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The Synthesis and Properties of EF-hand Type Calcium-binding Peptides[1)]

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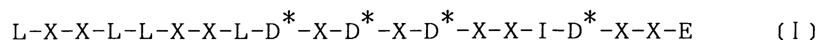
An eicosapeptide was synthesized in liquid phase, purified and characterized. It contains a N-terminal putative helical region (8 residues) and a loopregion (12 residues). This incomplete EF-hand peptide retained calcium-binding activity comparable with a native calmodulin fragment (107-148), when measured by the nitrocellulose membrane filtration method.

Many kinds of the proteins and peptides with calcium-binding activity have been characterized to date. Most interesting motif is EF-hand model proposed by Kretsinger and Tufty,[2)] which is realized in a family of proteins such as calmodulin, troponin C and so on.[3)] Calmodulin is a ubiquitous protein in all the eukaliotic cells and regulates the various functions in a calcium-dependent manner.[4)] Calmodulin molecule has four calcium-binding domains which are consisted of the highly homogeneous, repeated amino acid sequence;[5)] especially a common sequence, Asp-Gly-Asp-Gly locates in each calcium-binding loop.

In order to elucidate the detailed relationship between the structure and function of such calcium-binding proteins, we planed to synthesize the peptides with EF-hand structure, and characterized and subjected them to calcium-binding assay system recently developped by us.[6)]

At first, we have synthesized a peptide of H_8L_{12} which is constructed from a N-terminal putative α-helix (8 amino acid residues) and a loop region (12 residues). Its sequence was designed according to the EF-hand restriction[2)] and the amino acid sequence of bovine brain calmodulin;[5)] L, D* and X in the model peptide(I) are Phe, Asp and Gly, respectively.



L-X-X-L-L-X-X-L-D*-X-D*-X-D*-X-X-I-D*-X-X-E (I)

An outline of the strategy of synthesisis by the conventional liquid-phase

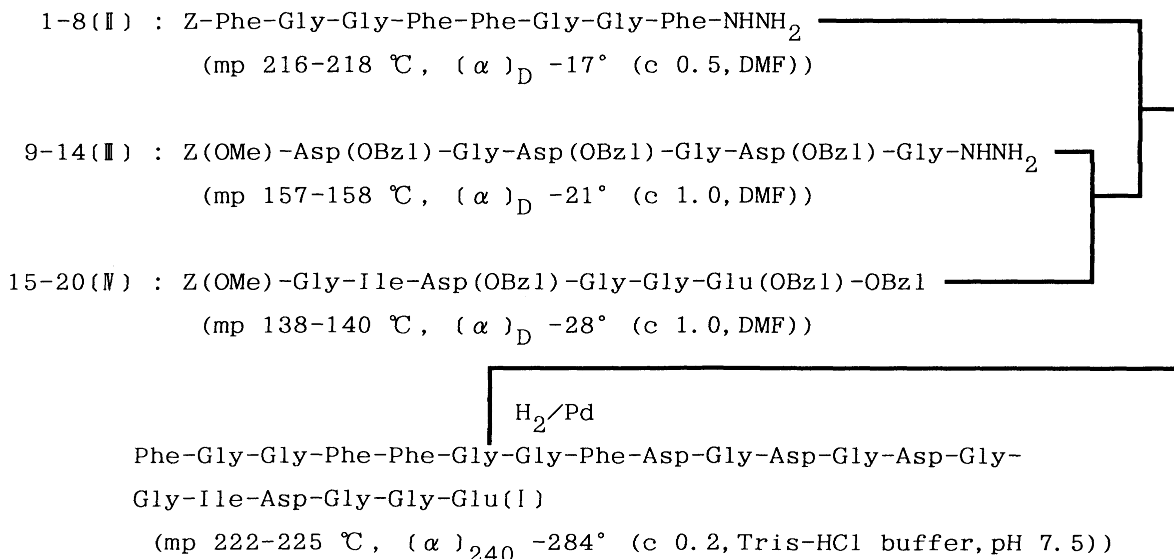
method is presented in Scheme 1, which shows the three peptide fragments selected as building blocks to construct the entire peptide backbone. In combination with the TFA-labile Z(OMe) group, amino acid derivatives bearing protecting groups removable by hydrogenolysis in the final stage were selected, i.e., Z-Phe, Asp(OBzl) and Glu(OBzl).

The N-terminal octapeptide hydrazide(II) was synthesized by azide condensation[7] of Z-Phe-Gly-Gly-Phe-$NHNH_2$ and a HBr-treated sample of Z-Phe-Gly-Gly-Phe-OMe followed by the usual hydrazine treatment of the resulting protected octapeptide ester.

The next fragment(III) was synthesized; Z(OMe)-Asp(OBzl)-Gly-ONPm[9] was treated with TFA and coupled twice with Z(OMe)-Asp(OBzl)-Gly according to DCC method, followed by the hydrazine treatment for only 10 min at 5 ℃. In the Asp(OBzl) containing peptide thus obtained no succinimide derivatives were observed.

The C-terminal hexapeptide(IV) was synthesized by DCC[8] condensation of Z(OMe)-Asp(OBzl)-Gly and a TFA-treated sample of Z(OMe)-Gly-Glu(OBzl)-OBzl, followed by azide condensation of Z(OMe)-Gly-Ile-$NHNH_2$.

The three fragments thus prepared were assembled successively onto a TFA-treated sample of the C-terminal fragment(IV) via the azide as shown in Scheme 1 by Honzl and Rudinger's azide procedure[7] in order to minimize racemization.



Scheme 1. Synthesis of the eicosapeptide(I).

All the protecting groups were removed by catalytic hydrogenation and the reduced product was purified by HPLC on TMS-250 column using gradient elution with acetonitrile(20-50%) containing 0.1% TFA. The purity of HPLC-purified sam-

ple was further confirmed by amino acid analysis and aminopeptidase M digestion.

Synthesized eicosapeptide was shown to have as much calcium-binding activity as a native calcium-binding peptide obtained from bovine brain calmodulin as described below.

The calcium-binding assay was carried out for synthesized peptides themselves or dinitrophenylated ones.[11] Dinitrophenylation was performed for the peptides of L_{12} and H_8L_{12}.[12] DNP-H_8L_{12} was retained up to about 3 μg on the membrane per assay. L_{12}, H_8L_{12}, and DNP-L_{12} were, however, not retained(Fig. 1).

The disociation constant of Ca^{2+} for DNP-H_8L_{12} was determined as 370 μM by double reciprocal plotting. On the other hand, a native calmodulin fragment[13] (107-148, calcium-binding site IV) had Kd of 250 μM when measured by the same assay method.[11]

CD spectra of H_8L_{12} were measured under the various conditions(Fig. 2). Ca^{2+} induced some conformation of H_8L_{12} in the presence of TFE, which seems to be different from α-helix or β-structure.

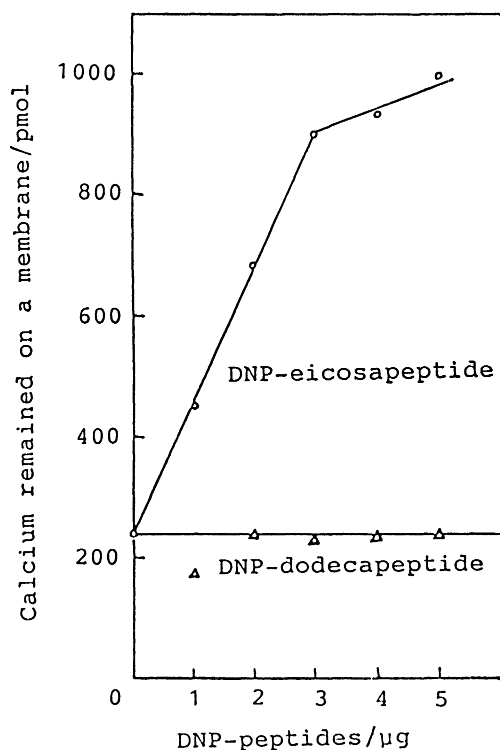


Fig.1. Binding of DNP-peptides to the nitrocellulose membrane. Each 1 to 5 μg of DNP-eicosapeptide or DNP-dodecapeptide was applied to the membrane in the presence of 500 μM $CaCl_2$ containing ^{45}Ca. Ca amount retained on the membrane was measured in a scintillation countor.

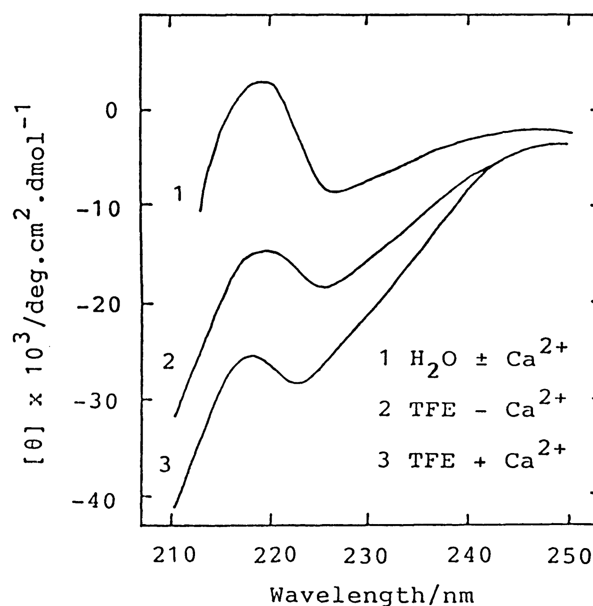


Fig.2. CD spectra of the eicosapeptide in various conditions. CD spectrum was measured in 25 mM Tris-Cl buffer(pH 7.5) and/or 50% TFE in the presence or absence of 5 mM $CaCl_2$.

In summary, an incomplete EF-hand type synthetic peptide was shown to retain the calcium-binding activity nearly equal to that of a native EF-hand type peptide obtained from calmodulin and to have a potentiality of folding into a structure different from α-helix or β-structure, which has recently suggested for a lipoprotein, lipocortin.[14)]

References

1) Amino acids used are of the L-configuration. The following abbreviations are used: Bzl=benzyl, DCC=dicyclohexylcarbodiimide, DMF=dimethylformamide, ONP^{m}=m-nitrophenoxy, TFA=trifluoroacetic acid, Z(OMe)=p-methoxybenzyloxycarbonyl, Z=benzyloxycarbonyl
2) R. M. Tufty and R. H. Kretsinger, Science, 187, 167(1975).
3) R. H. Kretsinger, Ann. Rev. Biochem., 45, 916(1976).
4) C. B. Kree, T. H. Crouch, and P. G. Richman, Ann. Rev. Biochem., 49, 489(1980).
5) H. Kasai, Y. Kato, T. Isobe, H. Kawasaki, and T. Okuyama, Biomed. Resear., 1, 248(1980).
6) H. Kawasaki, H. Kasai, and T. Okuyama, Anal. Biochem., 148, 297(1985).
7) J. Honzl and J. Rudinger, Collect. Czech. Chem. Commun., 26, 2333(1961).
8) J. C. Sheehan and G. P. Hess, J. Am. Chem. Soc., 77, 1067(1955).
9) H. Ito, Synthesis, 1979, 465.
10) M. Bodanszky and J. Z. Kwei, Int. J. Pep. Protein Res., 12, 69(1978).
11) Typically, a solution containing 1-3 μg of peptide and radioactive calcium ion was placed onto a nitrocellulose membrane (pH 7.9) and filtered under N_2 pressure. The residual amount of ^{45}Ca on the membrane was measured against a control in a liquid scintillation counter.
12) 160 μg of H_8L_{12} was dissolved in 80 μl of 1% $NaHCO_3$ solution and added with 16 μl of 5% DNFB ethanol solution. After 2 h reaction at room temperature, resultant water layer was washed with 20 μl x 3 of ether and then neutralized with 1 M HCl. The reaction mixture was subjected to HPLC column (TMS-250, 0.47ϕ x 250 mm). DNP-L_{12} and DNP-H_8L_{12} were obtained in a homogeneous form.
13) It was obtained from bovine brain calmodulin by the limited trypsin digestion.
14) M. J. Geisow, U. Fritsche, J. M. Hexham, B. Dash, and T. Johnson, Nature, 320, 636(1986).

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