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CADYSTIN A AND B, MAJOR UNIT PEPTIDES COMPRISING CADMIUM BINDING PEPTIDES INDUCED IN A FISSION YEAST-----SEPARATION, REVISION OF STRUCTURES AND SYNTHESIS

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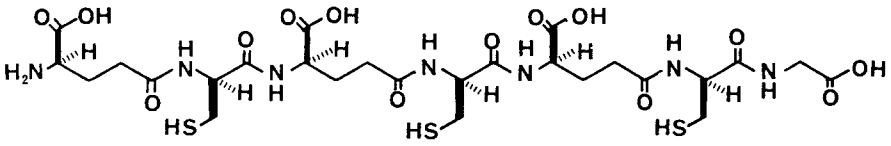
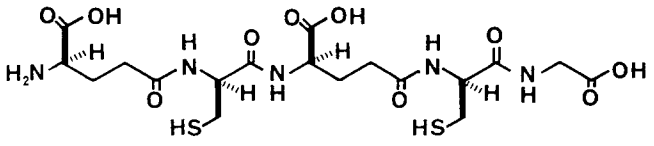
Abstract: The unit peptide, cadystin, of the cadmium-binding peptides occurring in a fission yeast was further separated into two major components, **cadystin A** and **B**, structures of which were determined to be **1** and **2**, respectively, and confirmed by synthesis. The structure previously reported for cadystin was thus revised.

Cadmium binding peptides, Cd-BP 1 and 2, in a fission yeast Schizosaccharomyces pombe, are metallothionein-like substances because of (i) induction by addition of Cd^{++}, (ii) high capacities of Cd-binding and (iii) a high content of Cys-residues. Cd-BP 1 and 2 are associated molecules (MW ca. 4,000 and 1,800, respectively) comprising the unit peptide, **cadystin**, and Cd^{++}.[1] We have recently reported the structure of cadystin to be H-γ-Glu-Cys-γ-Glu-Cys-Cys-γ-Glu-Gly-OH, in which one of the cystein is D form and all others including Glu are L form.[2] Since all of the possible isomers of cadystin, which were synthesized thereafter, behaved very similarly on reversed phase HPLC, but none of them was identical with the natural cadystin, and since we found that the natural cadystin could be separated by the HPLC into two major fractions, we have reinvestigated structural study on these two fractions, which we named **cadystin A** (a hepta-peptide) and **cadystin B** (a penta-peptide).



cadystin A (1)



cadystin B (2)

The cadystin-fraction used for the previous structural studies[2] was further purified by reversed phase HPLC (Fig. 1) to afford pure **cadystin A** (retention time: 7.5 min) (ca. 60% yield) and **cadystin B** (4.5 min) (ca. 40% yield). DeS-cadystin (desulfurized product of cadystin with Raney Ni)[2] was also separated by HPLC (ODS, 1.5% CH_3CN, 0.1% TFA, 1.0 ml/min) into **deS-cadystin B** (8 min) and **deS-cadystin A** (18 min) in 2:3 ratio. Amino acid analyses and

FAB-MS of **deS-cadystin A** and **B** showed Glu(3), Ala(3), Gly(1), m/z 772(M+1) and Glu(2), Ala(2), Gly(1), m/z 540 (M+1), respectively.[3] Amino acid composition of **cadystin A** is Glu(3), Cys-SH(3), Gly(1) and that of **cadystin B** is Glu(2), Cys-SH(2), Gly(1).

Cadystin B (2) did not receive the usual Edman degradation, but afforded DNS-Glu by dansylation followed by acid hydrolysis, and treatment of 2 with carboxypeptidase P afforded Gly in 85% yield. When **deS-cadystin B** was digested with carboxypeptidase P [for 96 hr at 35°C in 0.1M acetate, pH 5.2], it afforded not only Gly (in 92% yield) but also a dipeptide[4] containing Glu and Ala in 1:1 ratio. The latter was isolated with HPLC [ODS, 0.1% TFA-aq, 0.5 ml/min, detection 210 nm, retention time 15 min] in 168% yield judging from the amino acid analysis to indicate the production of two molar equivalents of this dipeptide in 84% yield. The dipeptide was esterified with MeOH and trimethyl orthoformate in the presence of H^+, reduced with $LiBH_4$ in THF and then hydrolyzed with 6 N HCl at 105°C for 12 hr. The hydrolysate was analyzed by an amino acid analyzer to give 4-amino-5-hydroxyvaleric acid and 2-aminopropanol in nearly quantitative yield without the formation of Ala or 2-amino-5-hydroxyvaleric acid; thus, the dipeptide should be H-γ-Glu-Ala-OH. Its stereochemistry was determined by identification [ODS, 0.1% TFA-aq, 0.5 ml/min, 205 nm, at 15°C] with synthetic H-γ-L-Glu-L-Ala-OH but not with H-γ-L-Glu-D-Ala-OH (see Fig. 2). In conclusion, carboxypeptidase P did hydrolyze two peptide bonds next to all the free carboxylic acid residues. By the way, **cadystin B** itself was also treated with carboxypeptidase P [for 96 hr at 30°C, 0.1M phosphate, pH 2.5], but the corresponding dipeptide (H-γ-L-Glu-L-Cys-OH) to the case of **deS-cadystin** was not detected due to a rapid air oxidation to form an S-S dimer, which was reduced with 1,2-ethanedithiol to obtain the dipeptide in 86% yield. The ratio of Gly (85%) and the dipeptide was 1.0:2.0 (see Fig. 3). Trimethyl ester of **deS-cadystin B** [prepared in MeOH and trimethyl orthoformate in the presence of H^+] was reduced with $LiBH_4$ and hydrolyzed with 6 N HCl to produce Ala, 4-amino-5-hydroxyvaleric acid and 2-aminoethanol in high yields but no 2-aminopropanol; thus, Gly connects with Cys but not with alpha-carboxyl group of Glu. **DeS-cadystin B** should have the sequence of H-γ-Glu-Ala-γ-Glu-Ala-Gly-OH.[5] It was concluded that **cadystin B** is H-γ-Glu-Cys-γ-Glu-Cys-Gly-OH, in which all amino acids are in L configuration.[6]

DeS-cadystin A was also treated with carboxypeptidase P to give Gly (81%) and H-γ-L-Glu-L-Ala-OH (3x80% yield). **DeS-cadystin A** was esterified [MeOH, H^+], reduced [$LiBH_4$], and hydrolyzed [H^+] to give Ala (3x40%), 4-amino-5-hydroxyvaleric acid (3x76%) and aminoethanol (1x77% yield); determining the sequence H-γ-Glu-Ala-γ-Glu-Ala-γ-Glu-Ala-Gly-OH. Digestion of **cadystin A** with carboxypeptidase P afforded Gly (71% yield) and H-γ-L-Glu-L-Cys-OH (3x71% yield) when analyzed after the treatment with 1,2-ethanedithiol; the relative ratio being 1.0:3.0. The structure of **cadystin A** has to be H-γ-Glu-Cys-γ-Glu-Cys-γ-Glu-Cys-Gly-OH, and all the amino acids were in L form,[6] which was concluded together with the result of the CD spectra (vide infra).

Both of the peptides, **cadystin A** and **B**, were synthesized in a stepwise manner as in Scheme 1. Physical constants of the protected pentapeptide, Tr-L-Glu-OBn[L-Cys(MOB)-L-Glu-OBn{L-Cys(MOB)-Gly-OBn}]: mp 107°C; $[\alpha]_D$= -1.05° (c= 1.05, $CHCl_3$); PMR (200 MHz in $CDCl_3$) δ 1.90-2.40 (8H, m), 2.60-2.90(4H, m), 3.4-3.6(1H, m), 3.66(4H, m), 3.73(3H, s), 3.74(3H, s), 3.95(1H, dd, J= 5.5, 18.0), 4.10(1H, dd, J= 6.0, 18.0), 4.39(1H, d, J= 12.5), 4.60(1H, d, J= 12.5), 4.40-4.65(3H, m), 5.09(2H, s), 6.20(1H, d, J= 7.0), 6.77.5(43H, m), 6.96(1H, d, J= 7.5). Physical constants of the protected heptapeptide, Tr-L-Glu-OBn[L-Cys(MOB)-L-Glu-OBn{L-Cys(MOB)-L-Glu-OBn(L-Cys(MOB)-Gly)}]: mp 131°C; $[\alpha]_D$= -12.4°(c= 1.02, $CHCl_3$); PMR (200 MHz, $CDCl_3$) δ 1.9-2.5(12H, m), 2.5-3.0(6H, m), 3.4-3.6(1H, m), 3.63(6H, brs), 3.72(9H, s), 3.95-4.16(2H, m), 3.88(1H, d, J= 12.0), 4.10(1H, d, J= 12.0), 4.5-4.8(5H, m), 4.95-5.20(6H, m),

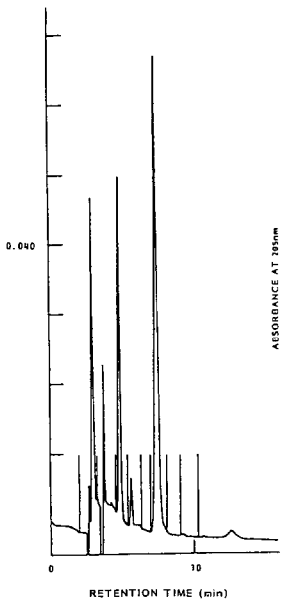


Fig. 1 CHROMATOGRAM OF CADYSTIN A AND B, column: Develosil ODS-5 (5 um, 4.6 x 250 mm), solvent: 9% CH_3CN, 0.1% TFA in H_2O, flow rate: 1.0 ml/min, detector: UV 210 nm

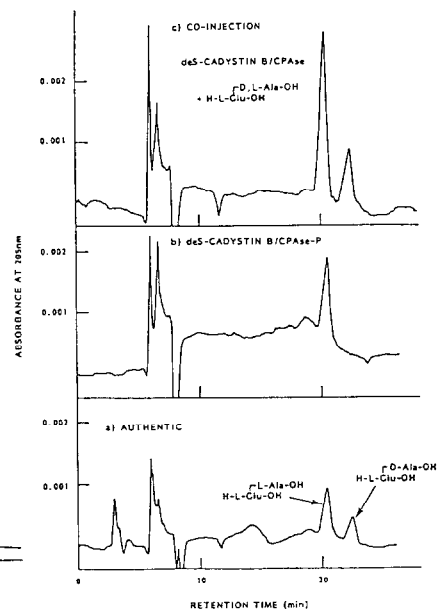


Fig. 2 IDENTIFICATION OF THE CPAse-P FRAGMENT OF DES-CADYSTIN B, column: ODS, solvent: 0.1% TFA in H_2O, flow rate: 0.5 ml/min, detector: UV 205 nm, temp: 15°C

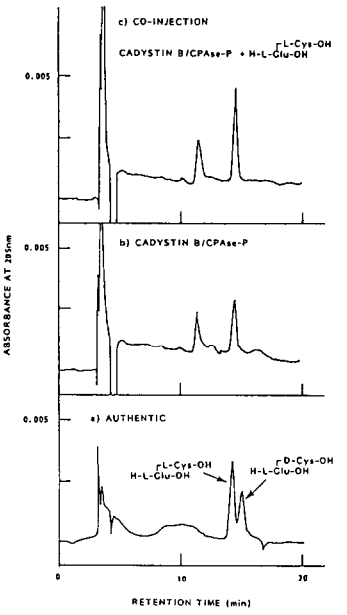


Fig. 3 IDENTIFICATION OF CPAse-P FRAGMENT OF CADYSTIN B FOLLOWED BY ETHANDITHIOL REDUCTION, column: ODS-5, solvent: 0.1% TFA in H_2O, flow rate: 1.0 ml/min, detector: UV 205 nm, temp: 11°C

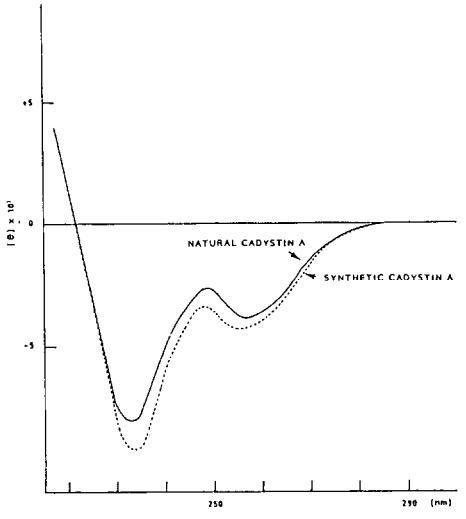


Fig. 4 CD SPECTRA OF SYNTHETIC AND NATURAL CADYSTIN A IN THE PRESENCE OF Cd++, solvent: 5 mM tris-HCl (pH 7.6), 10 mM KCl, 0.2 mM $CdCl_2$, cell length: 5 mm

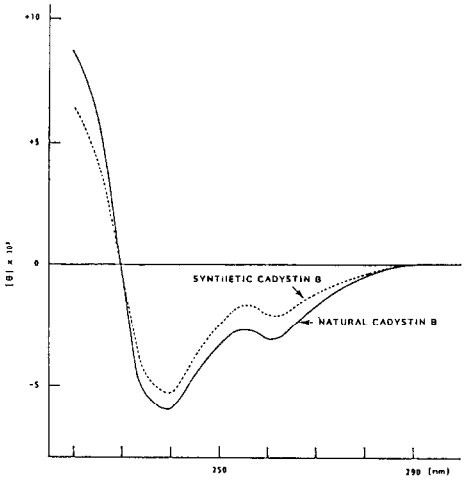
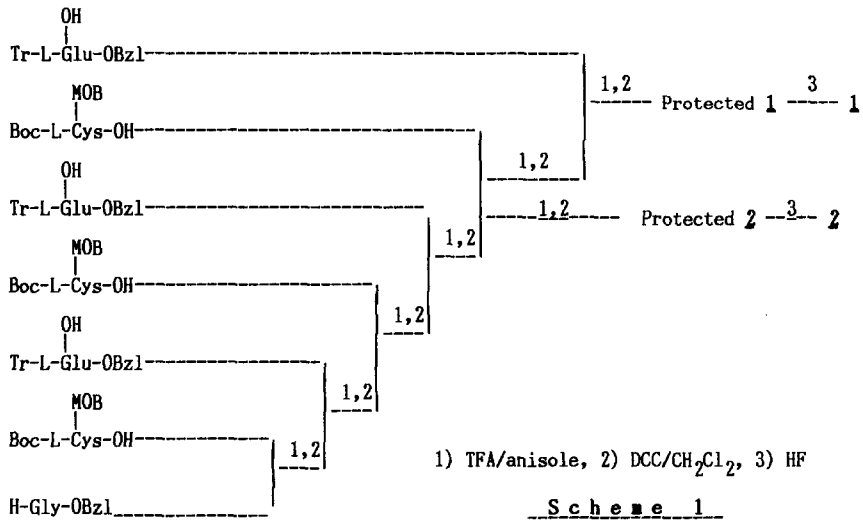


Fig. 5 CD SPECTRA OF SYNTHETIC AND NATURAL CADYSTIN B IN THE PRESENCE OF Cd++, solvent: 5 mM tris-HCl (pH 7.6), 10 mM KCl, 0.15 mM $CdCl_2$, cell length: 5 mm



Scheme 1

6.39(1H, d, J= 7.5), 6.6-7.6(52H, m), 6.96(1H, d, J= 7.5), 7.8(1H, d, J= 7.5); found C 66.84, H 6.20, N 5.72, calcd. for $C_{97}H_{103}N_7O_{17}S_3$ C 67.15, H 5.98, N 5.65. The protective groups of these synthesized products were removed by treatment with HF. Each of the synthesized peptides was purified by an ODS column, and identified with the natural peptides by means of HPLC, CD spectra in the presence of Cd^{++} (see Fig. 4 and 5), PMR spectra in 200 MHz, and FAB-MS, that concluded the structure of **cadystin A** and **B** to be **1** and **2** including stereochemistry.[7]

Biochemical aspects such as biosynthesis with reference to glutathion and chemical aspects such as molecular association concerning **Cd-BP1** are under investigation. These results will be reported elsewhere.

REFERENCES AND NOTES

1. A. Murasugi, C. Wada, Y. Hayashi, J. Biochem. **93**, 661 (1983), idem, ibid, **90**, 1561 (1981); idem, Biochem. Biophys. Res. Commun. **103**, 102 (1981); and the references cited therein.
2. N. Kondo, M. Isobe, K. Imai, T. Goto, A. Murasugi, Y. Hayashi, Tetrahedron Lett., **24**, 925 (1983). Cadystin-fraction was obtained from Cd-BP1 either by chromatography with Bio Gel P 2 [0.01M HCl/0.5M NaCl], or simply by treatment with 0.1% trifluroacetic acid.
3. N. Kondo, K. Imai, M. Isobe, T. Goto, Agric. Biol. Chem., **48**, #6 (1984).
4. The assignment of this product being a dipeptide was first referred to the condition suggested by J.L. Meek, Proc. Nat. Acad. Sci. U.S.A., **77**, 1632 (1980).
5. The sequence of Ala-Ala residue was suggested in the previous paper (see ref.# 2), based on the fact that the time course analysis of the amino acids indicated the delay of only Ala to reach the maximun value. This fact might arise during the acid hydrolysis via unusual transpeptidation.
6. Oxy-cadystin had been obtained from cadystin by treatment with performic acid, while ca. 60% cysteic acid in the product was racemized. This racemization led us to the previous assignment that one of the three Cys's was D form.
7. Des-cadystin A and B were also identical with synthetic samples.

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