Isolation of Plactins A, B, C and D, Novel Cyclic Pentapeptides that Stimulate Cellular Fibrinolytic Activity

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Four novel cyclic pentapeptides, designated plactins A, B, C and D, were isolated by solvent extraction and reverse-phase HPLC from mycelium of a fungal strain F165 that belongs to the order of Agonomycetales. By a combination of chemical and spectroscopic analyses and chemical synthesis, the structures of plactins A, B, C and D were determined to be *cyclo*(–D-Val–L-Leu–D-*allo*Ile–L-Tyr–D-Arg–), *cyclo*(–D-Val–L-Leu–D-Leu–L-Tyr–D-Arg–), *cyclo*(–D-Val–L-Leu–D-*allo*Ile–L-Phe–D-Arg–) and *cyclo*(–D-Val–L-Leu–D-Leu–L-Phe–D-Arg–), respectively. Plactins stimulated U937 cell-mediated degradation of ^{125}I-fibrin plates by 50% at a concentration of 7.5~32 μM.

Vascular endothelial cells play a crucial role in thromboregulation by virtue of a membrane-oriented fibrinolytic system. Cell-surface binding of fibrinolytic components, plasminogen and its activators, provide a mechanism for local regulation of fibrinolysis[1~4]. During the course of searching for microbial metabolites that stimulate cell-mediated fibrinolysis, we have found four novel cyclic pentapeptides as active compounds. The present paper describes the isolation, structural characterization and biological activity of these metabolites.

Materials and Methods

Materials

RPMI-1640 medium was purchased from Gibco and fetal bovine serum from Irvine Scientific, U.S.A. Carrier-free Na^{125}I was a product of Amersham. Human fibrinogen and thrombin were obtained from Sigma. 9-Fluorenylmethoxycarbonyl-amino acids (Fmoc-amino acids), 2-chlorotrithylchloride-resin, benzotriazol-1-yl-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP) and 1-hydroxybenzotriazole (HOBt) were purchased from Shimadzu Co., Japan. 9-Fluorenylmethyl *N*-succinimidyl carbonate (Fmoc-ONSu) was obtained from Peptide Institute, Japan, and *N*-methylmorpholine (NMM) from Wako Pure Chemical, Japan. Fmoc-D-*allo*Ile was prepared from Fmoc-ONSu and D-*allo*Ile as described by MILTON *et al.*[5] Human venous blood was drawn from healthy volunteers in 13 mM sodium citrate and centrifuged at 2,000 × *g* for 15 minutes at 4°C. The resulting platelet-poor plasma was stored at −80°C until use. Fibrinogen was radioiodinated by the iodine monochloride method[6] to a specific activity of 200~400 cpm/ng[7].

Microorganism

The producing strain F165, a fungal strain belonging to Fungi Imperfecti, was isolated from a soil sample collected in Itsukaichi-machi, Tokyo and subcultured on potato glucose agar slants.

Synthesis of Cyclic Pentapeptides

Linear pentapeptides were synthesized according to the Fmoc chemistry using the PyBOP-HOBt-NMM system and 2-chlorotrithylchloride-resin as described by NOKIHARA *et al.*[8]. Briefly, Fmoc-D-Leu, Fmoc-L-Leu and Fmoc-D-*allo*Ile linked to the resin were used as starting materials for the synthesis of linear pentapeptides. Peptide chains were elongated by consecutive cleavages of Fmoc groups and addition of Fmoc-amino acids. After removal of the Fmoc groups from amino-terminal residues, side chain-protected linear pentapeptide derivatives were released from the resin by treating with acetic acid - 2,2,2-trifluoroethanol - dichloromethane (1 : 1 : 8, by volume). The resulting linear peptide derivatives were dissolved in dimethylformamide (DMF) at a concentration of 1.43 mM and then subjected to cyclization by adding 3/7 volume of DMF containing 3.3 mM PyBOP, 3.3 mM HOBt and 3.3 mM NMM. The reaction was continued for 10 hours at 28°C. After removal of the protecting group by treating with trifluoroacetic acid - water - thioanisole - ethanedithiol - ethyl methyl sulfide - phenol (82 : 5 : 5 : 3 : 2 : 3, by volume), cyclic pentapeptides generated were precipitated with diethyl ether, washed with water and lyophilized.

Determination of the Configuration of Amino Acids

An acid hydrolysate of plactin B was fractionated by HPLC on an Inertsil PREP-ODS column (6 × 250 mm, GL Sciences, Japan) using 0.1% aq. formic acid as a mobile phase. Fractions containing Val, Leu, Tyr and

Arg were analyzed on a CrownPack CR(+) (4 × 150 mm, Daicel Chemical, Japan), which can resolve enantiomers of amino acids. The column was developed with aq. $HClO_4$ under the following conditions: for Leu and Tyr, at pH 2.0 and 25°C; for Arg, at pH 1.5 and 25°C, for Val, at pH 1.5 and 0°C. Identification of the absolute configuration of amino acids was carried out by co-chromatographies with authentic samples.

Cells

The human monocytoid cell line U937 was obtained from the Japanese Cancer Resources Bank. U937 cells bind plasminogen and produce plasminogen activators and their inhibitors, sharing these properties with vascular endothelial cells[9,10]. Cells were grown in RPMI-1640 medium supplemented with 10% of fetal bovine serum, 100 units/ml of penicillin and 100 μg/ml of streptomycin. For assays, cells were seeded at 1.5×10^5 cells/ml in 20 ml of the medium and grown for 2 days. Prior to use in experiments, cells were washed once with buffer A (0.15 M NaCl and 20 mM sodium phosphate, pH 7.4) and then suspended in buffer B (buffer A supplemented with 5 mg/ml calf skin gelatin).

Determination of Cellular Fibrinolytic Activity

^{125}I-Fibrin plate were prepared in wells of a 96-well microplate as described previously[7] using ^{125}I-fibrinogen (~1000 cpm/μg). The ^{125}I-fibrin plates were washed twice with 100 μl of buffer A supplemented with 0.1% Tween 80 and then once with 100 μl of buffer A. The ^{125}I-fibrin plates were then incubated at 37°C for 60 minutes in 200 μl of buffer B. After removing the buffer, the ^{125}I-fibrin plates received 35 μl of human citrated plasma and 35 μl of buffer B containing U937 cells (7.5×10^6/ml) and test samples and incubated at 37°C with occasional mixing. A portion (35 μl) of the incubation mixture was withdrawn after 2 hours to determine radioactivity released from the ^{125}I-fibrin plates.

General Procedures

UV spectra were taken on a 320 spectrometer (Hitachi, Japan). IR spectra were recorded on a Hitachi IR-810 spectrometer. Mass spectra were taken on a JMS-SX102A (JEOL, Japan) and a ZAB-HF (VG, Great Britain) spectrometers. Amino acid composition was analyzed on a model 835 amino acid analyzer (Hitachi). Peptide sequence was determined using 477A and 470A protein sequencers and a 120A PTH analyzer (Applied Biosystems, U.S.A.).

Results

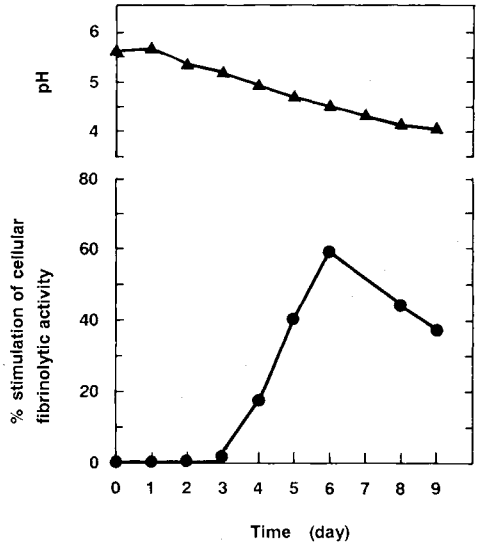
Taxonomy

Strain F165 showed good growth on potato glucose, yeast extract, Czapek-Dox and YpSs agar. Optimum growth was observed at pH 7~9 and ~20°C. Colonies on potato glucose agar attained a diameter of ~90 mm after two weeks at 25°C. They were flat and zonate with a velvety surface, characterized by a basal mycelium that was usually submerged but sometimes raised in central areas. Both the surface and the reverse were white. Hyphae were hyaline and 2~5 μm in width. Mycelium is composed of branched, non septated hyphae with a diameter of 2~5 μm. Most of hyphae bear enlarged amorphous cells at the tip: the cells were separated from the hyphae by septa. In some regions of the colony, aerial hyphae formed pale yellow or orange plectenchymas, which were 10~500 μm in diameter. There was no sclerotium or bulbil in the colony. Under the growth conditions tested, neither asexual nor sexual spores were formed. From these observations, it was concluded that strain F165 belongs to the order Agonomycetales (Mycelia Sterilia) in the class Hyphomycetes.

Fermentation

Strain F165 was cultivated aerobically at 25°C in 500-ml Erlenmeyer flasks containing 500 ml of fermentation medium which consisted of 3.5% glucose, 1% corn starch, 2% soybean meal, 0.5% peptone, 0.5% meat extract, 0.3% yeast extract, 0.2% NaCl, 0.05% KH_2PO_4, 0.05% $MgSO_4$ and 0.01% CB442 (antifoam, Nippon Oil and Fat Co., Japan) (pH 5.8). The flasks were rotated at 180 rpm with a 70-mm throw. A typical time course

Fig. 1. Time course for the production of plactins.
●, percent stimulation; ▲, pH.



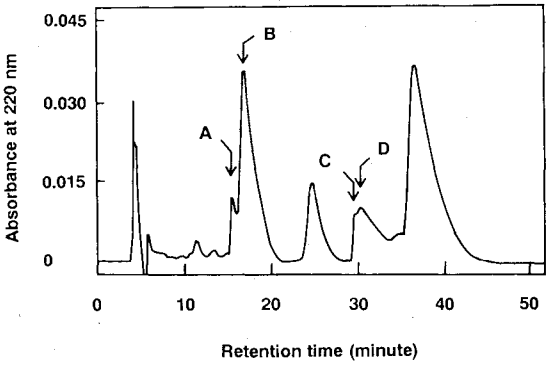
Strain F165 was grown aerobically as described in the text. One milliliter aliquot of the cultured broth was removed everyday and lyophilized. The lyophilized material was extracted with 1 ml of 90% aq. methanol, and 6-μl portions of the organic extracts were assayed in duplicate for the stimulation of cellular fibrinolysis.

of the fermentation is shown in Fig. 1, where the production of plactins was monitored by measuring stimulation of cellular fibrinolytic activity. The stimulating activity was produced after 4 days of cultivation, giving the maximum activity on day 6.

Isolation

Mycelium of strain F165 obtained from 11 liters of a 7-day culture was extracted with acetone (once with 3 liters and twice with 1.5 liters). The acetone extract was concentrated to 2 liters and kept overnight at 4°C. The precipitates formed were successively washed (twice with 1 liter of water and twice with 1 liter of ethyl acetate) and dried *in vacuo*. The resulting brownish powder (~5 g) was dissolved in 80 ml of hot methanol and subjected to preparative HPLC on an Inertsil PREP-ODS column (30 × 250, GL Sciences) in multiple batches. The column was developed at 40°C with acetonitrile-0.1% aq. phosphoric acid (32:68) at a rate of 25 ml/minute. Four active fractions (Fig. 2) were collected and re-chromatographed under the same conditions. Active fractions were neutralized with 1 M NaOH and then acetonitrile in the fractions was removed by evaporation. The resulting aqueous solutions were kept overnight at 4°C and precipitates formed were collected by centrifugation. After washing with water, the precipitates were suspended in water and then lyophilized, giving 13.7, 85.6, 0.2 and 26.6 mg of purified plactins A, B, C and D, respectively.

Fig. 2. HPLC elution profile of plactins.



A portion (22 mg) of partially purified plactins was applied to a silica ODS column as described in the text. Elution was monitored by UV absorption at 220 nm. Positions where plactins eluted are marked by arrows.

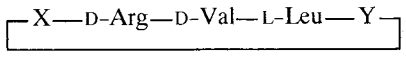
Structure Elucidation

The physico-chemical properties of plactins are summarized in Table 1. Plactins were soluble in dimethylsulfoxide and moderately soluble in methanol but insoluble or slightly soluble in diethyl ether, ethyl acetate, *n*-hexane and water. Both plactins A and B showed UV absorption maxima at 208, 223 and 276 nm, while a maximum at 206 was observed in the spectra of plactins C and D. The presence of intense bands at 1640 and 1540 cm^{-1} in the IR spectra suggested the involvement of peptide bond(s) in the structures of plactins. Upon treatment with 6 M HCl at 110°C for 20 hours, plactins A, B, C and D yielded the following amino acids (approximate molar ratio in parentheses): Arg, Val,

Table 1. Physico-chemical properties of plactins A, B, C and D.

	Plactin A	Plactin B	Plactin C	Plactin D
Appearance	White powder	White powder	White powder	White powder
Molecular formula	$C_{32}H_{52}N_8O_6$	$C_{32}H_{52}N_8O_6$	$C_{32}H_{52}N_8O_5$	$C_{32}H_{52}N_8O_5$
FAB-MS (*m/z*)	645 $(M+H)^+$	645 $(M+H)^+$	629 $(M+H)^+$	629 $(M+H)^+$
HRFAB-MS (*m/z*)				
Found $(M+H)^+$:	645.4114	645.4114	-	629.4175
Calcd:	645.4088 (for $C_{32}H_{53}N_8O_6$)	645.4088 (for $C_{32}H_{53}N_8O_6$)	-	629.4139 (for $C_{32}H_{53}N_8O_5$)
UV λ_{max}^{MeOH} nm (ϵ)	208 (5880), 223 (5050), 276 (1300)	208 (6130), 223 (5150), 276 (1610)	206 (5850)	206 (5870)
IR ν_{max} (KBr) cm^{-1}	2950, 2930, 1650, 1540, 1450, 1380	2960, 2930, 1650, 1540, 1460, 1380	-	3260, 2950, 2930 1640, 1540, 1080
Amino acid composition (molar ratio)	L-Tyr, D-Arg, D-Val, L-Leu, D-*allo*Ile (1:1:1:1:1)	L-Tyr, D-Arg, D-Val, L-Leu, D-Leu (1:1:1:1:1)	L-Phe, D-Arg, D-Val, L-Leu, D-*allo*Ile (1:1:1:1:1)	L-Phe, D-Arg, D-Val, L-Leu, D-Leu (1:1:1:1:1)

Fig. 3. The structures of plactins A, B, C and D.



Plactin	X	Y
A	L-Tyr	D-*allo*Ile
B	L-Tyr	D-Leu
C	L-Phe	D-*allo*Ile
D	L-Phe	D-Leu

Tyr, Leu and *allo*Ile (1:1:1:1:1); Arg, Val, Tyr and Leu (1:1:1:2); Arg, Val, Phe, Leu and *allo*Ile (1:1:1:1:1); Arg, Val, Phe and Leu (1:1:1:2), respectively. Edman degradation of plactins yielded no NH_2-terminal amino acids. From these observations, molecular formulae of plactins were determined as shown in Table 1.

The structure of plactins were determined by sequence analysis of partial acid hydrolysates. Plactins were treated with 12 M HCl at 37°C for 24 hours. The resulting lysates were fractionated by HPLC on a silica ODS column and each fraction was subjected to FAB-MS analysis. Fractions containing the largest fragment were collected and their peptide sequences were determined. Plactins A, B and D yielded the following linear peptides: H–Val–Leu–*allo*Ile–Tyr–Arg–OH (*m/z* 663, $(M+H)^+$) for A; H–Val–Leu–Leu–Tyr–Arg–OH (*m/z* 663, $(M+H)^+$) for B; H–Val–Leu–Leu–Phe–Arg–OH (*m/z* 647, $(M+H)^+$) for D. These results suggested plactins to be cyclic pentapeptides. The absolute configuration of amino acid residues in plactin B was determined chromatographically using a chiral column. The results indicated that the complete acid hydrolysate of plactin B contained D-Val, L-Tyr, D-Arg, L-Leu and D-Leu in an approximate molar ratio of 1:1:1:1:1. To determine the absolute configuration of the tandem leucine residues in plactin B, two cyclic pentapeptides, *cyclo*(–D-Val–L-Leu–D-Leu–L-Tyr–D-Arg–) and *cyclo*(–D-Val–D-Leu–L-Leu–L-Tyr–D-Arg–), were synthesized and compared chromatographically with the natural counterpart. The results showed that plactin B has the former structure. Similarly, structures of plactins A, C and D were identified to be *cyclo*(–D-Val–L-Leu–D-*allo*Ile–L-Tyr–D-Arg–), *cyclo*(–D-Val–L-Leu–D-*allo*Ile–L-Phe–D-Arg–) and *cyclo*(–D-Val–L-Leu–D-Leu–L-Phe–D-Arg–), respectively. These results are summarized in Fig. 3.

Biological Activity

When U937 cells were incubated with ^{125}I-fibrin in a plasma milieu at 37°C for 4 hours, approximately 0.8 μg of ^{125}I-fibrin was degraded. Plactins A, B, C and D potentiated this cellular fibrinolytic activity by 1.5-fold at concentrations of 32, 9.8, 24 and 7.5 μM, respectively. At higher concentrations (30 ~ 50 μM), the stimulation by plactins B and D ranged 3- to 4-fold.

Discussion

In the present study, we have isolated four novel cyclic pentapeptides, designated plactins A, B, C and D, as stimulators of cellular fibrinolytic activity. We had previously identified another activator of fibrinolysis. This compound (complestatin) stimulates the binding of plasminogen to U937 cells and fibrin[7]. The mechanism of plactin stimulation of fibrinolysis seems to be different from that of complestatin, since plactins failed to enhance plasminogen binding (data not shown).

Among four plactins, plactin D was most potent in activity. As compared with plactin D, plactin B, which has D-Tyr in place of D-Phe in plactin D, was slightly less active, and plactins A and C, which contain D-*allo*Ile in place of D-Leu in plactins B and D, respectively, showed approximately 1/3 the activity of the latter two. Thus, it is likely that D-Leu plays a key role in activating fibrinolysis. Results of detailed structure-activity study and the mechanism of action of plactins will be reported elsewhere.

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