鹿児島大学リポジトリ
Kagoshima University
Repository

Screening Test of Cucumisin Inhibitor (II)

著者	KAMADA Yoshiko, UCHIKOBA Tetsuya, YONEZAWA Hiroo, KANEDA Makoto
journal or publication title	鹿児島大学理学部紀要. 数学・物理学・化学
volume	25
page range	65-69
別言語のタイトル	ククミシンインヒビターの検索(II)
URL	http://hdl.handle.net/10232/6503

Screening Test of Cucumisin Inhibitor (II)

著者	KAMADA Yoshiko, UCHIKOBA Tetsuya, YONEZAWA Hiroo, KANEDA Makoto
journal or publication title	鹿児島大学理学部紀要. 数学・物理学・化学
volume	25
page range	65-69
別言語のタイトル	ククミシンインヒビターの検索(II)
URL	http://hdl.handle.net/10232/00000500

Rep. Fac. Sci. Kagoshima Univ., (Math., Phys. & Chem.),
No. 25, 65-69, 1992

Screening Test of Cucumisin Inhibitor (II)

Yoshiko KAMADA*, Tetsuya UCHIKOBA, Hiroo YONEZAWA
and Makoto KANEDA

Abstract

The activity of cucumisin was not affected by typical trypsin inhibitors such as TLCK, leupeptin, α_1-antitrypsin, ovomucoid, and STI. Pepstatin A of pepsin inhibitor could not inhibit also. The rate of autolysis of cucumisin decreased in the presence of leupeptin. α_2-Macroglobulin showed 45% inhibition for the original activity of cucumisin.

Key words: Cucumisin, Cucumisin inhibitor, Protease inhibitor

Introduction

Several recent reports from our laboratory have been concerned with the isolation and properties of proteases from Cucurbitaceous fruits[1-14]. These proteases are strongly inhibited by diisopropyl fluorophosphate (DFP) and phenylmethanesulfonyl fluoride (PMSF) but are unaffected by reducing compounds such as cysteine and 2-mercapto-ethanol. Therefore, these enzymes seem to be a serine protease[1]. The properties of these enzymes are homologus with each other. Among them, the properties of cucumisin [EC 3.4.21.25], isolated from the sarcocarp of prince melon has been investigated in more detail.

The inhibitors of these Cucurbitaceous proteases are still not found, except DFP and PMSF[15]. In this communication we describe the effects of the typical protease inhibitors against cucumisin.

Experimental procedures

Materials-The commercial products used were bovine pancreatic trypsin and α-chymotrypsin (Worthington), ovomucoid (Worthington), soybean trypsin inhibitor (STI, Worthington), α_1-antitrypsin (Boehringer Mannheim GmbH), α_2-macroglobulin (Boehringer Mannheim GmbH), N-p-tosyl-L-lysine chloromethyl ketone (TLCK, Sigma), pepsin (Nakarai Chemicals), casein (E. Merck), pepstatin A (Peptide Institute) and leupeptin (Peptide Institute). All other reagents were the highest grade available. Extract of seedlings was prepared as follows. Prince melon seeds was germinated for six days.

*Present address: Faculty of Dentistry, Kagoshima University, Kagoshima 890, Japan
Department of Chemistry, Faculty of Science, Kagoshima University Kagoshima 890, Japan

The whole body of seedlings, typically 31.6g, was homogenized in a mortar with 7ml of 1/15 M phosphate buffer, pH 7.3. The homogenate was centrifuged at 2,000g for 10 min and the supernatant was filtered through Toyo filter paper No. 5A. This filtrate was used for inhibitory test of cucumisin.

Estimation of the Inhibitor Effects of Various Inhibitor on the Caseinolytic Activity of Cucumisin

Test Solution-Cucumisin (0.03 or 0.15 mg) was dissolved in 0.5 ml of 1/15 M phosphate buffer, pH 7.3, and then mixed with 0.5 ml of inhibitor solution in the same buffer. The mixture was incubated at 30℃ for 20 min. After the preincubation, 1.0 ml of 2% casein solution in 1/15 M phosphate buffer, pH 7.3 was added. After incubation for 20 min the reaction was stopped by the addition of 3.0 ml of 5% trichloroacetic acid. After standing for 30 min at room temperature, the resulting precipitate was removed by filtration through Toyo filter paper No. 5C and the absorbancy at 280 nm of the trichloroacetic acid-soluble peptides formed was determined with Hitachi 100-60 spectrophotometer.

Control-Volumes of 0.5 ml of inhibitor solutions were mixed with 0.5 ml of cucumisin solution. After incubation for 20 min, 3.0 ml of 5% trichloroacetic acid solution was added to the mixture, and then 1.0 ml of 2% casein solution was quickly added, which was the stood for 30 min at room temperature.

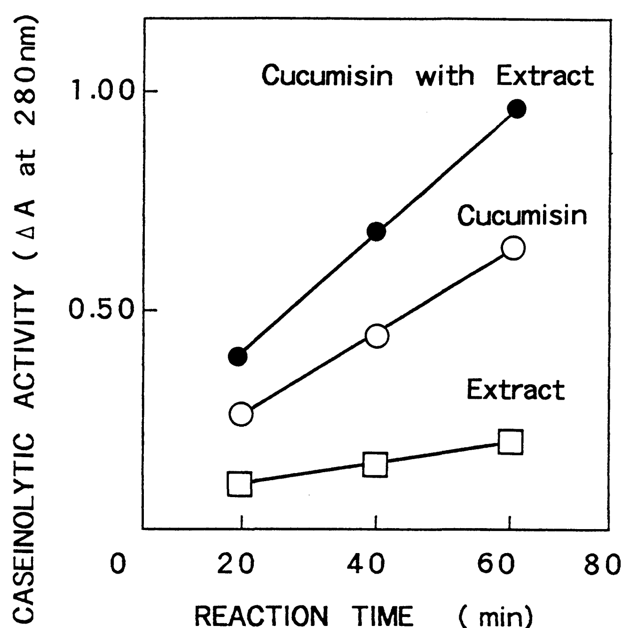

Fig. 1: Influence of extract from prince melon seedlings on the caseinolytic activity of cucumisin.

The experimental conditions were described in the text. Extract of prince melon seedlings was used in place of the "inhibitor solution" in the text. A buffer solution (1/15 M phosphate, pH 7.3) in place of "inhibitor solution" was used for assay of cucumisin activity. The mixture solution of 0.5 ml of extract and 0.5 ml of the buffer solution were used for protease activity of extract. Cucumisin (0.7 mg) was dissolved in 10 ml of 1/15 M phosphate buffer, pH 7.3.

Results and Discussion

Protein protease inhibitors are widely distributed in plants and animals. A number of these inhibitors have been purified and characterized. Previous studies from this laboratory on the effects of various generally potent sources of proteinase inhibitor on

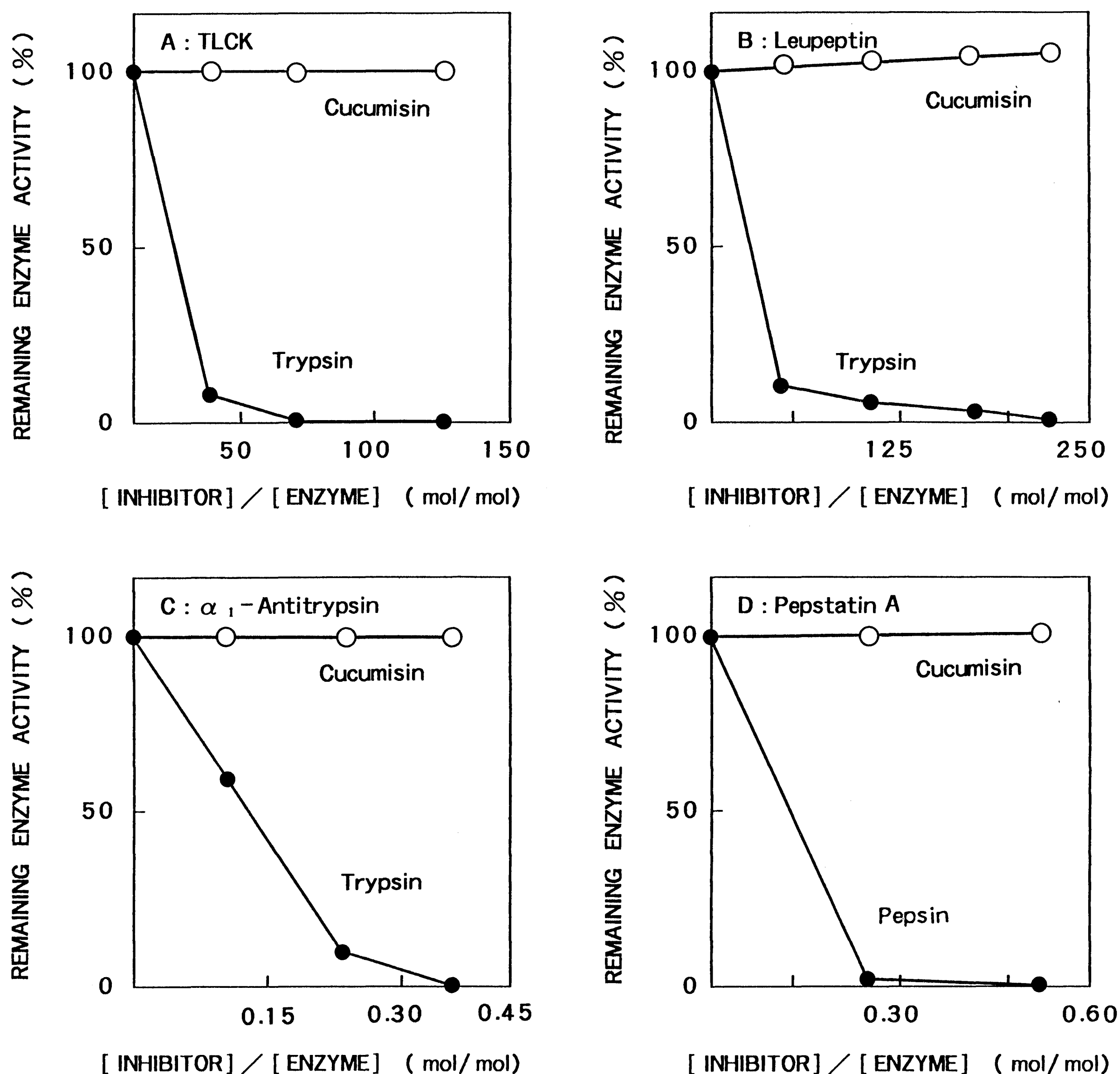

Fig. 2: Effect of inhibitors on cucumisin activity by different amounts of TLCK (A), leupeptin (B), α_1-antitrypsin (C) and pepstatin (D).

Cucumisin and trypsin were preincubated with different amounts of each inhibitor in 1/20 M Tris-HCl buffer, pH 7.5, except pepstatin in 1/20 M KCl-HCl buffer, pH 2.0, at 30℃, for 20 min. The amounts of cucumisin used were 0.7 mg in 10 ml of 1/20 Tris-HCl buffer, pH 7.5 (A, C and D) and 0.5 mg in 10 ml (B). The amounts of trypsin used were 1.2 mg in 10 ml of 1/20 M Tris-HCl buffer, pH 7.5 (A), 0.5 mg in 10 ml (B) and 1.0 mg in 10 ml (C), and pepstatin was 0.5 mg in 10 ml of 1/20 M KCl-HCl buffer, pH 2.0 (D). The amounts of inhibitors used were 3.5 mg of TLCK in 5 ml of 1/20 M Tris-HCl buffer, pH 7.5 (A), 2.0 mg of leupeptin in 10 ml (B), 0.9 mg of α_1-antitrypsin in 7 ml (C) and 0.1 mg of pepstatin A in 100 ml of 1/20 M KCl-HCl buffer, pH 2.0 (D).

the activity of cucumisin have shown that those materials did not contain the inhibitor of cucumisin[15]. As typical sources of proteinase inhibitor in previous report, pig liver and kidney, bovine serum, chicken egg-white, soy-bean, egg-plant, tomato, potato and mushroom were used for screening test. By the addition of the extract of prince melon seedlings, the activity of cucumisin was not affected (Fig. 1). Increasing of ΔA_{280} values on the extract indicated that any protease activity was present in it. Therefore, the

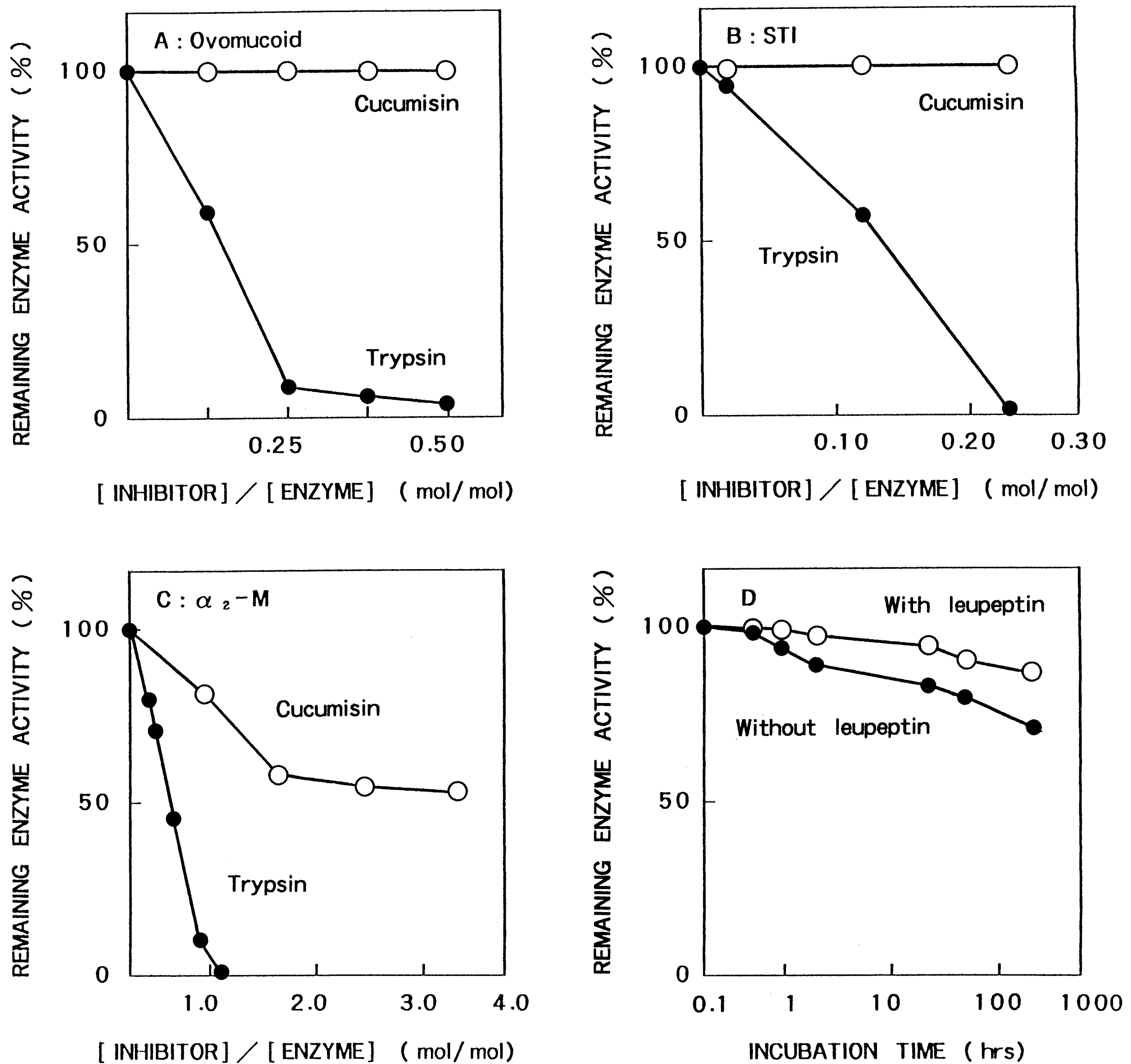

Fig. 3: Effect of inhibitors on cucumisin activity by different amounts of ovomucoid (A), STI (B), α_2-Macroglobulin (C) and for long time incubation with leupeptin (D).

Experimental conditions were described in the Fig. 2. The amounts of cucumisin used were 0.7 mg in 10 ml of 1/20 M Tris-HCl buffer, pH 7.5 (A and B) and 0.5 mg in 20 ml (C), Trypsin were 1.0 mg in 10 ml of 1/20 M Tris-HCl buffer, pH 7.5 (A and B) and 0.6 mg in 24 ml (C). The amounts of inhibitors used were 1.3 mg of ovomucoid in 10 ml of 1/20 M Tris-HCl buffer, pH 7.5 (A), 0.5 mg of STI in 10 ml (B) and 8 mg of α_2-Macroglobulin in 6 ml (C). The two systems contained the same concentration of cucumisin (1μM), and then 1 mM leupeptin was added to one of a pair indicated "with leupeptin" in Fig. 3 (D).

activity of mixture of cucumisin and the extract was higher than the case of extract free. As shown in Fig. 2 and 3, the caseinolytic activity of trypsin decreased markedly in the presence of TLCK (Fig. 2A), leupeptin (Fig. 2B), α_1-antitrypsin (Fig. 2C), ovomucoid (Fig. 3A) and STI (Fig. 3B). However, the activity of cucumisin was not affected by these typical trypsin inhibitors. Pepstatin A of pepsin inhibitor did not inhibit cucumisin activity (Fig. 2D). Increasing of additional amounts of leupeptin to cucumisin slightly increased cucumisin activity contrary to inhibitory action (Fig. 2B). It was confirmed in the long time incubation of cucumisin that the rate of autolysis of cucumisin with leupeptin was slower than the case of leupeptin free. α_2-Macroglobulin, a high-molecular mass plasma glycoprotein, has for a long time been known as an inhibitor of several serine proteinases. Trypsin was completely inhibited by α_2-macroglobulin, but inhibition of cucumisin was about 45% of original activity in spite of increasing of amount of α_2-macroglobulin (Fig. 3C).

References

1) Kaneda, M. & Tominaga, N. (1975) *J. Biochem.* **78**, 1287-1296.
2) Kaneda, M. & Tominaga, N. (1977) *Phytochemistry* **16**, 345-346.
3) Kaneda, M., Ohmine, H., Yonezawa, H. & Tominaga, N. (1984) *J. Biochem.* **95**, 825-829.
4) Kaneda, M., Sobue, A., Eida, S., & Tominaga, N. (1986) *J. Biochem.* **99**, 569-577.
5) Kaneda, M., Minematsu, Y., James C. Powers & Tominaga, N. (1986) *Agric. Biol. Chem.* **50**, 1075-1076.
6) Kaneda, M:, Minematsu, Y., James C. Powers & Tominaga, N. (1986) *Phytochemistry* **25**, 2407-2408.
7) Kaneda, M., Kamikubo, Y. and Tominaga, N. (1986) *Agric. Biol. Chem.* **50**, 2413-2414.
8) Kaneda, M., Kamachi, M. and Tominaga, N. (1987) *Phytochemistry* **26**, 365-366.
9) Kaneda, M. and Tominaga, N. (1987) *Agric. Biol. Chem.* **51**, 489-492.
10) Kaneda, M., Tomita, Y. and Tominaga, N. (1987) *Experientia* **43**, 318-319.
11) Kaneda, M., Uchikoba, T. and Tominaga, N. (1987) *Agric. Biol. Chem.* **51**, 1159-1161.
12) Kaneda, M., Nishimura, S. and Tominaga, N. (1988) *J. Dairy Science* **71**, 1132-1134.
13) Kaneda, M., Izumi, S., Fukuda, T., Uchikoba, T. & Tominaga, N. (1988) *Phytochemistry* **11**, 3661-3662.
14) Uchikoba, T., Horita, H. & Kaneda, M. (1990) *Phytochemistry* **29**, 1979-1981.
15) Kaneda, M., Sato, T., Nagata, H., Kitayama, T., Hatakeyama, N., Yonezawa, H. & Tominaga, N. (1983) *Rep. Fac. Sci. Kagoshima Univ.* **16**, 93-96.