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Megabalanein A; a Novel Cadmium-inducible Peptide from the Barnacle, *Megabalanus volcano*

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Abstract: A novel cadmium-inducible peptide, megabalanein A (CdIP1), was purified from the barnacle, *Megabalanus volcano*. This peptide is composed of 15 amino acids, five of which are acidic residues. Megabalanein A may be the smallest metal-inducible peptide that has been found in vertebrates and invertebrates. © 1998 Elsevier Science Ltd. All rights reserved.

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,[2-6] for detoxification purposes. With MT, cysteines are known to bind metals including cadmium, zinc, copper and other soft metals. We report here the isolation of a new metal-inducible peptide, megabalanein A, from the barnacle, *Megabalanus volcano*. 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. Megabalanein A may be the smallest metal-inducible peptide so far found in vertebrates and invertebrates.

Barnacles, *Megabalanus volcano* were collected from Izu (Japan) and were exposed to 200 μg of Cd/l of sea water for 4 weeks at room temperature. The internal organs were homogenized in 2 vol. of ice-cold 20 mM Tris/HCl at pH 8.0, and PMSF (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 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 FPLC column of 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 1ml were analyzed for cadmium and cadmium-binding fractions 19-26 were pooled and separated by reverse-phase HPLC (RP-HPLC; μBondasphere C4, 5μ, 300 Å, 3.9 x 150 mm; Waters), which was eluted over 45 min with a linear gradient from 5% to 48% of

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acetonitrile in 0.06% trifluoroacetic acid at a flow rate of 0.7 ml/min. It was resolved into four major peaks and one of the major peaks at a retention time of 18.1 min was concentrated by evaporation under reduced pressure to give a pure peptide.

Amino acid sequencing of the purified peptide was performed by degradation from the amino terminus with Edman reagent and phenyl isothiocyanate using a peptide sequencer (Fig. 1). The calculated molecular weight value from the sequence was in good agreement with the observed one from the MALDI-TOF mass spectrum (m/z 1703.90 [$(M+H)^+$; calcd for $C_{70}H_{119}N_{20}O_{29}$ m/z 1703.85]). This cadmium-inducible peptide is designated as megabalanein A (CdIP1).[7]

Glu-Ile-Glu-Lys-Arg-Ala-Glu-Glu-Leu-Ser-Gly-Gln-Ile-Asp-Ser

Fig. 1. Amino acid sequence of megabalanein A (CdIP1)

To determine the absolute configuration of megabalanein A, a peptide having the same sequence as that of megabalanein A 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[8] matched those of megabalanein A. The CD spectrum of the synthetic peptide, showing the specific band for a randam coil structure, was also the same as that of megabalanein A. These results confirmed the assignment of L-configuration to all of the megabalanein A residues.

For control purposes, same purification from unexposed barnacles was performed. Homogenate of the internal organs were similarly separated by gel fitration after adding cadmium, followed by Mono Q column and RP-HPLC purification. RP-HPLC chromatograms for cadmium-exposed and unexposed barnacle preparations are shown in Fig.2. In case of unexposed barnacles no remarkable peak could be detected, which indicates that megabalanein A is cadmium-inducible.

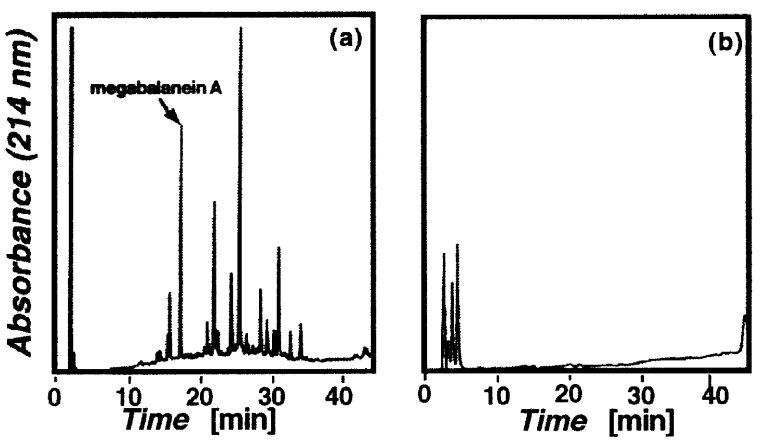

Fig. 2. RP-HPLC chromatograms of Cd-exposed (a) and unexposed barnacle (b) preparations

NMR data for megabalanein A is depicted in Table 1. The binding properties of megabalanein A to cadmium were examined by NMR spectroscopy. The ^{13}C-NMR chemical shift changes for amino acids of

megabalanein A were measured in the presence of $^{113}CdCl_2$ from 0.5 to 5 equivalents. Chemical shift changes in the presence of 5 equivalents of $^{113}CdCl_2$ are also shown in Table 1. Large chemical shift changes for methylene groups of acidic amino acids such as γ-CH_2 of Glu and β-CH_2 of Asp indicate the interaction between acidic amino acids and cadmium. ^{113}Cd-NMR spectra of megabalanein A in the presence of six different equimolar amounts of $^{113}CdCl_2$ were also measured (data not shown). Only one signal was observed in every $^{113}CdCl_2$ concentration, and 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. This data indicates that megabalanein A can bind to cadmium non-specifically and reversely.

Table 1. NMR data for megabalanein A (500MHz for ^1H, 125 MHz for ^{13}C, in D_2O at pH 7.0 adjusted with NaOD, and 30℃)

Residue		Chemical shift, ppm		Residue		Chemical shift, ppm	
		^1H	^{13}C (Δ*)			^1H	^{13}C (Δ*)
Glu-1	α CH	4.07(br.t, J=6.7Hz)	52.50(0.31)	Leu-9	α CH	4.40	52.03(0.08)
	β CH_2	2.05	22.88(0.59)		β CH_2	1.68, 1.62	39.04(0.00)
	γ CH_2	2.35	32.62(0.52)		γ CH	1.65	23.69(0.03)
	δ CO		180.18(0.16)		δ CH_3	0.84,	20.06(0.00)
Ile-2	α CH	4.20	57.87(0.06)			0.90	21.78(0.00)
	β CH_2	1.85	35.55(0.24)[a]	Ser-10	α CH	4.44(t, J=5.3Hz)	55.42(0.08)
	γ CH_2	1.42, 1.18	23.97(0.03)[b]		β CH_2	3.88(dd, J=5.3,12.0Hz)	60.63(0.06)
	γ CH_3	0.90	14.23(0.04)[c]			3.92(dd, J=5.3,12.0Hz)	
	δ CH_3	0.84	9.69(0.09)[d]	Gly-11	α CH_2	3.95(d, J=17.3Hz)	42.15(0.07)
Glu-3	α CH	4.28	53.36(0.20)			4.00(d, J=17.3Hz)	
	β CH_2	2.05, 1.97	26.84(0.02)	Gln-12	α CH	4.22	53.89(0.12)
	γ CH_2	2.24	33.02(0.64)		β CH_2	1.98, 2.09	26.26(0.01)
	δ CO		180.49(0.19)		γ CH_2	2.32	30.56(0.03)
Lys-4	α CH	4.32	42.70(0.18)		δ CO		177.28(0.19)
	β CH_2	1.76	29.89(0.05)	Ile-13	α CH	4.23	58.14(0.15)
	γ CH_2	1.43	21.46(0.06)		β CH_2	1.85	35.82(0.16)[a]
	δ CH_2	1.65	25.82(0.03)		γ CH_2	1.42, 1.18	24.07(0.03)[b]
	ε CH_2	2.97(t, J=7.5Hz)	38.90(0.02)		γ CH_3	0.90	14.30(0.00)[c]
Arg-5	α CH	4.34	52.70(0.18)		δ CH_3	0.84	9.73(0.05)[d]
	β CH_2	1.72, 1.87	27.78(0.07)	Asp-14	α CH	4.64(dd, J=5.1,8.5Hz)	51.23(0.23)
	γ CH_2	1.63	23.87(0.08)		β CH_2	2.59(dd, J=8.5,16.1Hz)	38.06(0.58)
	δ CH_2	3.19(t, J=6.8Hz)	40.12(0.02)			2.72(dd, J=5.1,16.1Hz)	
	ζ C		156.38(0.01)		γ CO		177.03(0.13)
Ala-6	α CH	4.22	49.86(0.09)	Ser-15	α CH	4.24	56.70(0.18)
	β CH_2	1.39(d, J=7.1Hz)	15.79(0.06)		β CH_2	3.84(d, J=5.0Hz)	61.79(0.06)
Glu-7	α CH	4.28	53.36(0.20)[e]				
	β CH_2	2.05, 1.67	27.00(0.12)[f]				
	γ CH_2	2.24	33.09(0.71)[g]				
	δ CO		180.62(0.08)				
Glu-8	α CH	4.32	52.90(0.14)[e]				
	β CH_2	2.05, 1.97	27.15(0.03)[f]				
	γ CH_2	2.24	33.18(0.53)[g]				
	δ CO		180.62(0.08)				

CO 170.65, 171.81, 171.92, 172.50(2×C), 172.57, 172.80, 172.91, 173.01, 173.32, 173.46, 174.23, 174.82, 175.28
Assignment could not be made.

* Change (absolute value) of the chemical shift by the addition of 5 equivalents of $^{113}CdCl_2$.

a-g, values with identical superscripts may be interchanged.

We have reported in this paper the isolation of a novel low-molecular-weight cadmium-inducible peptide, megabalanein A (CdIP1), from the barnacle, *Megabalanus volcano*. Megabalanein A lacked cysteine and was rich in acidic residues. Although "soft" metals like cadmium are thought to have high chemical affinity for S and "hard" metals like calcium to have high affinity for O, megabalanein A was found to be inducible by cadmium. However, its affinity for cadmium may be smaller than that for "hard" metals because it has many carboxyl groups. Future studies will analyze the binding properties of megabalanein A to various metals by NMR measurements.

The molecular weight of megabalanein A (1703.84) is much smaller than that of MT and other metal-binding/inducible proteins (about 6-10kDa).[1-6] It may thus be the smallest cadmium-binding peptide discovered. Details of the functions of these cadmium-binding/inducible proteins, including megabalanein A, are still being clarified. Megabalanein A is easy to synthesize, which will be efficient to investigate its functions further.

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7. Analytical data: CD λ_{max} 198.2 (-), 227.0 (-) nm; HRMS (FAB) found, m/z 1703.8446 ($M+H^+$) [$C_{70}H_{119}N_{20}O_{29}$ requires 1703.8452].
8. Analytical data: CD λ_{max} ($\Delta\varepsilon$) 198.0 (-17.67), 228.4 (-1.62) nm; IR (KBr) ν_{max} 3280, 3075, 2600, 1710(sh), 1630, 1530, 1415, 1200, 1180(sh), 1140 and 1070 cm^{-1}.