Enhancement of catalytic activities of serine proteases by tripeptides compounds

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Abstract The tripeptide compounds, Glu-Arg-Pro-amide (ERPm), D-Pro-Thr-Trp-amide (dPTWm) and thioproline-Thr-Trp (tPTW), were obtained by screening of synthetic peptides for growth-inhibitory activity toward cultured transformed cells. The effects of these peptide compounds on proteases were investigated and the results showed that these compounds enhanced the amidolytic activity of serine proteases despite the fact that each reaction was carried out under optimal conditions. ERPm stimulated the activities of trypsin, chymotrypsin, thrombin, plasmin urokinase and elastase. dPTWm also showed similar effects except that toward chymotrypsin. tPTW elevated the activity only of trypsin, chymotrypsin and thrombin. Stimulation of trypsin activity by these compounds was also confirmed by using casein as a substrate. None of these compounds affected the amidolytic activities of metalloproteinases (MMP-1 and MMP-9), cysteine proteinases (m- and μ-calpains, cathepsin B and papain) or an exopeptidase (leucine aminopeptidase). The activation was at least partly due to the stabilization of the catalytic activity of proteases as well as prevention of autolysis.

Key words: Serine protease; Protease-stimulatory tripeptide; Synthetic tripeptide

1. Introduction

We have previously reported the results of screening of bioactive peptides for growth-inhibitory activity toward transformed murine cells [1]. Glu-Arg-Pro-amide (ERPm), D-Pro-Thr-Trp-amide (dPTWm) and thioproline-Thr-Trp (tPTW) significantly inhibited the proliferation of v-*mos*- and v-*raf*-transformed rat fibroblasts although the action mechanism remains to be proved. Because some peptide compounds inhibit proteases [2,3], the effects of those tripeptides on proteases have been investigated. Unexpectedly, the results showed that the serine protease activity is enhanced by those peptides.

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Abbreviations: IC_{50}, half-maximal inhibition concentration; LAPase, leucine aminopeptidase; PBS, phosphate-buffered saline; DMSO, dimethylsulfoxide; PST, protease-stimulatory tripeptide; tPTW, thioproline-Thr-Trp; dPTWm, D-Pro-Thr-Trp-amide; ERPm, Glu-Arg-Pro-amide; Boc-, *t*-butyloxycarbonyl-; Suc-, succinyl-; MCA, 4-methylcoumaryl-7-amide; Pyr-, pyroglutamyl-; Z-, carbobenzoxy-; sP, sulfonated proline; tP, thioproline; aK, acetyllysine; dP, D-proline; dT, D-threonine; dE, D-glutamic acid; dR, D-arginine

2. Materials and methods

2.1. Materials

Bovine pancreatic trypsin and chymotrypsin, bovine plasma thrombin, bovine plasma plasmin, porcine pancreastic elastase, human kidney urokinase, bovine spleen cathepsin B, papain, and porcine kidney leucine aminopeptidase (LAPase) were purchased from Sigma Chemical Co. (St. Louis, MO). Porcine erythrocyte m- and μ-calpains were obtained from Nakarai Chemicals (Kyoto, Japan). The substrates of proteases were obtained from Peptide Institute Inc. (Osaka, Japan).

Peptide compounds were synthesized by conventional liquid phase fragment condensation and purified as described previously [1]. The purity of each peptide was analyzed by thin-layer or high-performance liquid chromatography. NMR spectroscopy, elemental analysis and specific rotation. All peptides were dissolved in dimethylsulfoxide (DMSO) at a concentration of 20 mg/ml.

2.2. Assay for protease activity

Amidolytic activities of serine and cysteine proteases were measured by incubation at 37°C for 10 min in the following reaction mixture (0.1 ml); trypsin (100 ng), 1×PBS, 1 mM EDTA and 10 μM Boc-Phe-Ser-Arg-MCA; chymotrypsin (25 ng), 1×PBS and 10 μM Suc-Leu-Leu-Val-Tyr-MCA; thrombin (1.1 ng), 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2.5 mM $CaCl_2$ and 10 μM Boc-Val-Pro-Arg-MCA; plasmin (20 μg), 1×PBS and 10 μM Boc-Val-Leu-Lys-MCA; elastase (15 μg), 50 mM Tris-HCl (pH 8.8), 1 mM EDTA and 10 μM Suc-Ala-Pro-Ala-MCA; urokinase (1 munit), 1×PBS, 1 mM EDTA and 10 μM Pyr-Gly-Arg-MCA; cathepsin B (10 μunits), 50 mM MES (pH 6.0), 2 mM EDTA, 2 mM DTT and 10 μM Z-Arg-Arg-MCA; papain (2.5 ng), 50 mM MES (pH 5.5), 2 mM EDTA, 2 mM DTT and 10 μM Z-Phe-Arg-MCA; LAPase (100 μunits), 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$ and 10 μM Leu-MCA. Each reaction was terminated by the addition of an equal volume of 100 mM sodium chloroacetate, 70 mM acetic acid and 30 mM sodium acetate [4,5]. Released 7-amino-4-methylcoumarin was measured by fluorescence spectroscopy with excitation and emission wavelengths of 345 and 440 nm, respectively.

Proteolytic activity of trypsin was determined in a mixture (200 μl) containing 1.5 μg of trypsin, 5 mg/ml casein, 1×PBS and 1 mM EDTA in the presence or absence of 200 μg/ml tripeptide compounds and incubated at 37°C for 20 min. The reaction was terminated by addition of 160 μl of 10% (w/v) ice-cold trichloroacetate. After centrifugation at 15000 rpm for 10 min, the absorbance at 280 nm of the supernatant was measured.

The activities of m- and μ-calpains were assayed by incubation at 37°C for 30 min in 50 mM Tris-HCl (pH 7.5), 10 mM 2-mercaptoethanol and 10 μM Suc-Leu-Val-Tyr-MCA. Activities of metalloproteinases, MMP-1.1 and -9, which had been purified from human epidermis and a human sarcoma cell line, respectively, were measured by using the assay kits (Yagai Corp., Yamagata, Japan) employing fluoresceine-labeled type I and IV collagen, respectively.

2.3. Assay for autolysis of chymotrypsin and trypsin

Chymotrypsin (2 μg) and trypsin (5 μg) were incubated at 37°C for 1 h in a mixture (20 μl) containing 1×PBS and 1 mM EDTA in the presence or absence of 1 mg/ml tPTW, ERPm or dPTWm. The reaction was terminated by addition of SDS-sample buffer followed by boiling at 100°C for 3 min. The samples were directly applied to a SDS-polyacrylamide (10%) gel.

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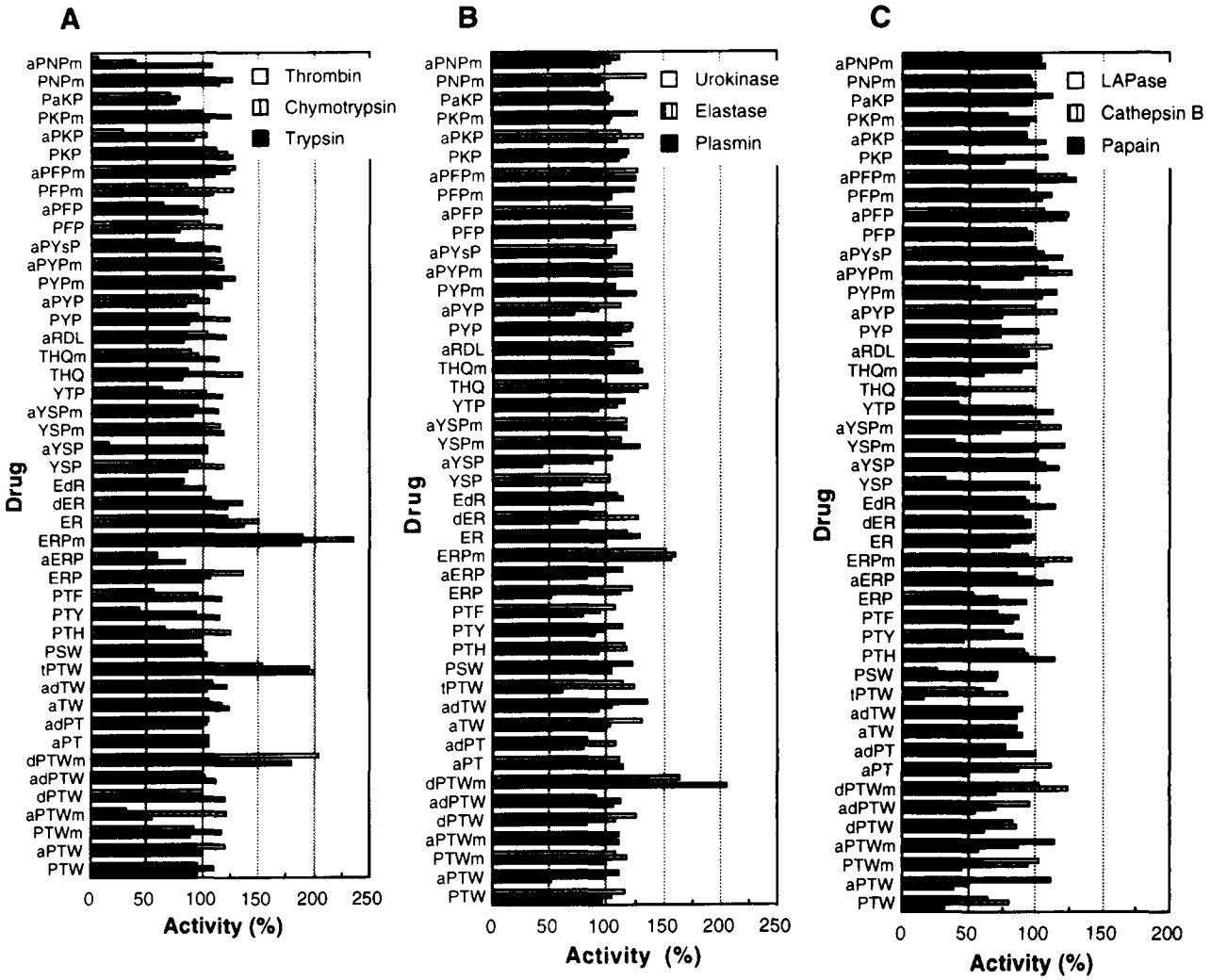

Fig. 1. Relative protease activities in the presence of synthetic peptides. Amidolytic activities of thrombin, chymotrypsin and trypsin (A), urokinase, elastase and plasmin (B) and LAPase, cathepsin B and papain were measured in the presence of synthetic peptides (200 μg/ml). The activity shown is the percentage of that in the absence of peptides.

3. Results

Fig. 1 shows the activities of various proteases in the presence of synthetic tripeptides. These peptides showed more or less growth-inhibitory activities toward transformed mouse fibroblasts and, among them, ERPm and tPTW were most inhibitory toward proliferation of SV40- and v-*mos*-transformed cells, respectively [1]. Fig. 1A shows the activities of serine proteases including trypsin, chymotrypsin and thrombin. The concentration of each peptide was 200 μg/ml, which might non-specifically affect the proteolytic activity. Some peptides could inhibit proteases by competing with the substrates. Thus, the activities between 50 and 150% of control might be insignificant. In contrast to our expectations, some peptides did not inhibit but rather enhanced the amidolytic activity. Activities of trypsin and thrombin were enhanced by ERPm, dPTWm and tPTW. Chymotrypsin activity was stimulated by ERPm and tPTW but not by dPTWm. None of the other peptide compounds showed such stimulation.

The activities of other serine proteases including urokinase, elastase and plasmin were then investigated and also stimulated by ERPm and dPTWm but not by tPTW (Fig. 1B). The activities of cysteine proteinases such as papain and cathepsin B and LAPase were not significantly altered by any peptide (Fig. 1C). However, tPTW showed some inhibitory activity toward papain. No significant effect of these peptides was observed on μ- and m-calpains and metalloproteinases such as MMP-1 and MMP-9 (data not shown).

Significant increase in urokinase activity was observed in the presence of ERPm at a concentration higher than 20 μg/ml (Fig. 2A). However, dPTWm and tPTW stimulated the amidolytic activities of urokinase and chymotrypsin, respectively, at higher than 200 μg/ml (Fig. 2A,C). It was of interest that the maximum levels of enhanced activities were almost the same in the presence of three different peptide compounds. The time course of the catalytic activities of urokinase and chymotrypsin in the presence of the peptides was also investigated (Fig. 2B,C). The control activity was gradually inactivated and reached saturation during incubation whereas the activity in the presence of ERPm, dPTWm or tPTW continued to hydrolyze the substrate for a longer period. Stimula-

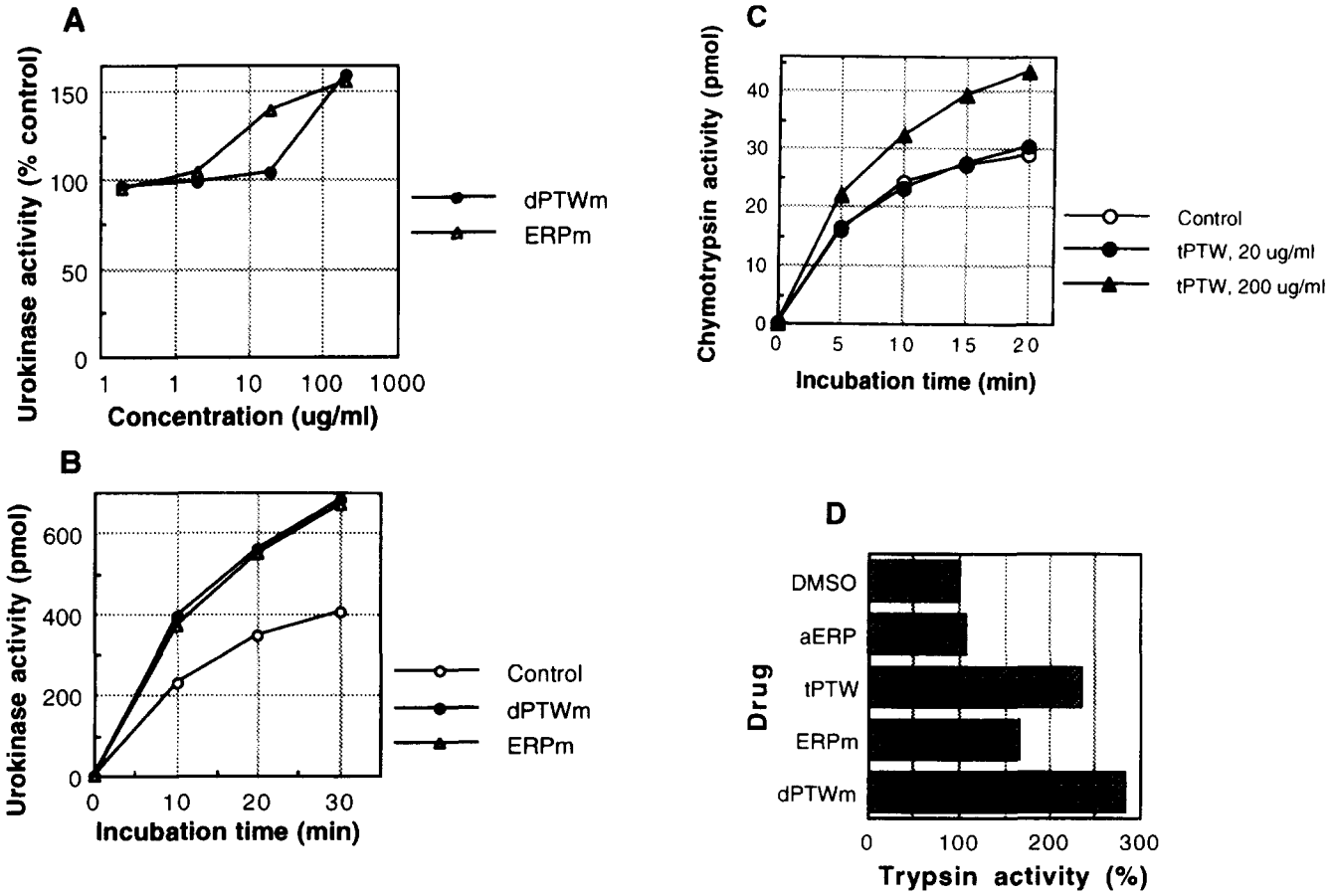

Fig. 2. Stimulatory effects of tPTW, ERPm and dPTWm on serine proteases. (A) The activity of urokinase was investigated after incubation at 37°C for 10 min in the presence of ERPm and dPTWm. Abscissa and ordinate represent the concentrations of ERPm and dPTWm and urokinase activity, respectively. (B) The urokinase activity was also measured during the incubation time in the presence or absence of 200 μg/ml ERPm or dPTWm. The ordinate represents total MCA produced by urokinase at each time point. (C) Chymotrypsin activity was measured likewise in the presence of 20 or 200 μg/ml tPTW. Control activities were measured in the presence of the solvent alone (DMSO, 1%). (D) Proteolytic activity of trypsin was measured in the presence of 200 μg/ml of aERP, tPTW, ERPm or dPTWm using casein as a substrate.

tion of other serine proteases by these compounds was similar to that shown in Fig. 2 (data not shown).

In order to confirm that the enhancement is not limited to amidolytic activity, the proteolytic activity of trypsin toward casein was also investigated. Proteolysis of casein was also stimulated by tPTW, ERPm and dPTWm although the extents of the stimulation by these compounds were not completely correlated to those of amidolytic activities (Fig. 2D). ERPm showed less stimulatory activity as compared to those of tPTW and dPTWm. As a negative control, aERP was without effect.

We then examined the effects of these compounds on the autolysis of serine proteases. Chymotrypsin and trypsin were incubated at 37°C for 1 h at high concentrations (100 and 250 μg/ml, respectively) in the presence or absence of the tripeptide compounds. Fig. 3 shows the results of SDS-polyacrylamide gel electrophoresis of the proteases with or without incubation. During incubation, both enzymes were rapidly degraded by autolysis. However, in the presence of tPTW or ERPm, the autolytic degradation of chymotrypsin was substantially suppressed. dPTW was ineffective in this suppression (Fig. 3, lane 6). The autolysis of trypsin was inhibited by tPTW, ERPm and dPTWm yet ERPm was less effective (Fig. 3, lanes 10–12). As a control, aERP was completely inactive in the suppression of autolysis of both proteases.

4. Discussion

Many protease inhibitors have been reported thus far including cystatins, serpins and TIMPs [6-8] yet little is known about stimulatory factors of proteolytic activities. Chen et al. previously reported that the amidolytic activity of a cysteine proteinase (gingipain) of *Porphyromonas gingivalis* was enhanced by glycine-containing dipeptides [9]. We have observed that tPTW, dPTWm and ERPm stimulated the amidolytic activities of several serine proteases. These compounds are hereafter referred to as protease-stimulatory tripeptides (PSTs).

Although we cannot rule out the possibility that the stimulation of proteolytic activities was due to minor contaminants in the peptide preparations, the direct effects of PSTs on proteases were suggested by the following observations. (i) Enhancement of protease activities was observed in only three compounds examined so far, two of which possess the same amino acid sequence, Pro-Thr-Trp. (ii) PSTs showed stimulatory effects only on serine proteases. (iii) Relative stimulation

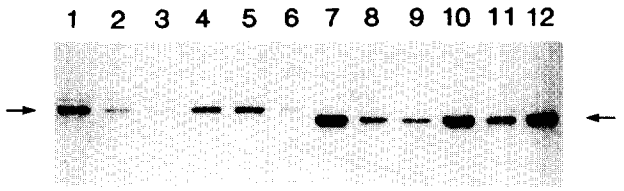

Fig. 3. Effects of tripeptide compounds on autolysis of chymotrypsin and trypsin. Chymotrypsin (lanes 1–6) and trypsin (lanes 7–12) were incubated at 0°C (lanes 1,7) or 37°C (lanes 2–6, 8–12) for 1 h in the presence of DMSO (5%) (lanes 1,2,7,8), or aERP (lanes 3,9), tPTW (lanes 4,10), ERPm (lanes 5,11) or dPTWm (lanes 6,12) (1 mg/ml). The Coomassie blue-staining profile of SDS-polyacrylamide gel electrophoresis is shown. Arrows at the left and the right indicate the positions of chymotrypsin and trypsin, respectively.

was different among PSTs; i.e. ERPm increased the amidolytic activities of all 6 serine proteases examined. dPTWm did not enhance chymotrypsin activity and tPTW stimulated only trypsin, chymotrypsin and thrombin. (iv) Each PST alone did not affect the emission of fluorescence of 7-amino-4-methylcoumarin (data not shown). (v) Stimulation of trypsin activity by ERPm, dPTWm and tPTW was similar when the substrate, Boc-Phe-Ser-Arg-MCA, was substituted by casein or by other synthetic peptide substrates such as Boc-Leu-Thr-Arg-MCA, Boc-Leu-Gly-Arg-MCA, Boc-Val-Leu-Lys-MCA and Boc-Gln-Lys-Lys-MCA (Fig. 2D, data not shown). It is thus unlikely that PSTs directly interact with the substrates. This was consistent with the results of the Lineweaver-Burk plot which showed that the major change induced by PSTs involved the V_{max} but not the K_m value for the substrate (data not shown).

The results of the time course study suggested that the stimulation of amidolytic activities of serine proteases by PSTs is at least partly due to stabilization of proteases (Fig. 2B,C). Consistently, PSTs suppressed the autolysis of trypsin and chymotrypsin (Fig. 3). Thus one possible explanation is that PSTs protect the autolytic cleavage sites of proteases.

Although PSTs were obtained by screening of cell-growth-inhibitory peptides [1], it remains obscure whether the enhancement of serine protease activities is obligatory for the inhibition of proliferation. It is noteworthy that endothelial cell growth factors-2a and -2b isolated from human hepatoma cells were identical with human pancreatic secretory trypsin inhibitor and urinary glycoprotein proteinase inhibitor, respectively [10]. Other inhibitors of metalloproteinases and cysteine proteinases have also been reported to simulate cell growth [11-14]. Thus, suppression of protease activities might be related to an elevated level of cell growth. Accordingly, it is not incompatible that stimulation of serine proteases results in decrease in cell growth.

There might be still many specific stimulators not only for proteases but also other enzymes. These enzyme stimulators could be promising candidates for use as therapeutic agents for some enzyme-defect diseases.

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