Vol. 86, No. 3, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 14, 1979 Pages 492-499

TRYPSIN INHIBITORY ACTIVITY OF A POLYPEPTIDE ISOLATED FROM RED KIDNEY BEANS, THAT ALSO ENHANCES LYMPHOCYTE STIMULATION

M. Harms-Ringdahl, Jane Forsberg, I. Fedorcsák* and L. Ehrenberg

Department of Radiobiology, Wallenberg Laboratory, University of Stockholm, S-105 91 Stockholm, Sweden

Received December 11, 1978

SUMMARY

A polypeptide isolated from red kidney beans, Phaseolus vulgaris, which has previously been shown to stimulate RNA synthesis in cultures of mouse spleen lymphocytes and plasmolyzed E. coli, is here shown to be a potent inhibitor of trypsin and α-chymotrypsin. This polypeptide is compared with commercially available trypsin inhibitors with regard to their capacity to inhibit some proteolytic enzymes and to stimulate in vitro cultures of lymphocytes. Similar to FV the lima been trypsin inhibitor was found to possess a stimulating effect on the RNA as well as the DNA synthesis in lymphocyte cultures.

INTRODUCTION

In an earlier paper we reported that extracts from red kidney beans (Phaseolus vulgaris), as well as certain phytohemagglutinin preparations, contained a low molecular weight protein called FV ("Fraction V") that stimulated RNA synthesis in plasmolyzed E. coli cells and in lymphocytes. A method for the purification of this protein was described (1).

Structural studies of FV revealed a molecular weight of about 10,000 and an unusually high content of cysteine (20 mole per cent) (2). It was pointed out (Wilson, K.A., and Laskowski, M., Jr., personal communication) that the amino acid sequence of FV (2) was very similar to that of certain trypsin inhibitors, especially the garden bean inhibitor II (3), lima bean inhibitor IV (4) and the Bowman-Birke soybean inhibitor (5, 6). The homology between large sections of the amino acid sequence of FV and the trypsin/α-chymotrypsin inhibitors from varieties of Phaseoulus vulgaris and other leguminous species, as well as the close similarity in amino acid composition of FV and trypsin inhibitors isolated from red kidney beans (7), led us to investigate the influence of FV on the activity of some proteolytic enzymes.

*Permanent address: Institute of Biochemistry, Department 1, Semmelweis University Medical School, Puskin u. 9, 1088 Budapest, Hungary

0006-291X/79/030492-08$01.00/0

In a recent publication in this journal (8), it was shown that a protease inhibitor of dipeptide nature enhanced the activation of small lymphocytes by concanavalin A (Con A). In this report we add further information on the stimulating properties of two naturally occuring trypsin inhibitors on resting and mitogen-stimulated lymphocytes.

MATERIALS AND METHODS

Biochemicals. 5-[^{3}H]-uridine and methyl-[^{3}H]-thymidine were purchased from Radiochemical Centre, Amersham. All media and medium supplements were from Flow Laboratories, Con A was purchased from Pharmacia Fine Chemicals and Phytohemagglutinin (PHA) from Wellcome Reagents Limited. Trypsin (from bovine pancreas, type III); α-chymotrypsin (from bovine pancreas, type II); protease (from Streptomyces griseus, type VI); trypsin inhibitor from lima bean (type II-L); trypsin inhibitor from soybean (type I-S); dithiothreitol (DTT); α-N-benzoyl-DL-arginine-p-nitroanilide HCl (BAPNA) and other organic chemicals were purchased from Sigma Chemical Co., St. Louis, Mo. Inorganic chemicals, analytical grade, were purchased from Merck, Darmstadt. FV was prepared and purified as described earlier (1).

Reduction of FV, soybean and lima bean trypsin inhibitors. FV or soybean inhibitor or lima been inhibitor (10 mg per ml in 10 mM $NaHCO_3$) were incubated with DTT (71 mM) at 37°C for 3 hours and then dialyzed against three changes of 10 mM $NaHCO_3$ in a nitrogen atmosphere at 4°C. Solutions with given amounts of FV, proteolytic enzymes and their inhibitors were perpared by measuring the protein content of these components with the Folin-Ciocalteus reagent (9).

Assay for trypsin, α-chymotrypsin and protease activity, and for the influence of inhibitors, using casein as substrate. Trypsin or α-chymotrypsin was preincubated with or without oxidized (i.e. unreduced) or reduced FV, soybean trypsin inhibitor or lima bean trypsin inhibitor in 0.1 M phosphate buffer, pH 7.6, for 3 minutes at 37°C in a volume of 1 ml. Phosphate buffer, 4 ml and 5 ml casein solution (Hammarsten) were then added to final concentrations of 0.1 M and 0.5%, respectively. After 20 minutes incubation at 37°C, 1 ml of the mixture was removed and added to 2 ml of 5% (w/v) ice-cold trichloroacetic acid, and after centrifugation the absorbance of the supernatant was measured at 280 nm (10). The activity of protease from Streptomyces griseus was measured as described above, except that Tris-HCl buffer, pH 7.6, in a final concentration of 0.1 M was used instead of phosphate buffer.

Assay for trypsin activity and for the influence of inhibitors using BAPNA as substrate. Trypsin was incubated with and without oxidized FV or soybean trypsin inhibitor or lima bean trypsin inhibitor in 0.16 M Tris-HCl, pH 7.8 at 25°C for 3 minutes in a final volume of 2 ml. Then 1 ml substrate (1 mg BAPNA/ml in H_2O) was added and the increase in absorbance at 405 nm was followed at 25°C for 5 minutes (11).

Cultivation of thymocytes and spleen lymphocytes and determination of ^{3}H-uridine and ^{3}H-thymidine incorporation. Thymus or spleen from a CBA-mouse (male, about 3 months old) was minced in PBS and forced several times through a Pasteur pipett. The tissue-free cell suspension was washed in PBS and cell cultures were prepared as described elsewhere (12). In brief, the cells ($2 \cdot 10^6$ cells/ml) were cultured under serum-free conditions in a medium consisting of GMEM supplemented with amino acids, vitamins, glutamine, antibiotics, uridine and thymidine at 37°C in 5% CO_2 atmosphere. When used, the final concentration of Con A or PHA were 0.5 µg/ml or 0.3 µg/ml, respectively. After appropriate times, the cells were filtered and washed with trichloroacetic acid on Whatman glass filter (GF/C, 2.5 cm) and the radioactivity of

Table 1.

Effect of FV, lima bean trypsin inhibitor and soybean trypsin inhibitor on the enzyme activity of trypsin, α-chymotrypsin and protease from S. griseus.

Inhibitor	Molar ratio inhibitor enzyme for 50% inhibition			
	trypsin		α-chymotrypsin	Protease from S. griseus
	with casein	with BAPNA		
FV	0.55	0.26	2.0	>2.0[b]
FV reduced	>2.0[b]	>2.0[b]	>2.0[b]	>2.0[b]
Lima bean trypsin inhibitor	0.21	0.16	1.0	>2.0[b]
Soybean trypsin inhibitor[a]	0.22-0.48	0.17-0.40	2-4	>2.0[b]

[a]The commercial sample of soybean trypsin inhibitor was prepared according to the method of Rackis and Anderson (17) and consists of two proteins with molecular weights about 8,000 and 22,000.

[b]No inhibition obtained.

insoluble material was determined in a liquid scintillator spectrophotometer at an efficiency of 29% (12).

Assay for the copper-catalyzed autoxidation of cysteine and the influence of reduced FV and reduced trypsin inhibitors on the rate of autoxidation. Cysteine (1 mM) was incubated in open test tubes at 37°C in 0.04 M Tris-HCl buffer, pH 7.2. At given times, 0.1 ml samples were transferred into 2.9 ml Ellman's reagent (13) and the optical density was measured at 412 nm. $CuCl_2$ (1 μM), was added to initiate the reaction in the presence or absence of oxidized or reduced FV or the reduced trypsin inhibitors.

RESULTS

Protease inhibition: The inhibitory activity of FV on the proteolytic enzymes trypsin, α-chymotrypsin and protease from S. griseus was compared with the activities of lima bean trypsin inhibitor and soybean trypsin inhibitor as shown in Table 1. The inhibitory effect of FV on trypsin and α-chymotrypsin is in the same range as obtained with lima bean trypsin inhibitor and soybean trypsin inhibitor. Like the two other inhibitors, FV did not affect the protease from S. griseus. FV in reduced form was devoid of inhibitory activity, as has also been observed for lima bean trypsin inhibitor (14) and Bowman-Birke soybean trypsin inhibitor (15).

Trypsin inhibitors chelate metal ions: The lymphocyte-stimulating properties of the polypeptide FV has been associated with the ability of reduced FV to counteract the autoxidation of thiols, presumably by chelation of certain metal ions (especially Cu^{2+}) that catalyze the oxidation of SH groups by molecular oxygen (16). The possibility that reduced lima bean trypsin in-

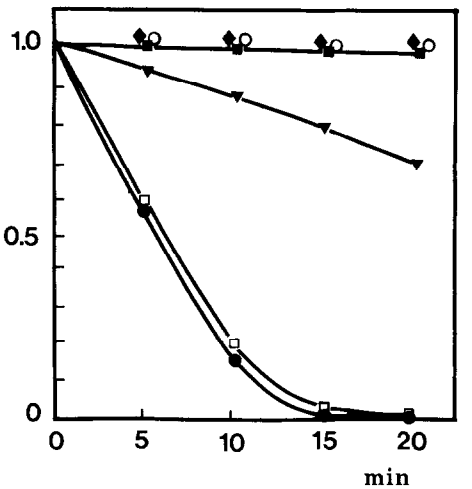
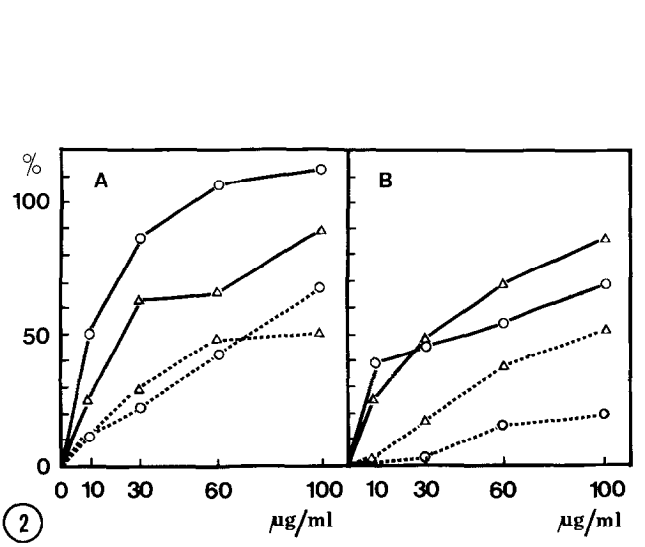


Figure 1. The influence of reduced trypsin inhibitors and FV on the rate of the copper-catalyzed autoxidation of cysteine.

Autoxidation of cysteine (1 mM) in the presence of $CuCl_2$(1 μM) (●—●), $CuCl_2$ (1 μM) and reduced FV (5 μg/ml) (■—■), $CuCl_2$ (1 μM) and reduced lima bean trypsin inhibitor (5 μg/ml) (◆—◆), $CuCl_2$ (1 μM) and reduced soybean trypsin inhibitor (10 μg/ml) (▼—▼), $CuCl_2$ (1 μM) and oxidized FV (5 μg/ml) (□—□) and with no additions of $CuCl_2$ and trypsin inhibitors (○—○).

hibitor or soybean trypsin inhibitor could inactivate the catalytic activity of metal ions in a similar way was studied and the results are presented in Fig. 1. In all assay systems used, special precautions were taken to avoid contaminating metal ions (16). As shown in Fig. 1, cysteine (1 mM) was autoxidized within 15 minutes in the presence of $CuCl_2$ (1 μM). When reduced FV (0.5 μM) or reduced lima bean trypsin inhibitor (0.5 μM) was added, a complete prevention of the catalytic activity of Cu^{2+} was achieved. The effect of reduced soybean trypsin inhibitor (0.5 μM) was lower although a clear reduction in the rate of autoxidation was obtained and oxidized FV did not effect the catalytic activity of Cu^{2+}.

Trypsin inhibitors enhance lymphocyte activation: Mouse spleen lymphocytes or thymocytes were cultured without mitogen in serum-free medium and the influence of FV or lima bean trypsin inhibitor on RNA or DNA synthesis was followed. Fig. 2 shows that increasing concentrations of the two protease inhibitors, when present from the start of the cultures, enhance the RNA synthesis in both cell types. The largest response was obtained with the reduced form of the inhibitors and an increase by more than 100% was seen with lima bean trypsin inhibitor at a concentration of 60 μg/ml in cultures of spleen cells (Fig. 2A).



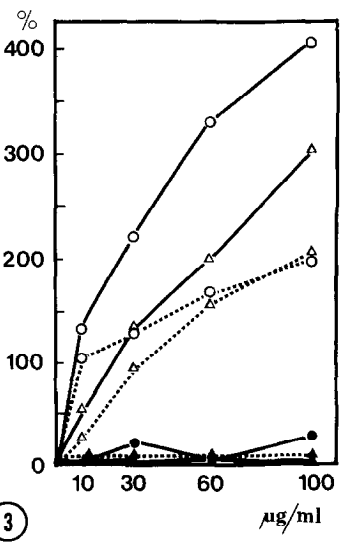


Figure 2. Enhancement of RNA synthesis by trypsin inhibitors in mouse spleen lymphocytes (A) and mouse thymocytes (B) cultured in the absence of mitogen.

The lymphocytes were pulsed with ^{3}H-uridine 0.1 µCi/ml (29 Ci/mmole) between 24-48 hours of culture. RNA synthesis was determined according to Materials and Methods. The degree of stimulation is presented as the increase, in per cent, over control. At the start of cultures the following additions were made to the cultures: lima bean trypsin inhibitor oxidized (○--○) or reduced (○—○), FV oxidized (△--△) or reduced (△—△). The absolute values for the control cultures (0% enhancement) are 5,300 cpm/2•10^{6} cells for spleen cells and 1,060 cpm/2•10^{6} cells for thymocytes.

Figure 3. The influence on DNA synthesis by trypsin inhibitors in mouse spleen lymphocytes and mouse thymocytes cultured in the absence of mitogen.

The lymphocytes were pulsed with ^{3}H-thymidine 0.1 µCi/ml (21 Ci/mmole) between 48 and 72 hours of culture and the TCA insoluble activity was determined as described in Materials and Methods. The degree of stimulation is presented as the increase, in per cent, over the control. Open symbols represent mouse spleen lymphocytes, filled symbols thymocytes. Solid lines represent the reduced form of the inhibitor and dotted lines the oxidized form. Lima bean trypsin inhibitor (○,●) and FV (△,▲). The absolute values for the controls are 6,500 cpm/2•10^{6} cells for spleen cells and 120 cpm/2•10^{6} for thymocytes.

Thymocytes also responded with a dose-dependent increase in RNA synthesis that reached a maximal increase of 85% with the highest tested concentration of reduced FV (100 µg/ml) (Fig. 2B). A different response is observed when DNA synthesis is followed (Fig. 3). In the spleen cells a four-fold or three-fold increase in the DNA synthesis is obtained in the presence of reduced lima bean trypsin inhibitor or reduced FV, respectively, demonstrating that the increased RNA synthesis observed after 24 hours of culture has led to blast transformation. However, the thymocyte response seen after 24 hours did not lead to initiation of DNA synthesis. Both

Table 2

The synergistic action of FV and of lima bean trypsin inhibitor with Con A or PHA on RNA and DNA synthesis in mouse spleen lymphocytes or thymocytes.

Polypeptide 100 µg/ml	Lymphocytes from spleen				Thymocytes			
	RNA		DNA		RNA		DNA	
	Con A	PHA	Con A	PHA	Con A	PHA	Con A	PHA
None (cpm)	100 (26,800)	100 (30,000)	100 (30,000)	100 (21,800)	100 (10,200)	100 (7,400)	100 (5,700)	- -
FV ox.	117	109	117	153	261	127	200	-
FV red.	119	96	100	138	279	127	259	-
Lima bean ox.	105	105	90	128	258	112	131	-
Lima bean red.	99	90	97	130	263	112	212	-

Cultivation of lymphocytes was done as described in Materials and Methods. RNA and DNA synthesis was measured as described in legends to figures two and three. The values represent (changes in) activities expressed in per cent of the respective controls (None given as 100%). The absolute values for control samples, in cpm/$2 \cdot 10^6$ cell, are given within brackets.

trypsin inhibitors, in oxidized as well as reduced form, were ineffective in this respect.

The trypsin inhibitors were also tested together with the mitogens Con A and PHA with respect to influence on RNA and DNA synthesis (Table 2). In the spleen cell cultures this treatment caused only minor changes in response. A synergistic effect of PHA and the inhibitors is, however, indicated, by the 53% increase of the DNA synthesis observed as maximal response to oxidized FV. Thymocyte growth is clearly enhanced when the trypsin inhibitors are combined with Con A. DNA as well as RNA synthesis increased 100-180% except in combination with oxidized lima been trypsin inhibitor. However, in combination with PHA only a minor increase of RNA synthesis was observed in the presence of the trypsin inhibitors.

DISCUSSION

The trypsin inhibitory activity of FV (Table 1) and its chemical similarities with cystine-rich trypsin inhibitors motivate the classification of FV as a trypsin inhibitor. Its ability to inhibit protease activity is confined to the oxidized form of the polypeptide (Table 1, refs. 14, 15), i.e. its state in equilibrium with air.

The ability of FV and other cystine-rich trypsin inhibitors to enhance the growth of lymphocytes *in vitro* appears to be related to at least two

mechanisms: a) in reduced form they protect essential SH-groups on membranes by eliminating, evidently through chelation, the catalytic action of copper ions on autoxidation of thiols (Fig. 1) and b) in oxidized form they protect against proteolytic enzymes. However, other effects on the biological activities of lymphocytes are possible (8) and should be considered in a total evaluation of the role of protease inhibitors on lymphocyte proliferation.

The enhancement of growth was initially demonstrated for reduced FV under conditions when it could be ascribed to a prevention of the generation of peroxide in the autoxidation of cysteine catalyzed by metal impurities in the media (16). The present series of experiments, which were carried out without thiols added to the cultivation media, show a stimulating activity of both the reduced and the oxidized form of FV and lima bean trypsin inhibitor (Figs. 2, 3). In spleen cells and thymus cells the response was greatest with reduced polypeptides, a fact that might tentatively be explained by a combined action of two effects advantageous to cell growth: a protection against autoxidation of essential SH-groups in membranes and an anti-proteolytic action. Under our experimental conditions the reduced polypeptide becomes oxidized, and regains its trypsin inhibitory activity, within three to six hours: (unpublished data).

A role of anti-protease activity in lymphocyte stimulation was suggested by Saito et al. (8) who found that a protease inhibitor ("Bestatin", a dipeptide) enhanced the stimulation by Con A of peripheral guinea pig lymphocytes. They assumed that the combined treatment increases the number of T-cells responding to Con A. The present results show a synergism of trypsin inhibitors and Con A, especially in thymocytes (Table 2), and are related to those of Saito et al. (8), but the effect is certainly not restricted to T-cells since spleen lymphocytes respond strongly to the trypsin inhibitors alone (Figs. 2, 3). These results suggest that discussion of the mechanism of action of protease inhibitors on in vitro cultures of lymphocytes should take into account both the synergistic action of the inhibitor and of the mitogen on thymocytes, as well as the effect of the protease inhibitors alone on a fraction of cells obtained from the spleen.

ACKNOWLEDGMENT

The authors are grateful to Ms. Inga-Lise Kinell for skillful technical assistance. The investigation was supported by the Swedish Natural Science Research Council and the Gesellschaft für Strahlen- und Umveltforschung, FRG.

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