INHIBITION STUDIES OF AN INTRACELLULAR INHIBITOR ON THIOL PROTEINASES

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1. Introduction

Recently, much attention has been paid to intracellular inhibitors isolated from leucocyte cells [1–5]. In the course of our further studies, two types of specific inhibitors of neutral proteinases were isolated from leucocyte [6] and from spleen [7] cells. We reported that the inhibitor with a molecular weight of 40 000 inhibits elastase and chymotrypsin-like neutral proteinase, whereas the inhibitor with a molecular weight of about 15 000 inhibits only the latter proteinase.

This paper reports studies on the inhibition effect of the low molecular weight inhibitor isolated from leucocytes, as well as from spleen, on the activity of cathepsin B, papain and a thiol proteinase, similar to cathepsin H [8].

2. Material and methods

Leucocyte inhibitor was isolated from the soluble phase of disrupted pig peripheral leucocytes, freed of granules by centrifugation, named post granular supernatant, as previously described [6]. In the present work we used the inhibitor of molecular weight of about 15 000, designated as LNPI-1 (leucocyte neutral proteinase inhibitor 1). Spleen inhibitor, SNPI-1, with the same molecular weight, was prepared from disrupted spleen cells [7].

Purified samples of cathepsin B (EC 3.4.12.3) were prepared in our laboratory by two methods: (a) from bovine spleen by the method of Otto [9]; (b) from lymph nodes, prepared by covalent coupling to commercial thiol Sepharose (unpublished). Papain (EC 3.4.22.2) was supplied by Worthington Biochemical Corporation (New Jersey, USA). Thiol proteinase similar to cathepsin H was prepared in our laboratory.

The activities of cathepsin B, papain and cathepsin H, were determined using *N*-benzoyl-DL-arginine-2-naphthylamide (BANA), from Sigma, USA, as a substrate, according to Barrett's procedure [10].

2.1. *Inhibition studies*

Two systems were used when the action of thiol proteinases and LNPI-1 or SNPI-1 were being studied. In the first, the amount of enzyme was maintained at a constant level (25 μl) in 0.2 N K–Na phosphate buffer at pH 6.0, with the addition of variable quantities of inhibitor (5–50 μl). The total volume of buffer, enzyme and inhibitor was 0.5 ml. After 15 min preincubation at room temperature, the samples were assayed on BANA substrate.

The effect of pH on the inhibition of cathepsin B by LNPI-1 and SNPI-1 was also studied. The inhibition tests with constant concentration of cathepsin B (25 μl) and inhibitor (25 μl) were performed in buffer solutions from pH 4.5 to pH 7.0. 0.1 M Na acetate and 0.2 M K–Na phosphate buffers were used in these experiments. Cathepsin B and inhibitors in this experiment were also preincubated for 15 min, at room temperature prior to their assay on BANA substrate.

In the second system, the effect of time of preincubation at 22°C on the stability of the complex between cathepsin B and LNPI-1 was measured. Cathepsin B was preincubated with inhibitor in buffer solution at pH 6.0 from 2 min to 4 h prior to its assay on BANA test.

2.2. *pH stability*

The effect of pH on the denaturation of the inhibitor LNPI-1 was tested by preincubating the inhibitor sample at 37°C in buffer solutions in the pH range of pH from pH 4.5 to 7.0. After 1 h preincubation, cathepsin B was added for determination of the residual inhibitor activity.

Disc gel electrophoresis of inhibitor samples was performed by the method of Davies [11], on 6 cm cylindrical gels of 7% acrylamide in 25 mM Tris, 0.2 M glycine, pH 8.4. Gels were stained for protein with Coomassie blue.

Protein was assayed by the method of Lowry [12].

3. Results

The results of fig.1 show that the purified inhibitor samples of LNPI-1 and SNPI-1 are almost identical.

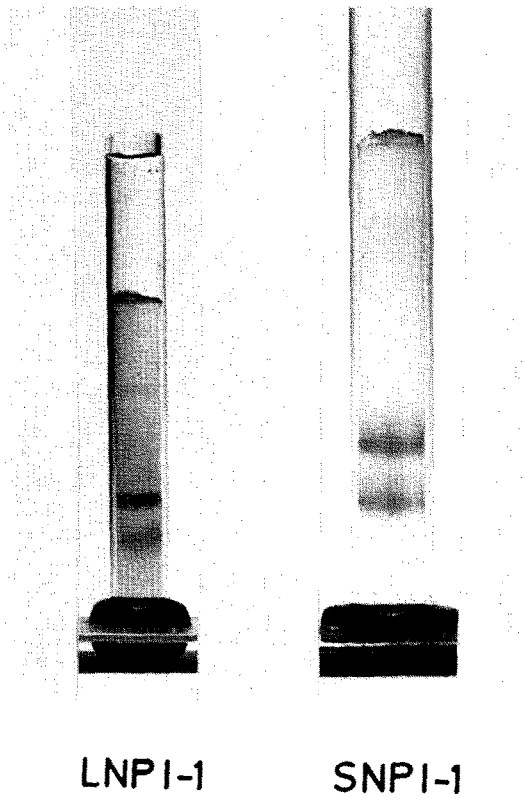

Fig.1. Gel electrophoretograms of inhibitor sample LNPI-1 and SNPI-1.

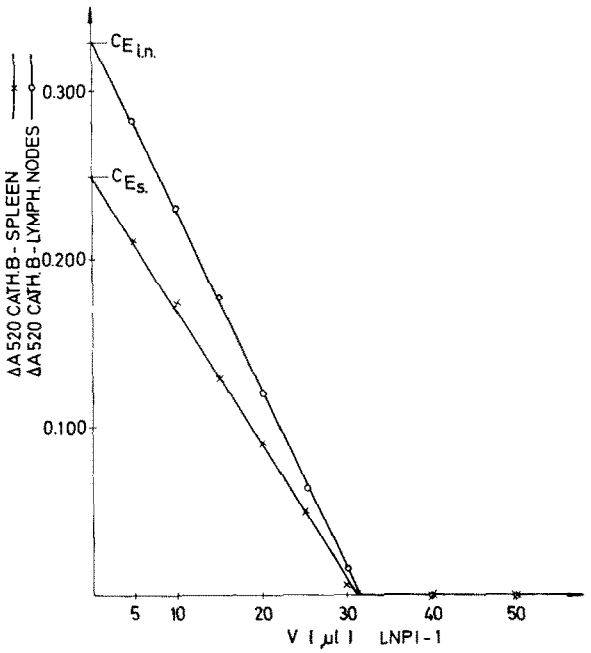

Fig.2. (A) Inhibition of cathepsin B from lymph nodes (25 µl) by addition of different quantities of LNPI-1 (5–50 µl). Residual enzyme activity was assayed on BANA substrate. (B) Inhibition of cathepsin B from spleen (25 µl) by addition of different quantities of LNPI-1 (5–50 µl). Residual enzyme activity was assayed on BANA substrate. C_E, enzyme control: 25 µl of cathepsin B (from lymph nodes or from spleen) + 475 µl of buffer of pH 6.0, were preincubated for 15 min at room temperature and then assayed on BANA substrate. LNPI-1, 0.76 mg/ml; cathepsin B lymph nodes, 0.17 mg/ml; cathepsin B spleen, 0.13 mg/ml.

Figure 2 shows the inhibition of cathepsin B from lymph nodes (A) and from spleen (B) by increasing quantities of LNPI-1. It is evident in both cases that the degree of inhibition increases linearly with concentration of inhibitor to complete inhibition. At a volume ratio of 1(E): 1(I) (25 µl: 25 µl) inhibition was 80% in both cases. If the values are given in µg, then the corresponding quantities of cathepsin B (A, B) and LNPI-1 are: 4.2 µg (A), 3.2 µg (B) : 19 µg (LNPI-1). At the volume ratio of 1 (E) : 1.3 (I), the inhibitor concentration (24 µg) was sufficient to produce complete inhibition of cathepsin B (A, B).

Figure 3 shows the inhibition of papain by inhibitor isolated from leucocytes, LNPI-1 and from spleen, SNPI-1. Complete inhibition of 2 µg of

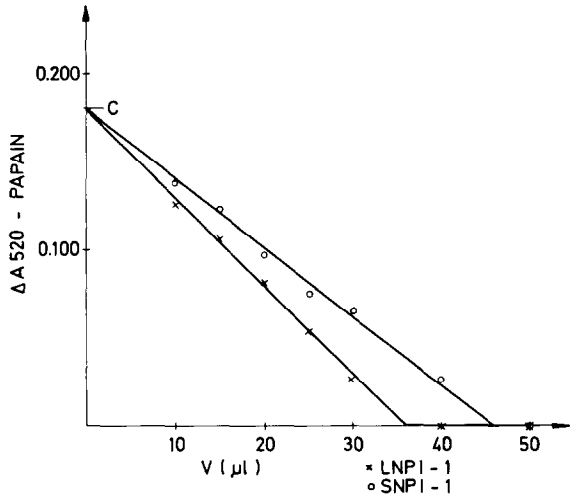

Fig.3. Inhibition of papain (25 μl) by addition of different quantities (10–50 μl) of LNPI-1 and SNPI-1. Residual enzyme activity was assayed on BANA substrate. C_E, enzyme control: 25 μl of papain + 475 μl of buffer of pH 6.0 were preincubated for 15 min at room temperature and then assayed on BANA substrate. LNPI, 0.76 mg/ml; SNPI-1, 0.75 mg/ml and papain, 0.080 mg/ml.

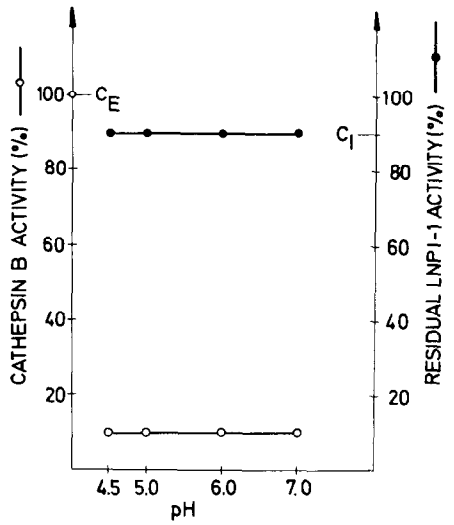

Fig.4. Effect of pH on the activity of LNPI-1. 450 μl of buffer (pH 4.5–7.0) + 25 μl of LNPI-1 were maintained for 1 h at 37°C. 25 μl of spleen cathepsin B was then added to each tube and preincubated for 15 min at room temperature and assayed on BANA substrate. C_E, control of spleen cathepsin B activity (100%): 475 μl of buffer of pH 6.0 + 25 μl of cathepsin B assayed on BANA substrate. C_I, control of inhibitor activity (90%): 450 μl of buffer of pH 6.0 + 25 μl of cathepsin B + 25 μl of LNPI-1 preincubated for 15 min at room temperature and assayed on BANA substrate.

papain (25 μl) was obtained with 28 μg (37 μl) of LNPI-1 and with 36 μg (48 μl) of SNPI-1.

The effect of thiols on both inhibitor samples prior to adding the enzyme (cathepsin B or papain) was tested. The addition of 2 mM cysteine and 1 mM dithioerythritol to the inhibitor was without effect. No regeneration of enzyme activity was noted.

Inhibition tests on cathepsin H were done with the same concentrations of LNPI-1 and SNPI-1 as in the case of papain. LNPI-1 and SNPI-1 concentrations sufficient to produce complete inhibition of this enzyme were the same as in case of cathepsin B.

Figure 4 shows the pH stability of LNPI-1. From the diagram presented, it is evident that LNPI-1 is quite stable in the tested pH region from pH 4.5–7.0. The stability of the complex of cathepsin B and LNPI-1 or SNPI-1 in buffer solutions from pH 4.5–7.0 was also studied. From the results presented in fig.5, it is evident that the inhibition of cathepsin B by inhibitor samples was not dependent on pH.

The effect of preincubation time on the extent of inhibition was also studied. The stability of the complex of cathepsin B and LNPI-1 was not depen-

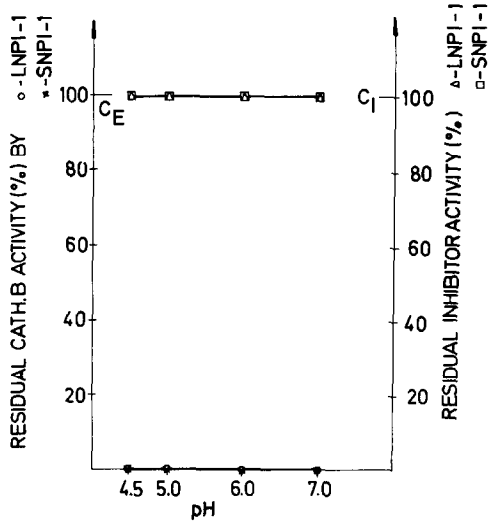

Fig.5. Effect of pH on the stability of the complex of spleen cathepsin B and LNPI-1 or SNPI-1. 450 μl of buffer (pH 4.5–7.0) + 25 μl of cathepsin B + 25 μl of inhibitor sample were preincubated for 15 min at room temperature and then assayed on BANA substrate. C_E and C_I of inhibitors were performed in buffer of pH 6.0.

Table 1
Temperature dependence of LNPI-1

Temperature °C	BANA assay A_{520}		Inhibition %
38	C_E	0.180	
38	C_I	0.048	74
50		0.050	74
60		0.048	74
70		0.050	74
80		0.050	74
90		0.140	24

25 μl of LNPI-1 + 450 μl of buffer pH 6.0 were incubated for 10 min at various temperatures. The remaining inhibitory activity was tested after cooling on room temperature on papain (25 μl) and then assayed on BANA substrate. C_I, control inhibition test, performed under the same condition, at 38°C; C_E, enzyme activity of papain (25 μl)

dent upon the preincubation time, followed from 2 min to 4 h. The complex was stable over the entire time range tested. No reversibility of enzyme inhibition was observed even when enzyme and inhibitor were given simultaneously to substrate, without preincubation.

LNPI-1 was also tested for stability at various temperatures (table 1). The inhibitor showed thermal stability in the temperature interval from 38°C to 80°C. Significant loss of activity was noted at 90°C, its activity being diminished by 50%, in comparison to the control value.

4. Discussion

Until now there was no work published dealing with the isolation and characterization of the thiol proteinase inhibitor of leucocyte cells or from spleen. There was only a study of Davies et al. [13], who found that the recovery of cathepsin B activity from the leucocyte subcellular fractions was in excess of that found in whole cell homogenate, probably due to the presence of an inhibitor in cytoplasm. The inhibition of cathepsin B and other thiol proteinases by our inhibitor samples was pH independent, in the tested interval from pH 4.5–7.0. Moreover, the present experiments demonstrate that the inhibition of thiol proteinases is not time consuming; no preincubation of enzyme with inhibitor is needed for complex formation. According to its inhibitory ability in acid, as well as in neutral pH, our inhibitor samples are similar to skin 'BANA-hydrolase' inhibitor II [14]. Protein inhibitors of thiol proteinases with this molecular weight have previously been detected also in chicken egg white [15], rabbit skin [16] and bovine nasal cartillage [17]. For all these inhibitors, their high thermal stability is characteristic.

The latest studies on cathepsin B showed that not only is its intracellular function (lysosomal) important, but also its extracellular one. Recent investigations indicated that the activity of α_1 antitrypsin is regulated by acid proteinases. Namely, Sandhous and Janoff [18] observed that hepatocyte granule extract, at acid pH, produced degradation of α_1 antitrypsin, and Johnson and Travis [19] later confirmed that cathepsin B was the enzyme which inactivates α_1 antitrypsin, by specific peptide bond hydrolysis. The extracellular function of cathepsin B is also important in its ability to activate latent collagenase into active collagenase [20]. It has also been proposed that cathepsin B may play some role in cellular detachment, invasive growth and the primary step of metastases formation [21]. With reference to these possible roles of cathepsin B, its intracellular inhibitor is of great interest. The regressive effect of leucocyte intracellular inhibitors on tumour growth and metastases formation was already confirmed [22]. An inhibitory effect on tumour growth was observed previously with a cartillage inhibitor [23].

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