

Enhancement of Reciprocal Activation of Prourokinase and Plasminogen by the Bacterial Lipopeptide Surfactins and Iturin Cs

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(Received for publication June 20, 2002)

Urokinase-type plasminogen activator (u-PA) is a serine protease that cleaves Arg⁵⁶¹-Val⁵⁶² bond in plasminogen to convert it to the active serine protease plasmin, which is involved not only in blood clot dissolution but also in a variety of physiological and pathological processes requiring localized proteolysis¹. u-PA is synthesized and secreted as a single-chain zymogen form (pro-u-PA)². Pro-u-PA is proteolytically activated (by cleavage at Lys¹⁵⁸-Ile¹⁵⁹) into a two-chain form (tcu-PA) by plasmin^{2,3}. It is postulated that pro-u-PA has a slight intrinsic proteolytic activity to convert plasminogen to plasmin^{4,5}. The reciprocal activation of pro-u-PA and plasminogen provides a mechanism for initiation and localized propagation of fibrinolysis and matrix proteolysis. We screened microorganisms for their ability to produce low molecular weight compound that enhances the reciprocal activation of pro-u-PA and plasminogen and identified surfactin C as an active compound. Surfactin C enhances the reciprocal reaction by modulating plasminogen conformation. This effect leads to elevation of fibrinolysis both *in vitro* and *in vivo*⁶. In this study, we have identified and characterized several other bacterial lipopeptides (isohalobacillin and iturins C₂ and C₄, as well as four surfactins).

Materials and Methods

Materials

Spectrozyme UK (carbobenzoxy-L- γ -glutamyl (α -*t*-butoxy)-glycyl-arginine-*p*-nitroanilide) was purchased from American Diagnostica (Greenwich, CT, USA) and H-valyl-leucyl-lysine-*p*-nitroanilide (VLK-*p*NA) from Bachem (Bubendorf, Switzerland). Purified human pro-u-PA (with 0.23% intrinsic amidolytic activity toward Spectrozyme UK) was obtained from Welfide Corporation (presently,

Mitsubishi Pharma Corporation, Osaka, Japan), human tcu-PA from JCR Pharmaceuticals (Kobe, Japan) and human plasmin from Sigma (St. Louis, MO, USA). Plasminogen was isolated by lysine-Sepharose affinity chromatography as described⁷. Amidolytic activity of the purified plasminogen toward VLK-*p*NA was below the limit of detection (<0.01% of plasmin). Five surfactin congeners (surfactins A, B₁, B₂ and C, and [Ile⁷]surfactin) were isolated from a culture of *Bacillus* sp. A9184 by solvent extraction and reverse-phase HPLC. The structure was determined by a combination of spectroscopic analyses and chemical degradation. The results were consistent with those of surfactin congeners reported in literatures^{8,9}. Isohalobacillin was isolated from a culture of *Bacillus* sp. A1238 and iturins C₂ and C₄ from *Bacillus* sp. A2822 as described previously^{10,11}. Iturins A₂ and A₄¹² were kindly provided by Higeta Shoyu, Co. (Chiba, Japan).

Reciprocal Activation of Pro-u-PA and Plasminogen

u-PA activity generated from pro-u-PA in incubation with plasminogen was determined as an index of the reciprocal activation. Pro-u-PA (20 nM) and plasminogen (20 nM) was incubated with 0.1 mM Spectrozyme UK (synthetic substrate for u-PA) in 50 μ l of TBS/BSA (50 mM Tris-HCl, 100 mM NaCl and 2 mg/ml bovine serum albumin, pH 7.4) at 37°C for up to 120 minutes. The hydrolysis of Spectrozyme UK was measured continually using a BioRad model 450 microplate reader at 405 nm. Each assay was repeated at least twice in triplicate.

Other Enzyme Assays

The following enzyme activities were determined at 37°C in 50 μ l of TBS/BSA using a 96-well microplate. The release of *p*NA was measured continually for up to 120 minutes at 405 nm. The concentrations of enzyme (and/or zymogen) and chromogenic substrate were as follows: (i) plasminogen activation by tcu-PA, 20 nM plasminogen, 20 nM tcu-PA and 100 μ M VLK-*p*NA; (ii) pro-u-PA activation by plasmin, 20 nM pro-u-PA, 1 nM plasmin and 100 μ M Spectrozyme UK; (iii) amidolytic activities of tcu-PA, 3 nM tcu-PA and 100 μ M Spectrozyme UK; (iv) amidolytic activity of plasmin, 20 nM plasmin and 100 μ M VLK-*p*NA. Each assay was repeated at least twice in triplicate.

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Results and Discussion

As a result of screening microbial cultures for agents that enhance reciprocal activation of pro-u-PA and plasminogen, we identified seven metabolites in addition to surfactin C, which was previously characterized in detail⁶). Of these, five compounds belonged to surfactin family (surfactins A, B₁ and B₂, [Ile⁷]surfactin and isohalobacillin) and two to iturin family (iturins C₂ and C₄) (Fig. 1). When the reciprocal activation was measured as the generation of u-PA activity from pro-u-PA, activity appeared after a 20-minutes lag time and increased rapidly thereafter. The addition of surfactins A, B₁ and B₂, [Ile⁷]surfactin and isohalobacillin at 3~10 μM caused marked enhancement of pro-u-PA activation (Fig. 2, A and B). The dose response curve for each compound was sigmoidal and half maximal effect was observed at 4~7 μM, suggesting an involvement of a cooperative mechanism in the action of these compounds. These effects were very similar to that of

Fig. 1. Structures of surfactins, isohalobacillin and iturins.

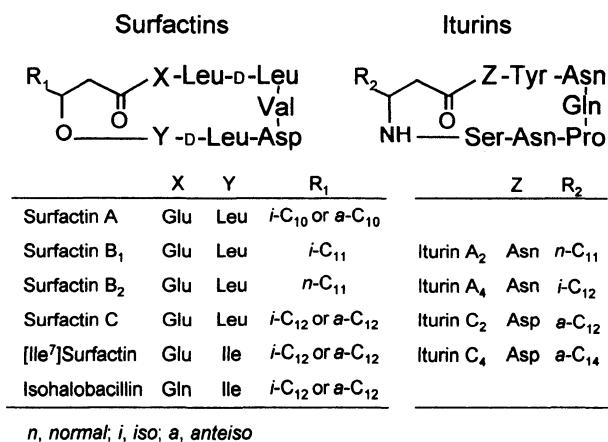
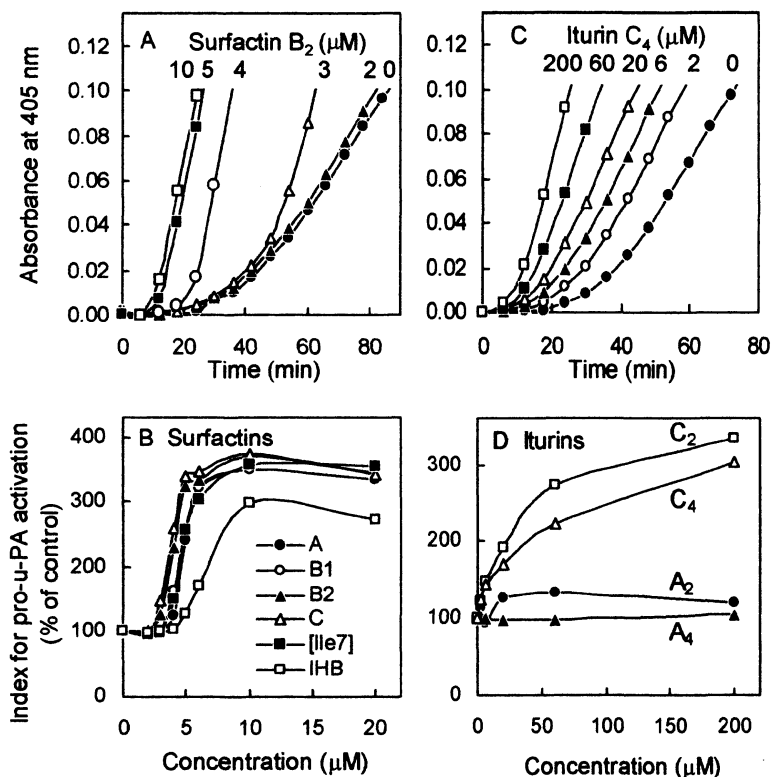
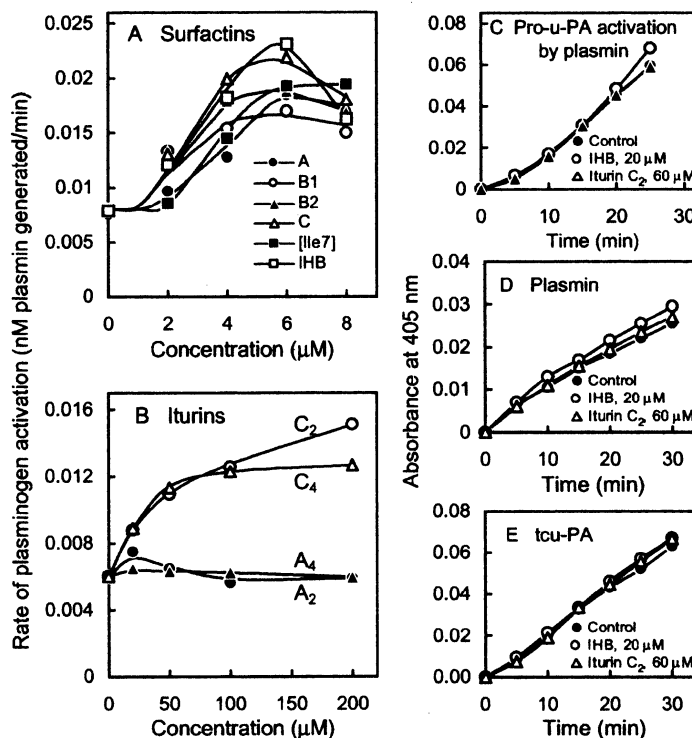


Fig. 2. Effects of various lipopeptides on reciprocal activation of pro-u-PA and plasminogen.



Reciprocal activation of pro-u-PA and plasminogen was determined in the presence of the indicated concentrations of the lipopeptides shown. (A) and (C), time courses for the effects of surfactin B₂ and iturin C₄, respectively. (B) and (D), dose-response curves for surfactins (A, B₁, B₂, C, [Ile⁷] and IHB) and iturins (A₂, A₄, C₂ and C₄), respectively, in which the index for pro-u-PA activation is given in ordinate as percent of control value. The index was calculated by dividing time required for the pNA release to reach 75 mAU in the absence of drug (control) by that in the presence of drug. Each value represents the average of triplicate determinations. IHB, isohalobacillin.

Fig. 3. Effects of various lipopeptides on plasminogen activation by tcu-PA (A and B), pro-u-PA activation by plasmin (C) and activities of plasmin (D) and tcu-PA (E).



Each assay was performed in the presence of the indicated concentrations of lipopeptides shown. Each value represents the average of triplicate determinations. IHB, isohalobacillin.

surfactin C⁶). The enhancement by iturin Cs was observed at as low as 2 μM , while it required 60~200 μM to exert maximal effect (Fig. 2, C and D). In addition, the dose response curves for iturin Cs were not sigmoidal but hyperbolic. These results suggest that, unlike surfactins, iturin Cs do not act cooperatively. Two iturin C congeners, iturins A₂ and A₄, which have Asn¹ in place of Asp¹ in the heptapeptide moiety of iturins C₂ and C₄, were not active at concentrations up to 200 μM (Fig. 2, D).

Surfactin C enhances the reciprocal activation by modulating plasminogen conformation⁶). This effect can be assayed by measuring plasminogen activation, since conformational relaxation renders the plasminogen molecule to be activated by tcu-PA at higher rate. In this plasminogen activation assay, the four surfactins and isohalobacillin caused marked enhancement at concentrations similar to those required for elevating the reciprocal activation (Fig. 3, A). Similarly, iturins C₂ and C₄ were also active in increasing tcu-PA-catalyzed plasminogen activation (Fig. 3, B). These seven agents did

not affect activities of plasmin and tcu-PA as well as plasmin-catalyzed pro-u-PA activation (part of the results are shown in Fig. 3, C~E). These results suggest that the modulation of plasminogen is the common mechanism for the enhancement of the reciprocal activation by these agents, whereas detailed action of iturins C₂ and C₄ may be distinct from that of surfactins, as suggested by the results shown in Fig. 2. Iturins A₂ and A₄ were inactive in enhancing tcu-PA-catalyzed plasminogen activation (Fig. 3, B), consistently with the results obtained with the reciprocal activation assay.

The present results show that two structurally distinct families of bacterial lipopeptides, the surfactins and iturins, enhance reciprocal activation of pro-u-PA and plasminogen. Surfactins A, B₁ and B₂, as well as surfactin C, have in common the Glu-Leu-D-Leu-Val-Asp-D-Leu-Leu heptapeptide, which is connected with a β -hydroxy fatty acid with variable carbon number (C₁₃~C₁₅) to construct a cyclodepsipeptide structure. The difference in β -hydroxy fatty acyl chain within such range causes no significant

change in potency. The substitution of Leu⁷ by Ile does not affect activity (surfactin C vs. [Ile⁷]surfactin), while the substitution of Glu¹ by Gln slightly lowers activity ([Ile⁷]surfactin vs. isohalobacillin). The latter observation suggests a partial importance of Glu¹ for activity. Iturins belong to another family of lipopeptides. Iturin Cs have Asp-Tyr-Asn-Gln-Pro-Asn-Ser in the heptapeptide moiety, which is connected with an β -amino fatty acid (C₁₅~C₁₇) to form a cyclopeptide structure. Again the difference in chain length in β -amino fatty acyl moiety does not affect activity (iturin C₂ vs. C₄), while the substitution of Asp¹ by Asn abolishes activity (iturin Cs vs. iturin As). This observation demonstrates a crucial role of Asp¹ in activity of iturins. As mentioned above, the dose-response curves for iturin Cs are hyperbolic whereas those of surfactins are sigmoidal. The major difference between the two families is at the heptapeptide moiety: iturin Cs contain one acidic residue and five uncharged polar residues, while surfactins have two acidic residues and five bulky aliphatic residues. The NMR and molecular modeling studies have revealed that surfactin exhibits a compact "horse saddle" topology in which the acidic side chains of Glu¹ and Asp⁵ form a minor polar domain¹³). On the other hand, polar groups in iturin Cs distribute evenly in the heptapeptide moiety. This difference in chemical nature and/or conformation of the heptapeptide may account for the apparently distinct effects. Although lipopeptides have surface-activating potential, agents with such activity in general do not enhance reciprocal activation of pro-u-PA and plasminogen. The ratio of effective dose to critical micelle concentration in active agents are at 1:1 to 5:1, while iturin A₄ is inactive even at a ratio of 8:1. In addition, we have previously shown that some nonionic and anionic detergents such as Tween 80, Triton X-100 and sodium deoxycholate are rather inhibitory⁶).

Acknowledgments

We are grateful to Drs. AKIRA ENDO and NOBUHIRO TAKAHASHI for encouragement and Welfide Corporation for pro-u-PA. This work was supported in part by a grant from the Takano Life Science Research Foundation.

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