Inhibition of *Streptomyces chromofuscus* Phospholipase D by Antifungal Lipopeptides from *Bacillus subtilis*

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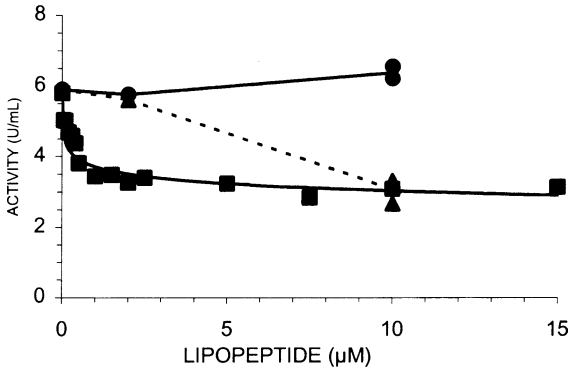
Plipastatins (PL), as bacillomycins L (BL) and surfactins (SF), are lipopeptide antibiotics produced by *Bacillus subtilis*. All these cyclic antibiotics contain D and L-series amino acids[1]. SF and BL are heptapeptides linked to a β-hydroxy (for SF) or β-amino (for BL) fatty acid. PL are decapeptides with a lactone linkage between the carboxyl group of the *C*-terminal residue and the phenyl hydroxyl of Tyr_3. PL were isolated from *Bacillus cereus* as an inhibitor of phospholipase A2, but they also inhibit phospholipase D from cabbage[2]. The effects of SF or BL on phospholipases D (PLD) have not been yet studied. It was only shown that SF inhibits cytosolic phospholipases A2[3]. In this study, on the contrary of the previous studies on phospholipase inhibitors[2,3], we used a soluble substrate of phospholipase D from *S. chromofuscus* (PLDsc). In these conditions, only the PLDsc inhibition by the lipopeptides would be studied and possible interactions between the lipopeptides and the phospholipidic substrate (*i.e.* liposomes) would be excluded. More precisely, we investigated the effects of PL, BL and SF on PLDsc by measuring its activity by using a rapid method involving bis(*para*-nitrophenyl)phosphate [bis(*p*NP)P][4]. PLDsc was purchased from Sigma Chemical Co. (St. Louis, MO) and lipopeptides were prepared from *B. subtilis* cultures as described in References[1,5,6].

The influence of SF, BL and PL on PLDsc activity was tested at 10 μM. PLDsc inhibition was observed only with PL and BL, giving rise in both cases 50% inhibition at ~10 μM. On the contrary of BL, significant inhibition was obtained with PL at very low concentrations (~30% at 500 nM) but it was impossible to obtain a 100% inhibition (Fig. 1). It is interesting to compare this PL concentration with the PLDsc concentration in the assay (about 170 nM); this means that the inhibition of PLDsc began when the PL concentration reached the enzyme concentration. Besides, PLDsc requires the presence of calcium for its catalytic process[4,7]. Since PL and BL contain Glu residues able to complex divalent ions, the influence of Ca^{++} on PLD inhibitions was tested. When Ca^{++} concentration varied from 60 μM to 1 mM, PLDsc activity varied from 5.9 to 7.2 U/ml without lipopeptide, from 3 to 5.4 U/ml with 10 μM BL, corresponding to an inhibition decrease (from 49 to 25%), and from 3.5 to 3.8 U/ml with 4 μM PL, indicating no inhibition decrease (from 41 to 46%). The inhibition kinetic of PLDsc by PL was then studied. Fig. 2A shows that the inhibition is uncompetitive and Fig. 2B gives a *Ki* of ~3.9 μM, indicating a greater affinity of PLDsc for PL than for bis(*p*NP)P (*Km* ~0.6 mM)[4]. This means that inhibition occurs through reversible association of PL with the PLD-bis(*p*NP)P complex.

In conclusion, we identified a new inhibitor of PLDsc (BL) and showed that PLDsc inhibition by BL could be due to the complexation of calcium by its Glu residue[6], while the inhibition by PL was not. PL is the first identified inhibitor of PLDsc for which the effect does not depend on calcium.

Fig. 1. Influence of the lipopeptide concentrations on PLDsc activity.



Enzyme activity was measured at 37°C in a 10 mM. Tris HCl buffer, pH 8.0, containing 150 mM NaCl, 60 μM $CaCl_2$ and 2.5 mM bis(*p*NP)P. Substrate hydrolysis which gives para-nitrophenol absorbing at 420 nm was measured spectrophometrically. Plipastatin (squares), bacillomycin L (triangles) and surfactin (circles).

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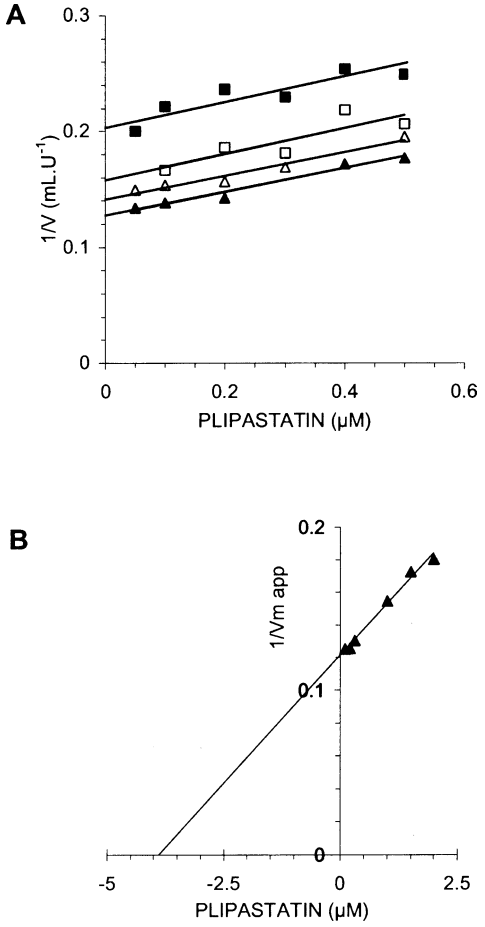
Fig. 2. Dixon (A) and secondary (B) plots of the inhibitions of PLDsc by plipastatin.



Bis(*p*NP)P concentrations were 0.5 mM (full squares), 1 mM (open squares), 2 mM (open triangles) and 2.5 mM (full triangles).

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