

Interleukin-8 processing by neutrophil elastase, cathepsin G and proteinase-3

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Abstract Activated neutrophils secrete two forms of IL-8 with 77 and 72 amino acids, IL-8(77) and IL-8(72), along with proteinases that could process these cytokines. Significant conversion of IL-8(77) to more potent, N-terminally truncated forms was observed upon incubation with neutrophil granule lysates and purified proteinase-3. IL-8(72) was considerably more resistant to proteolytic processing than IL-8(77). The present observations indicate that neutrophil proteinases released in inflamed tissues convert IL-8 to more active forms and therefore tend to conserve or enhance, rather than decrease IL-8 activity.

Key words: IL-8; Chemokine; Neutrophil activation; Neutrophil proteinase; Processing

1. Introduction

Interleukin-8 (IL-8) belongs to a family of small chemotactic cytokines, now called chemokines, which are released by phagocytes and a variety of tissue cells upon challenge with inflammatory stimuli [1,2]. IL-8 and several analogs, NAP-2 [3], GRO α , GRO β and GRO γ [4], and ENA-78 [5], activate neutrophil leukocytes via common heptahelical receptors coupled to GTP-binding proteins, and induce three main responses: shape change and migration, exocytosis of enzymes and other storage proteins, and the respiratory burst [2]. IL-8 is generated as a precursor of 99 amino acids, and is secreted after cleavage of a signal peptide of 20 residues. The mature protein is processed at the N-terminus by extracellular proteinases yielding several biologically active forms the most abundant of which consist of 77 and 72 amino acids [2]. Neutrophils were shown to release IL-8 during phagocytosis [6] and after stimulation, e.g. with fMLP [7], or LPS [6,8], but the role of neutrophil proteinases in IL-8 processing is unknown.

Activated neutrophils release a variety of proteinases with broad substrate specificity that can process chemokines and modify their biological activity. We have determined the forms of IL-8 released by neutrophils, and have studied the processing of IL-8 and its analogs, NAP-2 and GRO α , by neutrophil proteinases. Our results show that the proteinases tend to enhance IL-8 activity by conversion into derivatives that are more potent and more resistant to proteolysis.

2. Materials and methods

2.1. Materials

IL-8(72) and IL-8(77), NAP-2 and GRO α were prepared by stepwise chemical synthesis by Dr. Ian Clark-Lewis (University of British

Columbia, Vancouver, Canada) [9]. Bovine pancreatic trypsin and chymotrypsin (Boehringer Mannheim) were active-site titrated using p-nitrophenyl-p'-guanidinobenzoate [10] and 4-nitrophenylacetate [11], respectively. Active-site titrated human leukocyte elastase, cathepsin G and α 1-proteinase inhibitor were provided by Dr. J. Bieth (INSERM U.237, Strasbourg, France), and purified proteinase-3 [12,13] by Dr. B. Gray (University of Minnesota, Minneapolis, MN, USA). Bovine neck ligament elastin, bovine plasma fibrinogen and bovine achilles tendon collagen I were purchased from Sigma.

2.2. Purification of IL-8 from supernatant of stimulated neutrophils

Neutrophils were suspended in 1000 ml MEM supplemented with 2% (v/v) plasma protein solution (Central Laboratory of the Swiss Red Cross) at 5×10^7 cells/ml, and stimulated with 1 μ g/ml lipopolysaccharide for 18 h at 37°C/5% CO₂. The medium was diluted with an equal volume of buffer (20 mM potassium phosphate buffer, pH 7.2, containing 20 mM NaCl, 1 mM EDTA and 5% glycerol), and loaded onto a 13-ml phosphocellulose column (Whatman P11), and elution was performed with 120 ml of a linear NaCl gradient (0.02 to 2 M). Fractions with IL-8 activity were pooled and further purified by reversed-phase HPLC on a C4 column (10 \times 100 mm, 20 μ m, Aquapore, Applied Biosystems) which was eluted at 2 ml/min with a gradient of 0 to 80% acetonitrile in 0.1% trifluoroacetic acid (TFA). IL-8 activity was rechromatographed on a microbore C4 column (2.1 \times 100 mm, 7 μ m, Aquapore, Brownlee Labs) which was eluted at 0.5 ml/min with a gradient of 0 to 75% acetonitrile in 0.1% TFA at an increment of 1.25% per min. Active fractions were rerun on a CN-propyl column (2.0 \times 100 mm, 7 μ m, Nucleosil 300-7 μ m, Macherey-Nagel) as described for the C4 column.

2.3. Reduction of IL-8(72)

20 μ g IL-8(72) were treated with 7 M guanidine and 10 mM dithiothreitol in 2 ml 0.3 M Tris/HCl, pH 8.5, and then alkylated for 2 h at room temperature with 22 mM iodoacetamide. The preparation was dialyzed overnight against 50 mM Tris/HCl, pH 8.3. Reduced IL-8(72) was devoid of biological activity.

2.4. Neutrophil granule lysate

Human neutrophils were isolated from buffy coats of donor blood (Central Laboratory of the Swiss Red Cross) [14]. The cells (10^8 /ml) were suspended in PBS, pH 7.4 and disrupted by nitrogen cavitation (20 min, 30 bar). The homogenate was centrifuged at $250 \times g$ for 10 min at 4°C, and a granule pellet was obtained by centrifugation of the supernatant at $100,000 \times g$ for 30 min. Granule enzymes were extracted by resuspending the pellet in 0.2 M glycine, pH 3.0, containing 0.2% Triton X-100. The lysate was stored at -20°C in portions corresponding to 2×10^7 cells. The lysate from 5×10^6 neutrophils contained 15 μ g elastase, as estimated by comparison with purified, active-site titrated neutrophil elastase using N-methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide as substrate, 3 μ g vitamin B₁₂-binding protein estimated by the binding of cyano-[⁵⁷Co]cobalamin, and β -glucuronidase at a specific activity of 5 nmol/min/mg [15,16].

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Abbreviations: HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline. IL-8(77), IL-8(72), IL-8(70), N-terminally truncated forms of IL-8 with 77, 72 and 70 amino acids, respectively.

2.5. Proteolytic degradation

Mixtures containing granule lysate or a purified proteinase, a chemokine as substrate and additions as indicated, were incubated in PBS for 0.5 to 24 h at 37°C. The reaction was stopped by freezing at –70°C for later assays of biological activity, separation by HPLC, or SDS-PAGE analysis.

2.6. Fragment analysis

Proteolytic fragments were separated by SDS-PAGE (18% polyacrylamide containing 7.2 M urea) under reducing conditions (2% β -mercaptoethanol), and visualized by silver staining. For sequence analysis, proteins were separated by HPLC on a Brownlee Aquapore Butyl-column (2.1 mm \times 10 cm) using a gradient of 60% acetonitrile. NH_2 -terminal sequencing was performed by automated Edman degradation with a gas-phase sequencer (Model 477A, Applied Biosystems, Inc.).

2.7. Bioassays

The biological activity of chemokines was assessed in human neutrophils by determining enzyme release [14,16] or H_2O_2 formation during the respiratory burst [17].

3. Results

3.1. Neutrophil IL-8

A major peak of biologically active IL-8 was obtained by reversed-phase HPLC from the supernatants of LPS-stimulated neutrophils. Two forms were identified in this peak by N-terminal sequencing: About 60% of the total activity corresponded to IL-8(77) and 40% to IL-8(72). IL-8(77) is the predominant form found in cultures of fibroblasts, endothelial cells and synovial cells [18–21], while human monocytes largely process IL-8 to the IL-8(72) form [22,23] which is considerably more active [24].

3.2. Processing of IL-8(77) by granule enzymes

It was of interest to study possible modifications of IL-8(77), the predominant form synthesized in neutrophils, by a lysate of neutrophil granules, which contains serine-, thiol-, carboxy- and metallo-proteinases [12], and corresponds to the mixture of proteolytic activities that are liberated at sites of inflammation. As shown by SDS-PAGE, a cleavage product of IL-8(77) was obtained which comigrated with IL-8(72) (Fig. 1a). The conversion of IL-8(77) (AVLPSAKELRC...) to IL-8(72) (SAKELRC...) was confirmed by sequence analysis. It resulted

in a progressive increase in respiratory burst activity over 5 h (Fig. 1b). No intermediate sequence or further truncations were found after incubation for 5 h. The processing of IL-8(77) was prevented by diisopropyl fluorophosphate or $\alpha 1$ -proteinase inhibitor suggesting that a serine proteinase was involved. EDTA and leupeptin, which block metallo- and thiol-proteinases, respectively, had no effect.

3.3. Processing of IL-8(77) by purified proteinases

The effect of single neutrophil serine proteinases was also studied. No change in respiratory burst activity was observed after exposure of IL-8(77) to elastase or cathepsin G, but a marked increase was obtained with proteinase-3. This process was relatively slow, with a maximum increase of activity after 5 h (Fig. 2). Edman degradation analysis yielded the N-terminal sequence KELRC... indicating that proteinase-3 generates IL-8(70), the potent 70 amino acid form of IL-8. About 20% of IL-8(77) was converted to IL-8(70) in 5 h.

3.4. Processing of IL-8(72)

IL-8(72) was also exposed to a neutrophil granule lysate and purified proteinases, and changes in size and biological activity were monitored. Incubations of IL-8(72) with the granule lysate at pH values between 4 and 8 for up to 24 h, did not lead to the formation of cleavage products that could be assessed by SDS-PAGE. The biological activity of IL-8(72) decreased only slightly, i.e. on average by 7% after 5 h and 25% after 24 h (not shown). IL-8(72) was also incubated at neutral pH with samples of granule lysate that had been treated with aminophenylmercuric acetate to fully activate the metalloproteinases [25], but no changes in biological activity were observed, although the enzyme preparation readily degraded *N*-methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide (11.4 $\mu\text{mol/min}/\mu\text{g}$ granule lysate) and azocoll (3.2 $\text{mg/min}/\mu\text{g}$ granule lysate).

No cleavage products were apparent on SDS-PAGE, after incubation of IL-8(72) with human neutrophil elastase or cathepsin G, while degradation into smaller peptides was observed in the presence of trypsin or chymotrypsin (Fig. 3a). Despite the absence of detectable degradation products, elastase and cathepsin G led to a relatively slow, gradual loss of biological activity (Fig. 3b). By contrast, proteolytic degrada-

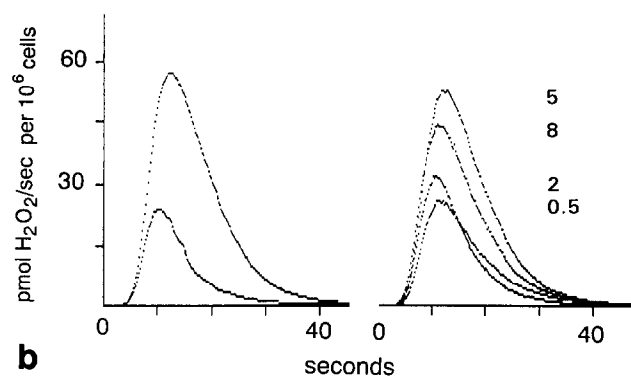
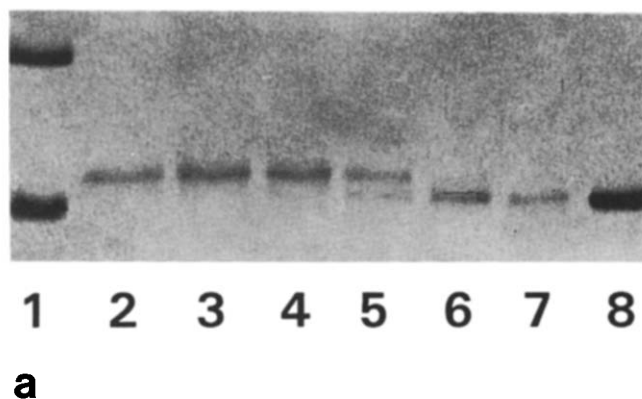


Fig. 1. Conversion of IL-8(77) to IL-8(72). 2 μM IL-8(77) was incubated with granule lysate (60 μg protein) in 1 ml PBS. (a) SDS-PAGE of samples withdrawn at time 0 (lane 2), and after 10 min (lane 3), 30 min (lane 4), 2 h (lane 5), 8 h (lane 6) and 24 h (lane 7). Lane 1, aprotinin (6.5 kDa) and lysozyme (14.4 kDa); lane 8, IL-8(72) standard. (b) Respiratory burst activity obtained with 10 μl samples withdrawn after 0.5, 2, 5 and 8 h (right panel), and of 10 μl 2 μM IL-8(72) and IL-8(77) (top and bottom, left panel). Separate experiments showed that granule lysate does not influence the respiratory burst response of neutrophils to IL-8(72) or IL-8(77). Similar results were obtained in four additional experiments.

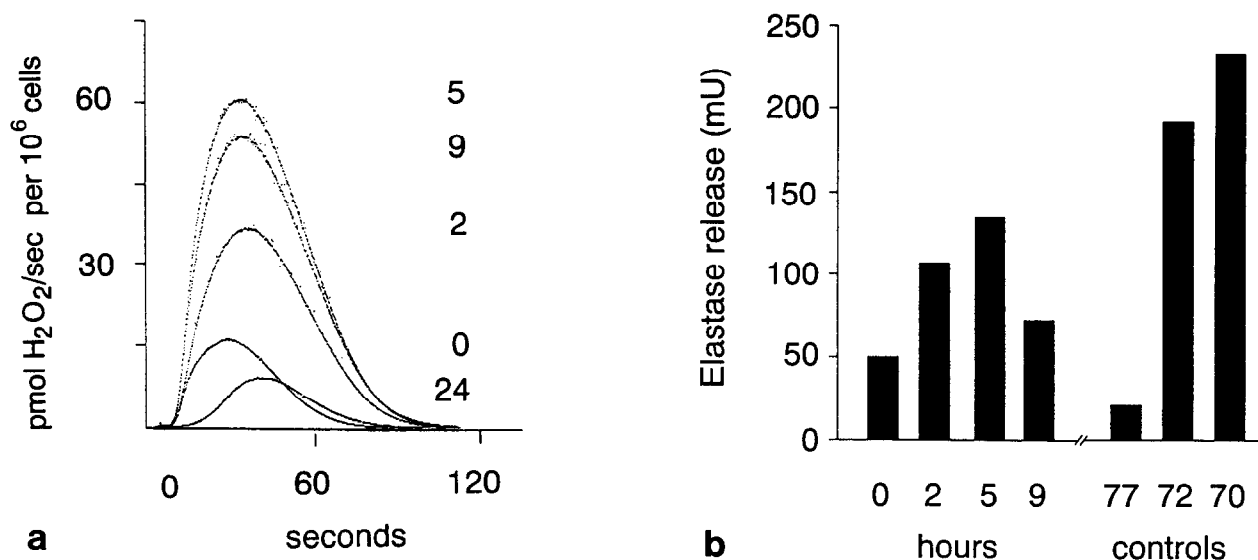


Fig. 2. Conversion of IL-8(77) to IL-8(70) by proteinase-3. 2 μ M IL-8(77) was incubated with 50 nM purified proteinase-3 in 1 ml PBS. (a) Respiratory burst activity obtained with 10 μ l samples withdrawn after 0, 2, 5, 9 and 24 h. (b) Elastase release induced in neutrophils by 1 μ l samples withdrawn after 2, 5 and 9 h. Control experiments were performed with 1 μ l samples of 2 μ M solutions of IL-8(77) (77), IL-8(72) (72) and IL-8(70) (70).

tion was very rapid and complete after denaturation of IL-8(72) by reduction and alkylation of the disulfide bonds. This result indicates that the tertiary structure confers to native IL-8(72) its marked resistance to proteolysis.

The proteolytic inactivation of IL-8(72) and the two related chemokines, NAP-2 and GRO α , was then compared. The biological activity of all three chemokines decreased as a first-order function of time of incubation. As shown by the half-life values, IL-8(72) was more susceptible than NAP-2 and GRO α to elastase and cathepsin G, but more resistant to trypsin and proteinase-3 (Table 1). NAP-2 was degraded rapidly by proteinase-3 and trypsin, but was not affected by chymotrypsin.

3.5. Competing substrates and inhibitors

To gain more information about the efficiency of chemokine degradation in inflamed tissues, the inactivation of IL-8(72) was assessed in the presence of natural substrates as well as α 1-proteinase inhibitor, which abound in the extracellular space. As shown in Fig. 4, inactivation of IL-8(72) by elastase and cathepsin G was prevented by fibrinogen and was retarded considerably by elastin. By contrast type-I collagen, which has only low affinity for elastase and is not a substrate of cathepsin G, inhibited the degradation of IL-8(72) by elastase only slightly, and did not influence degradation by cathepsin G.

Expectedly, the effect of elastase, cathepsin G, trypsin and chymotrypsin was prevented by α 1-proteinase inhibitor which is known irreversibly to block serine proteinases (not shown).

4. Discussion

Proteinases present in neutrophil granule lysates were found to convert IL-8(77) into IL-8(72) resulting in enhanced biological activity. N-terminal processing of IL-8(77) was also observed with proteinase-3 which, however, generated IL-8(70), a form that is about 10-fold more potent [24]. The conversion of the 77 into the 70 amino acid form of IL-8 implies the cleavage of an Ala-Lys bond, which is in agreement with the results of a former study showing that proteinase-3 acts preferentially on bonds that link small aliphatic amino acids to other residues [26]. Although elastase shares with proteinase-3 a substantial degree of active site homology and a similar substrate specificity [26], it did not convert IL-8(77) to more potent derivatives. The conversion of IL-8(77) to IL-8(70) was not observed with neutrophil granule lysates, and no IL-8(70) was found in supernatants of neutrophils stimulated with LPS. This suggests that proteinase-3 may not be present in sufficient amounts in the lysate or may be inactivated by other enzymes or inhibitors. The neutrophil granule enzymes elastase, cathepsin G and

Table 1
Interaction of proteinases with IL-8, NAP-2 and GRO α *

	Cathepsin G		Elastase		Chymotrypsin		Trypsin		Proteinase-3	
	<i>k</i> (h ⁻¹)	<i>t</i> _{1/2} (h)	<i>k</i> (h ⁻¹)	<i>t</i> _{1/2} (h)	<i>k</i> (h ⁻¹)	<i>t</i> _{1/2} (h)	<i>k</i> (h ⁻¹)	<i>t</i> _{1/2} (h)	<i>k</i> (h ⁻¹)	<i>t</i> _{1/2} (h)
IL-8	0.306	2.26	0.174	3.98	0.076	9.34	0.067	10.35	0.040	17.03
NAP-2	0.083	8.24	0.101	6.94	—**	—	2.650	0.26	2.650	0.22
GRO- α	0.029	24.40	0.086	7.94	0.104	6.67	0.418	1.66	0.310	2.26

*Apparent first order inactivation rate constants (*k*) and half times of inactivation (*t*_{1/2} = 0.693/*k*) of IL-8, NAP-2 and GRO α by proteinases. 50 nM enzyme was incubated with 200 nM substrate in 1 ml PBS. Samples of 100 μ l were withdrawn at times between 0 and 24 h at 37°C, and activity was assessed as release of elastase or *N*-acetyl- β -glucosaminidase.

**NAP-2 is not affected by chymotrypsin.

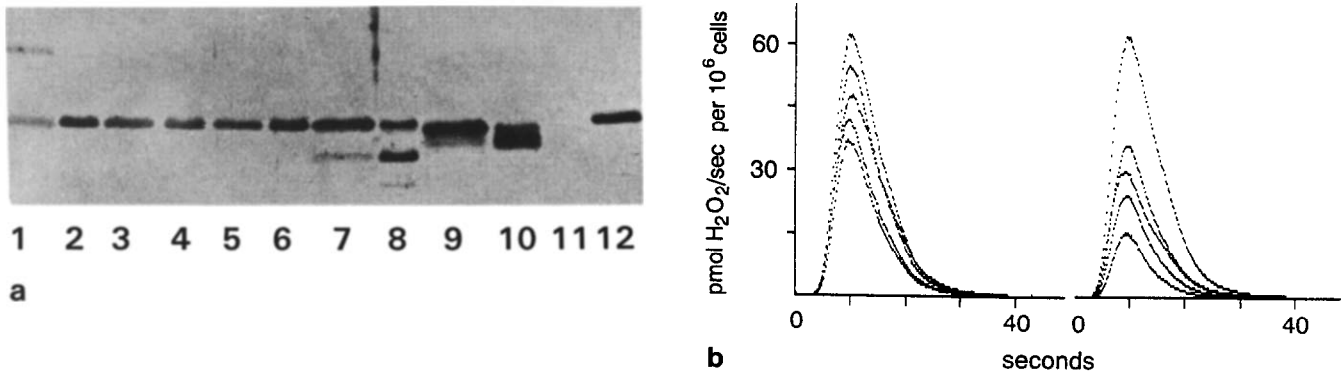


Fig. 3. Effect of proteinases on IL-8(72). 5 μ M IL-8(72) was incubated with 50 nM of the purified enzymes in 0.1 ml PBS. (a) SDS-PAGE analysis of 50 μ l samples withdrawn at time 0 (lane 2); 5 h (lanes 3, 5, 7, 9, 11) or 24 h (lanes 4, 6, 8, 10). The enzymes were: elastase (lanes 3, 4), cathepsin G (lanes 5, 6), chymotrypsin (lanes 7, 8) and trypsin (lanes 9, 10). Lane 11, reduced IL-8(72) incubated with 50 nM elastase; lane 12, reduced IL-8(72) without proteinase. Lane 1, aprotinin (6.5 kDa) and lysozyme (14.4 kDa). (b) Respiratory burst activity obtained with 10 μ l samples withdrawn after 5 h (left) or 24 h (right) incubation with PBS, trypsin, chymotrypsin, elastase or cathepsin G (top to bottom). Similar results were obtained in two additional experiments.

proteinase-3 also inactivated NAP-2 and GRO α . Inactivation by granule lysates was markedly slower than that observed with pure proteinases, suggesting that enzymes and other proteins present in the lysate may act as competing substrates.

The relatively rapid processing of IL-8(77) and relatively slow inactivation of IL-8(72) suggest that the enzymes released in inflamed tissues by activated neutrophils tend to enhance, rather than decrease the activity of IL-8 and possibly other chemokines. In addition to tissue cells and mononuclear phagocytes, stimulated neutrophils themselves have the capacity to produce IL-8 and thus to reinforce their own recruitment [6,7]. The conversion of IL-8(77) to IL-8(72) and/or IL-8(70) will amplify chemotactic activity, and further enhance the process of neutrophil recruitment.

The three-dimensional structure of IL-8 comprises a short, conformationally disordered N-terminus followed by three antiparallel β -strands leading, via a β -turn, to a C-terminal α -helix [27,28]. Several putative cleavage sites for elastase, cathepsin G and proteinase-3 are present in the region of the β -strands, between the second and fourth cysteine (Fig. 5). They are unlikely to be accessible to proteolytic attack in the folded molecule, but become exposed when IL-8 unfolds, explaining the rapid degradation observed upon reduction of the disulfide bonds.

The N-terminal region is less protected than other parts of the IL-8 molecule, and this could explain the relatively efficient conversion of IL-8(77) to IL-8(72) and IL-8(70) by neutrophil proteinases. It has been shown that the sequence Glu-Leu-Arg preceding the first cysteine is essential for IL-8 receptor binding and biological activity [24,29]. It is interesting to note that neutrophil proteinases readily process the extended N-terminus of IL-8(77), but are much less active on IL-8(72), and are apparently unable to cleave the receptor-binding Glu-Leu-Arg domain. This region must be sterically protected since the Leu-Arg bond, a putative cleavage site of cathepsin G, remains intact. The N-terminal domain of other chemokines related to IL-8 also appears to be particularly susceptible to proteolytic attack. In a former study, we showed that connective tissue-activating peptide III is readily converted to neutrophil-activating proteins (NAP-2 and variants) by N-terminal processing through serine proteinases from neutrophils and monocytes

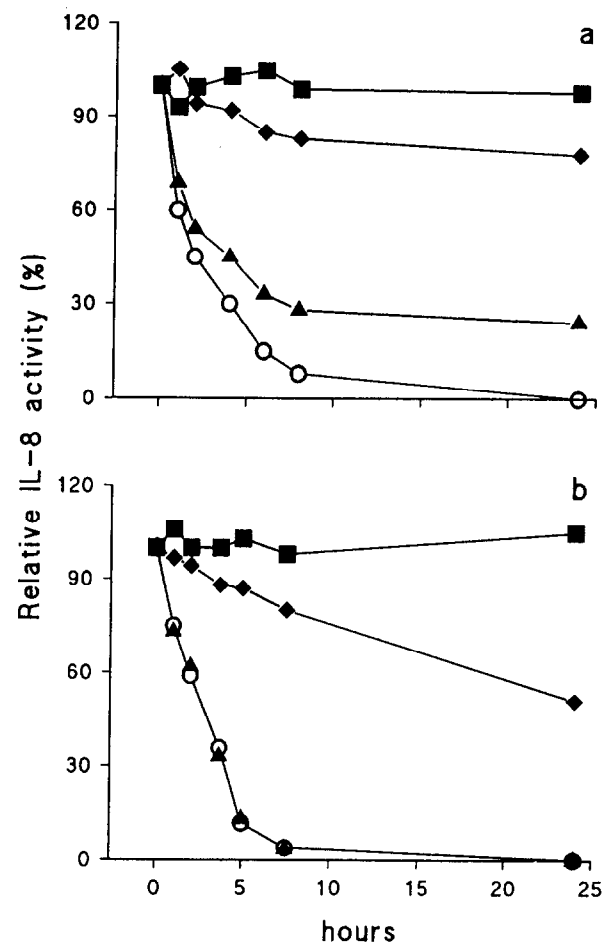


Fig. 4. Effect of added proteinase substrates on IL-8(72) degradation. 50 nM elastase (a) or cathepsin G (b) were incubated with 8 mg proteinase substrate and 200 nM IL-8(72) in 1 ml PBS. Exocytosis of N-acetyl- β -glucosaminidase (a) or elastase (b) induced in neutrophils by 1 μ l samples withdrawn at the times indicated. The proteinase substrates were: Fibrinogen (■), elastin (◆), type-I collagen (▲). Control incubations were performed in the absence of additional substrate (○). Separate experiments showed that fibrinogen, elastin and type-I collagen did not influence the response of neutrophils to IL-8(72). Similar results were obtained in four additional experiments.

Cleavage sites

► Elastase + Proteinase-3

▷ Proteinase-3

➤ Cathepsin G

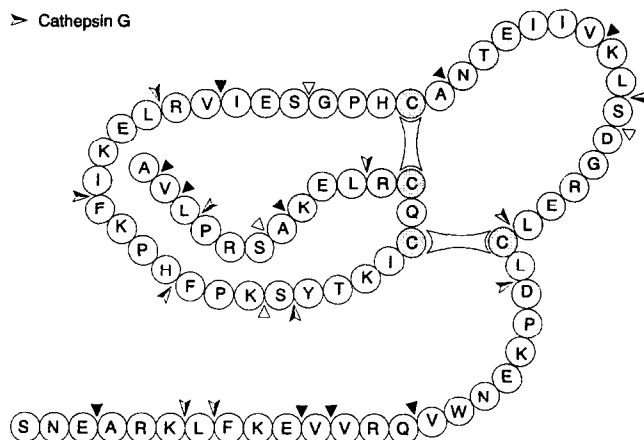


Fig. 5. Structure of IL-8(77). Amino acid sequence and putative cleavage sites for elastase, cathepsin G, proteinase-3 and neutrophil granule enzymes.

[30]. Furthermore, it was recently shown that IL-8(77) is converted to IL-8(72) by thrombin [21] and plasmin [31].

The present study was not designed to assess the proteolytic degradation of the C-terminal helix. It is known, however, that the α -helix can be shortened by two turns without loss of activity and that IL-8 remains biologically active even upon elimination of the complete C-terminal sequence following the last cysteine [24].

Two properties qualify IL-8 as particularly suited as a mediator of inflammation, its production by cells of inflamed tissues and by immigrant leukocytes, and its local persistence in active form. In this respect, IL-8 and related chemokines differ radically from other chemotactic agonists, like C5a, fMet-Leu-Phe, LTB₄ and PAF, which are rapidly inactivated by oxidation or hydrolysis. The present evidence for the low effectiveness of neutrophil proteinases in the inactivation of IL-8, and for their potential role in the formation of biologically more potent forms by N-terminal processing, uncovers a mechanism for conserving IL-8 dependent chemotactic activity at sites of inflammation.

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