fitration after adding cadmium (Fig. 1), followed by Mono Q column (Fig. 2) and RP-HPLC purification (Fig. 3 (b)). In the case of unexposed barnacles no remarkable peak could be detected in RP-HPLC purification, which indicates that the peptides obtained from cadmium-exposed barnacles are cadmium-inducible.

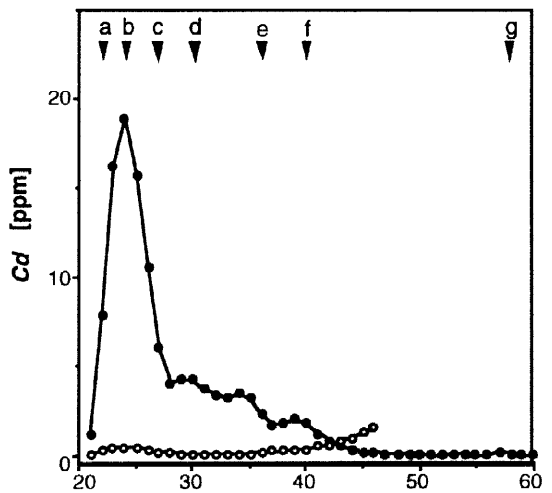


Fig. 1. Gel-filtration chromatography of the extracts from cadmium-exposed ( ● ) and unexposed (○) barnacles, *M. volcano*. The supernatant of the homogenate from *M. volcano* was applied to a HiLoad 16/60 Superdex 75 pg column (1.6 x 60 cm; Pharmacia). Elution was performed with 20 mM Tris/HCl at pH 8.0 at a flow rate of 0.5 ml/min. Fractions of 2 ml were analysed for cadmium by an ICP spectrometer (Shimadzu ICPS-1000IV). Arrows represent the elution positions of the molecular-weight markers. a, 2000 ; b, 158 ; c, 67 ; d, 43 ; e, 25 ; f, 13.7 ; g, 0.204 kDa.

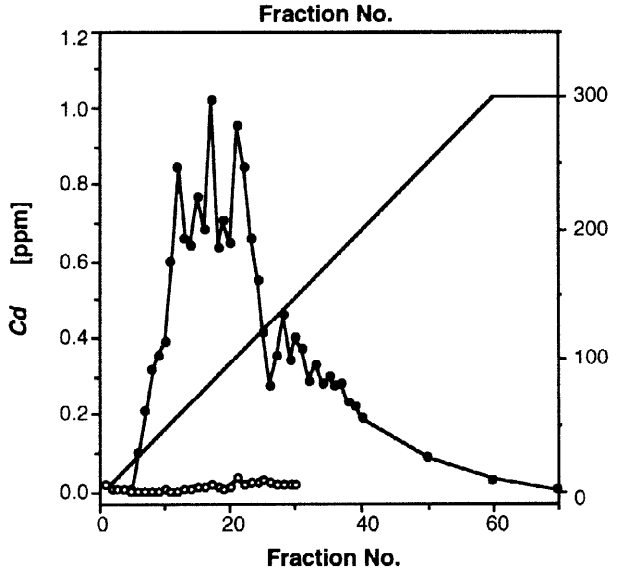


Fig. 2. Anion-exchange chromatography of the fractions corresponding to the low-molecular-mass cadmium-binding region (apparent molecular mass = 10 kDa) after gel filtration chromatography, obtained from cadmium-exposed ( ● ) and unexposed (○) barnacles, *M. volcano*. Fractions of Superdex 75 corresponding to the low-molecular-mass, cadmium-binding region were pooled and diluted with distilled water to a concentration of 3 mM Tris. The diluted cadmium-binding fractions were chromatographed on an anion-exchange FPLC column Mono Q HR 5/5 (0.5 x 5 cm; Pharmacia) that had been equilibrated with 3 mM Tris/HCl at pH 8.0. The column was developed with a linear gradient (-) from 3 mM to 300 mM Tris/HCl at pH 8.0 at a flow rate of 1.0 ml/min. Fractions of 1 ml were analyzed for cadmium contents by an ICP spectrometer.

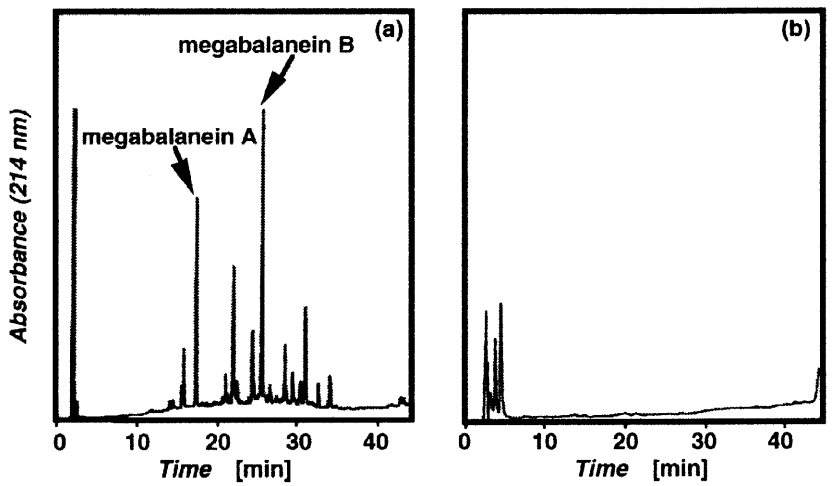


Fig. 3. Reverse-phase HPLC chromatograms of the cadmium-binding fractions of anion-exchange chromatography, obtained from cadmium-exposed (a) and unexposed (b) barnacles, *M. volcano*. Cadmium-binding fractions of Mono Q (Fr. 19-26) were pooled and analysed by reverse-phase HPLC (RP-HPLC ; μBondasphere, C4, 5 μ, 300 Å, 3.9 x 150 mm; Waters), which was eluted over 45 min with a linear gradient from 5% to 48% acetonitrile in 0.06% trifluoroacetic acid at a flow rate of 0.7 ml/min.

*Amino acid sequences and mass spectra*

Amino acid sequencing of the purified peptides with retention times of 18.1 and 26.4 was performed (Fig. 4). The calculated molecular weight values from the sequences were in good agreement with the observed ones from the MALDI-TOF mass spectra (*m/z* 1703.90 [(M+H)$^+$; calcd for $C_{70}H_{119}N_{20}O_{29}$ *m/z* 1703.85] ; *m/z* 2004.07 [(M+H)$^+$; calcd for $C_{70}H_{119}N_{20}O_{29}$ *m/z* 2004.12]). These cadmium-inducible peptides are designated as megabalanein A (CdIP1) [8] and megabalanein B (CdIP2), respectively. Amino acid sequences of megabalaneins A and B were similar to each other. Megabalanein B consists of megabalanein A with four more amino acids added to the amino-terminal end.

(a) Glu-Ile-Glu-Lys-Arg-Ala-Glu-Glu-Leu-Ser-Gly-Gln-Ile-Asp-Ser

(b) Ser-Gly-Val-Gly-Glu-Ile-Glu-Lys-Arg-Ala-Glu-Glu-Leu-Ser-Gly-Gln-Ile-Asp-Ser

Fig. 4. Amino acid sequences of megabalanein A (a) and megabalanein B (b). Amino acid sequencing of megabalaneins A and B was performed by degradation from amino terminus with the Edman reagent, phenyl isothiocyanate, using a protein sequencer PSQ-2 (Shimadzu).

*Comparison between natural and synthetic megabalanein B*

To determine the absolute configuration of megabalanein B, a peptide having the same sequence as that of megabalanein B was synthesized by starting from Fmoc L-amino acids. The mass spectrum, $^1$H-NMR spectrum, and RP-HPLC retention time of the synthetic peptide were identical to those of the natural megabalanein B. The CD spectrum of the synthetic peptide, showing the specific band for a random coil

structure, was also the same as that of the natural megabalanein B. These results confirmed the assignment of L-configuration to all of the megabalanein B residues.

*$^{1}$H- and $^{13}$C-NMR spectra of megabalanein B*

Chemical shifts of $^{1}$H- and $^{13}$C-NMR spectra of megabalanein B are depicted in Table 1. The sequence was determined by COSY, HSQC, HMBC, ROESY and HOHAHA experiments. This sequence is completely the same with the sequence determined by amino acid sequence analysis and mass spectrometry. We predicted that the cadmium-binding property of megabalanein B is probably the same as that of megabalanein A because their acidic amino acids are considered to provide binding sites to cadmium and an additional Ser-Gly-Val-Gly portion is probably not involved in cadmium-binding.

*$^{113}$Cd-NMR spectra of megabalanein A*

$^{113}$Cd-NMR spectra of megabalanein A in the presence of six different equimolar amounts of $^{113}CdCl_2$ were measured, and only one signal was observed in every $^{113}CdCl_2$ concentration. The sample was dissolved in $D_2O$ at pH 7.4 adjusted with NaOD. The pH of the sample decreased slightly in proportion to the amount of $^{113}CdCl_2$. The pH of the sample in the presence of 5 equivalents of $^{113}CdCl_2$ was 6.4. The signal of $^{113}$Cd with megabalanein A was shifted upfield in the range of 9-17 ppm as compared with the signal of $^{113}CdCl_2$ only at the same concentration (Fig. 5). This tendency didn't change in the presence of 5 equivalents of $^{113}CdCl_2$ when pH was adjusted to 7.4 with NaOD. These data indicate that megabalanein A can bind to cadmium non-specifically and reversibly.

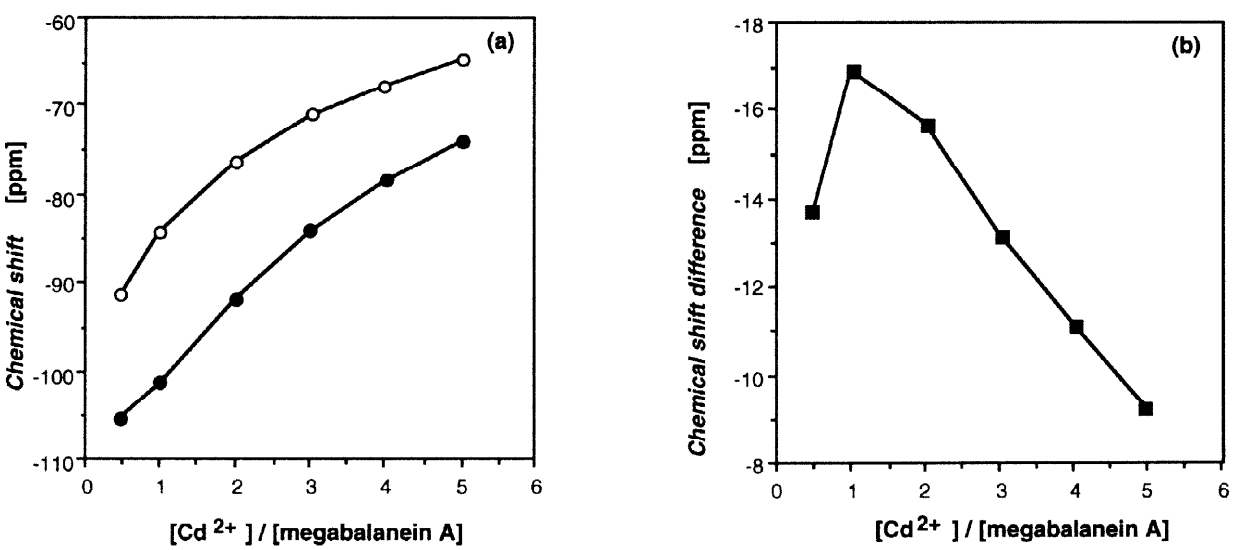


Fig. 5. Chemical shifts of $^{113}$Cd-NMR spectra of megabalanein A in the presence of six different equimolar amounts of $^{113}CdCl_2$ (●) as compared with the signal of $^{113}CdCl_2$ only (○) at the same concentration (a) and their differences of the chemical shifts (b). $^{113}$Cd-NMR spectra of megabalanein A were measured on a Varian spectrometer at 110 MHz.

Table 1 NMR data for megabalanein B (500 MHz for $^1H$, 125 MHz for $^{13}C$, in 90% $H_2O$ and 10% $D_2O$ at pH 5.2 adjusted with NaOD, and 25 ℃)

| Residue | | Chemical shift, ppm | | Residue | | Chemical shift, ppm | |
|---|---|---|---|---|---|---|---|
| | | $^1H$ | $^{13}C$ | | | $^1H$ | $^{13}C$ |
| Ser-1 | α CH | 4.04 | 55.03 | Glu-11 | α CH | 4.06 | 54.60 |
| | β $CH_2$ | 3.84 | 60.62 | | β $CH_2$ | 1.80, 1.90 | 27.54[h] |
| | α NH | - | | | γ $CH_2$ | 2.15 | 33.51[c] |
| | CO | | 168.77 | | δ CO | | 180.76 |
| Gly-2 | α $CH_2$ | 3.93 | 42.58 | | α NH | 8.37 | |
| | α NH | 8.64(t, J=5.8Hz) | | | CO | | 173.82[i] |
| | CO | | 171.43 | Glu-12 | α CH | 4.08 | 54.30 |
| Val-3 | α CH | 3.96 | 60.32 | | β $CH_2$ | 1.70, 1.80 | 27.59[h] |
| | β $CH_2$ | 1.95 | 30.00 | | γ $CH_2$ | 2.12 | 33.51[c] |
| | γ $CH_3$ | 0.81 | 17.84 | | δ CO | | 180.93 |
| | | 0.80 | 18.52 | | α NH | 8.35 | |
| | α NH | 8.19 | | | CO | | 173.85[j] |
| | CO | | 174.38 | Leu-13 | α CH | 4.22 | 52.82 |
| Gly-4 | α $CH_2$ | 3.79 | 42.80[a] | | β $CH_2$ | 1.46, 1.54 | 39.76 |
| | α NH | 8.50(t, J=5.8Hz) | | | γ CH | 1.50 | 24.62[f] |
| | CO | | 171.59 | | δ $CH_3$ | 0.71 | 20.78 |
| Glu-5 | α CH | 4.13 | 53.67 | | | 0.76 | 22.53 |
| | β $CH_2$ | 1.78, 1.88 | 27.01[h] | | α NH | 8.09 | |
| | γ $CH_2$ | 2.08 | 33.45[c] | | CO | | 175.03 |
| | δ CO | | 181.12 | Ser-14 | α CH | 4.26 | 56.22 |
| | α NH | 7.99(d, J=6.6Hz) | | | β $CH_2$ | 3.75 | 61.35 |
| | CO | | 174.11[d] | | α NH | 8.14(d, J=5.6Hz) | |
| Ile-6 | α CH | 3.92 | 59.03 | | CO | | 172.59 |
| | β $CH_2$ | 1.71 | 36.19[e] | Gly-15 | α $CH_2$ | 3.80 | 42.89[a] |
| | γ $CH_2$ | 1.04, 1.32 | 24.71[f] | | α NH | 8.26(t, J=5.8Hz) | |
| | γ $CH_3$ | 0.75 | 15.00[g] | | CO | | 171.40 |
| | δ $CH_3$ | 0.71 | 10.33[h] | Gln-16 | α CH | 4.19 | 53.23 |
| | α NH | 8.10 | | | β $CH_2$ | 1.83, 1.93 | 27.77 |
| | CO | | 174.11[d] | | γ $CH_2$ | 2.18 | 31.30 |
| Glu-7 | α CH | 4.11 | 53.88 | | δ $NH_2$ | 6.72, 7.49 | |
| | β $CH_2$ | 1.84, 1.94 | 27.38[h] | | δ CO | | 178.03 |
| | γ $CH_2$ | 2.14 | 33.45[c] | | α NH | 8.05(d, J=7.1Hz) | |
| | δ CO | | 180.90 | | CO | | 173.29 |
| | α NH | 8.21 | | Ile-17 | α CH | 4.05 | 58.61 |
| | CO | | 174.27[d] | | β $CH_2$ | 1.71 | 36.53[e] |
| Lys-8 | α CH | 4.13 | 54.13 | | γ $CH_2$ | 1.03, 1.32 | 24.93[f] |
| | β $CH_2$ | 1.62 | 30.53 | | γ $CH_3$ | 0.75 | 15.03[g] |
| | γ $CH_2$ | 1.28 | 22.23 | | δ $CH_3$ | 0.71 | 10.47[h] |
| | δ $CH_2$ | 1.52 | 26.57 | | α NH | 8.15(d, J=7.6Hz) | |
| | ε $CH_2$ | 2.83 | 39.65 | | CO | | 173.25 |
| | ε $NH_2$ | 7.15 | | Asp-18 | α CH | 4.54(dd, J=4.7,8.7Hz) | 51.84 |
| | α NH | 8.29 | | | β $CH_2$ | 2.48(dd, J=8.7,16.0Hz) | 38.52 |
| | CO | | 173.77 | | | 2.62(dd, J=4.7,16.0Hz) | |
| Arg-9 | α CH | 4.11 | 54.24 | | γ CO | | 177.43 |
| | β $CH_2$ | 1.62, 1.74 | 28.43 | | α NH | 8.36 | |
| | γ $CH_2$ | 1.50 | 24.44[i] | | CO | | 172.59 |
| | δ $CH_2$ | 3.06 | 40.89 | Ser-19 | α CH | 4.09 | 57.42 |
| | ζ C | | 157.10 | | β $CH_2$ | 3.69(d, J=4.2Hz) | 62.48 |
| | NH | 6.62, 7.43 | | | α NH | 7.77(d, J=7.3Hz) | |
| | α NH | 8.22 | | | COOH | | 175.97 |
| | CO | | 173.99 | | | | |
| Ala-10 | α CH | 4.07 | 50.73 | | | | |
| | β $CH_3$ | 1.26 | 16.45 | | | | |
| | α NH | 8.30 | | | | | |
| | CO | | 175.72 | a-j, values with identical superscripts may be interchanged. | | | |

## DISCUSSION

The present study describes the characteristics of two novel cadmium-inducible peptides derived from the barnacle, *M. volcano*. They lack cysteine and contain high amounts of acidic residues such as glutamic acid and aspartic acid. Among invertebrates, cadmium-binding/inducible proteins with low cysteine content have been reported in molluscs,[2-4] annelids[5-7] and insects,[9] most of which are rich in acidic amino acids. Megabalaneins A and B contain 33 and 26% acidic amino acids, respectively, which are considered to be involved in cadmium-binding. Molecular weights of megabalaneins A and B are 1703.9 and 2004.1, respectively, which are quite smaller than those of MT and other cadmium-binding/inducible proteins (about 6-10 kDa).[1-7,9] They may thus be the smallest cadmium-inducible peptides discovered. Details of the functions of these cadmium-binding/inducible proteins, including megabalaneins A and B, are still being clarified.

$^{113}$Cd-NMR spectra of megabalanein A in the presence of $^{113}CdCl_2$ indicate that megabalanein A can bind to cadmium non-specifically and reversibly. This binding property and the fact that these peptides are cadmium-inducible may indicate that the main role of these peptides is detoxification of metals.

Now functional investigations of megabalaneins A and B including their cDNA sequence analysis and northern blot analysis are being performed. Primers were designed from the amino acid sequences of megabalaneins A and B, polymerase chain reaction was carried out, and cDNA sequences are being analysed by a rapid amplification of cDNA ends method.

## EXPERIMENTAL SECTION

*General Methods*

NMR measurements of megabalanein B were performed on a Bruker spectrometer at 500 MHz for $^{1}$H-NMR spectra and at 125 MHz for $^{13}$C-NMR spectra. Samples were dissolved in 90% $H_2O$ and 10% $D_2O$ at pH 5.2 adjusted with NaOD. $^{113}$Cd-NMR spectra of megabalanein A were measured on a Varian spectrometer at 110 MHz. Mass spectra were obtained on a MALDI-TOF mass spectrometer. CD spectra were taken with a JASCO J-600 spectropolarimeter and IR spectra were obtained with KBr method on a JASCO FT/IR-7000 Fourier transform infrared spectrometer. Cadmium contents were analyzed with a Shimadzu ICP spectrometer ICPS-1000IV.

*Animals*

Barnacles, *M. volcano*, were collected in Izu (Japan).

Except for the control, barnacles were exposed to 200 μg of Cd/l of sea water for 4 weeks at room temperature.

*Isolation of cadmium-inducible peptides from M. volcano*

The internal organs of cadmium-exposed barnacles were homogenized in 2 vol. of ice-cold 20 mM Tris/HCl pH 8.0, and phenylmethylsulfonyl fluoride was added to the homogenate to a final concentration of 0.1 mM. The homogenate was centrifuged at 39,900 x *g* for 60 min at 4 °C. The resulting supernatant was concentrated with an ultrafiltration membrane (amicon YM 1) and fractioned by a gel filtration FPLC column of HiLoad 16/60 Superdex 75 pg (1.6 x 60 cm; Pharmacia) eluting with 20 mM Tris/HCl at pH 8.0 and at a flow rate of 0.5 ml/min. Fractions of 2 ml were analysed for cadmium by an ICP spectrometer. Fractions corresponding to the low-molecular-mass, cadmium-binding region (apparent molecular mass = 10 kDa) were pooled and diluted with distilled water to a concentration of 3 mM Tris. The diluted cadmium-binding fractions were chromatographed on an anion-exchange FPLC column Mono Q HR 5/5 (0.5 x 5 cm; Pharmacia) that had been equilibrated with 3 mM Tris/HCl at pH 8.0 and the column was developed with a linear gradient from 3 mM to 300 mM Tris/HCl at pH 8.0 and at a flow rate of 1.0 ml/min. Fractions of 1 ml were analyzed for cadmium and cadmium-binding fractions were pooled and separated by reverse-phase HPLC (RP-HPLC; μBondasphere, C4, 5 μ, 300 Å, 3.9 x 150 mm; Waters), which was eluted over 45 min with a linear gradient from 5% to 48% acetonitrile in 0.06% trifluoroacetic acid at a flow rate of 0.7 ml/min. Major peaks were individually pooled and concentrated by evaporation under reduced pressure. For control purposes, the same purification from unexposed barnacles was performed. The homogenate of the internal organs were similarly separated by gel fitration after adding cadmium, followed by Mono Q column and RP-HPLC purification.

Spectral data for megabalanein A (CdIP1; 73 μg) was reported previously.[8]

Spectral data for megabalanein B (CdIP2; 106 μg): CD $\lambda_{max}$ 198.6 (-), 224.6 (-) nm.

*N-terminal amino acid sequences*

N-terminal amino acid sequencing of megabalaneins A and B was performed by degradation from the amino terminus with the Edman reagent, phenyl isothiocyanate, using a protein sequencer PSQ-2 (Shimadzu).

*Spectral data for synthetic megabalanein B*

A peptide having the same sequence as that of megabalanein B was synthesized by starting from Fmoc L-amino acids (Sawady Technology). IR (KBr) $\nu_{max}$ 3280, 3075, 2640, 1710(sh), 1660, 1540, 1420, 1200, 1180(sh), 1140 and 1070 $cm^{-1}$; CD $\lambda_{max}$ ($\Delta\varepsilon$) 200.0 (-54.77), 224.2 (-15.78) nm.

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