Note

Staurosporine, a Prolyl Endopeptidase Inhibitor

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Staurosporine was first isolated from *Streptomyces staurosporeus* as an antifungal alkaloid antibiotic[1] and was reevaluated as a potent inhibitor of protein kinase C[2] (Fig. 1). Staurosporine induced neurite outgrowth in the rat pheochromocytoma cell line, PC12h,[3,4] and had an anti-amnesic effect on Alzheimer's rats.[5] In PC12h cells staurosporine blocked NGF-induced neurite outgrowth at low doses, but at higher doses PC12h cells responded to staurosporine by rapid generation of neurites.[4] The mechanisms of neurite outgrowth prompted by staurosporine might be different from that of NGF, dB-cAMP, or infection with a retrovirus carrying the v-src oncogene.[4] It was also reported that protease inhibitors such as Ac-Leu-Leu-Nle-al also induced neurite outgrowth in the presence of NGF and endogenous protease of a new type is involved in restricting neurite outgrowth in PC12h cells.[6]

On the other hand, one of the serine proteases, prolyl endopeptidase (post-proline cleaving enzyme) [EC 3.4.21.26] was first discovered in human uterus as an oxytocin-degrading enzyme.[7] This enzyme has been purified from various animals, plants, and microorganisms.[8-10] This is an active neuropeptide-metabolizing enzyme that cleaves peptide bonds at the carboxyl side of proline residues. Vasopressin, one of these biologically active peptides susceptible to the enzyme, has been suggested to be concerned with learning and memory processes.[11] So specific inhibitors for prolyl endopeptidase are expected to have anti-amnesic effects, and many inhibitors were synthesized as anti-amnesic drugs.[12,13] Very recently, it was shown that the activity of prolyl endopeptidase increased in patients with Alzheimer's disease.[14]

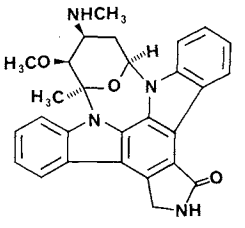

Fig. 1. Chemical Structure of Staurosporine.

In this experiment, we examined whether staurosporine would inhibit the activity of prolyl endopeptidase or not. Staurosporine is a natural product inhibiting prolyl endopeptidase and has an indolocarbazole chromophore unlike the synthesized inhibitors.

Staurosporine was purchased from Kyowa Medex Co., Ltd., and dissolved in methanol. Prolyl endopeptidase from

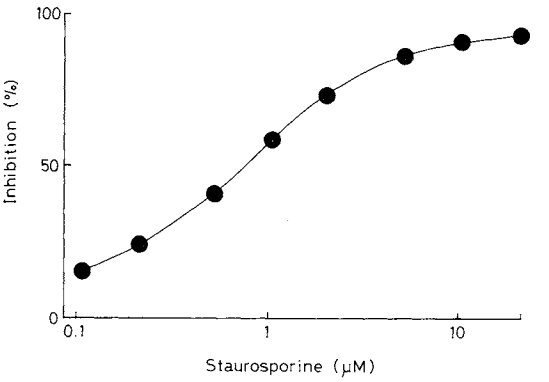

Fig. 2. Effects of Staurosporine on the Prolyl Endopeptidase Reaction.

The inhibitor was incubated for 5 min at 30°C with the enzyme and the reaction was started by addition of Z-Gly-Pro-*p*NA as described.

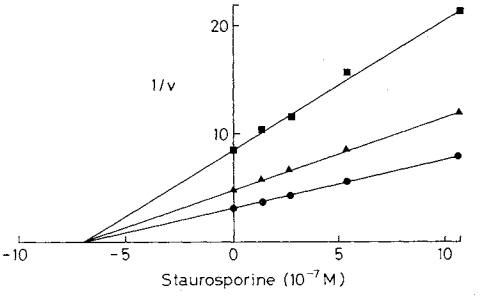

Fig. 3. Dixon Plots of the Inhibition of Prolyl Endopeptidase by Staurosporine.

The inhibitor was incubated for 5 min at 30°C with the enzyme and the reaction was started by addition of Z-Gly-Pro-*p*NA as described. $1/v$ was defined as $1/\Delta$ 410 nm.

—■—, Z-Gly-Pro-*p*NA 0.05 mM; —▲—, 0.1 mM; —●—, 0.2 mM.

Abbreviations. NGF, nerve growth factor; dB-cAMP, N^6,2′-O-dibutyryl adenosine 3′:5′-cyclic monophosphate; Ac-Leu-Leu-Nle-al, acetyl-leucyl-leucyl-norleucinal; Z, benzyloxycarbonyl; *p*NA, *p*-nitroanilide.

Flavobacterium and substrate (Z-Gly-Pro-*p*NA) were purchased from Seikagaku Kogyo Co., Ltd. Z-Pro-prolinol, one of the synthetic inhibitors, was synthesized by the method of Wilk and Orlowski[15] and dissolved in methanol. The enzyme activity was assayed by the method of Yoshimoto *et al.*,[16] in which 0.1 M Tris–HCl, pH 7.0 (0.84 ml), prolyl endopeptidase (0.05 ml of 0.1 unit/ml) and test samples in methanol (0.01 ml) were mixed and incubated at 30°C for 5 min, then the reaction was started by adding 0.1 ml of 2 mM Z-Gly-Pro-*p*NA (in 40% 1,4-dioxane). After incubation at 30°C for 30 min, 0.5 ml of stop solution (10 g Triton X-100/95 ml 1 M acetate buffer, pH 4.0) was added and the absorbance was measured at 410 nm.

IC_{50} values were measured by this method with various concentrations of test compounds. The percentage of inhibition can be calculated from the absorbance with (A) and without (B) inhibitor, by the following equation:

$$\text{Percentage of inhibition} = [(B-A)]/B \times 100$$

Staurosporine inhibited prolyl endopeptidase in a dose-dependent manner and IC_{50} was 0.77 μM as shown in Fig. 2. Under the same conditions, Z-Pro-Prolinol inhibited prolyl endopeptidase by 50% at 0.57 mM.

Many synthetic inhibitors of prolyl endopeptidase such as Z-Pro-Ala, Z-Ala-Pro, Z-Pro-Pro, and Z-Pro-Prolinol containing proline residues appear to be competitive inhibitors.[15] On the other hand, one of the potent synthetic inhibitors, Z-Pro-Prolinal appears to have a noncompetitive inhibition.[15] A kinetic study of staurosporine was done and Dixon plots[17] for prolyl endopeptidase inhibition are shown in Fig. 3. Staurosporine was a noncompetitive inhibitor of prolyl endopeptidase with the inhibition constant (*Ki*) of 0.70 μM.

We have not examined the prolyl endopeptidase activity in PC12h cells and in Alzheimer's rats yet. But the most tempting explanation is that the effectiveness of staurosporine in Alzheimer's rats is due to the inhibition of prolyl endopeptidase. The relationship between prolyl endopeptidase inhibition and the induction of neurite outgrowth is now being investigated.

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