Synthesis of a trihexacontapeptide corresponding to the sequence 8–70 of eglin c and studies on the relationship between the structure and the inhibitory activity against human leukocyte elastase, cathepsin G and α-chymotrypsin

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A trihexacontapeptide corresponding to the sequence 8–70 of eglin c and its related peptides were synthesized by the conventional solution method and their inhibitory activity against human leukocyte elastase, cathepsin G and α-chymotrypsin was examined. Although synthetic eglin c (41–49) inhibited cathepsin G and α-chymotrypsin ($K_i = 4.0 \times 10^{-5}$ M and 2.0×10^{-5} M, respectively) but not leukocyte elastase, the synthetic trihexacontapeptide potently inhibited cathepsin G, α-chymotrypsin and leukocyte elastase ($K_i = 1.8 \times 10^{-9}$ M, 1.4×10^{-9} M and 2.2×10^{-9} M, respectively). The relationship between the stucture of eglin c and the inhibitory activity against the above enzymes is also described.

Eglin c related peptide; Chemical synthesis; Inhibitory activity; Leukocyte elastase; Cathepsin G; Structure–activity relationship

1. INTRODUCTION

Eglin c, isolated from the leech *Hirudo medicinalis*, consists of 70 amino acid residues [1] and inhibits chymotrypsin and subtilisin as well as leukocyte elastase and cathepsin G. The latter two enzymes have attracted our interest due to their possible involvement in connective tissue turnover and diseases such as inflammation, rheumatoid arthritis and emphysema [2]. Therefore, eglin c is a potential candidate for the therapeutic treatment of emphysema and inflammation. Rink et al. prepared N^{α}-acetyleglin c by means of genetic techniques [13], although its molecular weight is too large for practical therapeutic use. Previously, Bode et al. [4] and McPhalen and James [5] reported that the nine residues of the binding loop (40–48) of eglin c are involved in direct contact with subtilisin as a result of the determination of the crystal structure of the complex formed between eglin c and subtilisin Carlsberg by X-ray analysis. It was also reported that eglin c present in the complex with subtilisin is shortened N-terminally by 7 amino acid residues [4]. This observation indicates that eglin c derivatives shortened from the N-terminus by up to 7 amino acid residues should be active as an inhibitor. In fact, eglin c derivatives consisting of positions 5–70 and 7–70 which were prepared enzymatically using cathepsin C, exhibited the same equilibrium dissociation constants for the interaction of eglin c derivatives with chymotrypsin as that of eglin c [6].

This paper deals with the synthesis of a trihexacontapeptide corresponding to the sequence 8–70 of eglin c and its related peptides and the examination of their inhibitory activity against leukocyte elastase, cathepsin G and α-chymotrypsin in order to study the relationship between the structure of eglin c and inhibitory activity.

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Abbreviations: Z, benzyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; OBzl, benzyl ester; Mts, mesytilenesulphonyl; Bom, benzyloxymethyl; Suc, succinyl; *p*NA, *p*-nitroanilide

2. MATERIALS AND METHODS

A trihexacontapeptide was synthesized by the fragment condensation method employing the minimal protection method. According to the route shown in Fig. 1, 14 peptide fragments (1–14) were prepared by a stepwise condensation method. A benzyl ester group on β- or γ-carboxyl group of Asp or Glu was removed by catalytic hydrogenation prior to use for fragment condensation. Each peptide fragment was condensed successively by the azide method to minimize racemization [7]. The protected trihexacontapeptide and related peptides were purified by reprecipitation from DMF/MeOH and/or column chromatography on Sephadex LH-60 and their homogeneity was ascertained by TLC, amino acid analysis and elemental analysis. The final deprotection was performed by the HF method [8] using thioanisole and *m*-cresol as scavengers [9]. The product obtained as the HF salt was converted into an acetate form by treatment with Amberlite IRA 45 (acetate form) and was purified by gel-filtration on Sephadex G-50 using 3% AcOH as an eluant, followed by preparative HPLC. The homogeneity of the peptides obtained was ascertained by amino acid analysis and HPLC. Full details of the synthesis and characterization of the peptides will be published elsewhere (Y. Okada et al., in preparation). N^{α}-acetyleglin c was a generous gift from Drs. H.H. Peter and K. Scheibli of CIBA-GEIGY Ltd., Basel.

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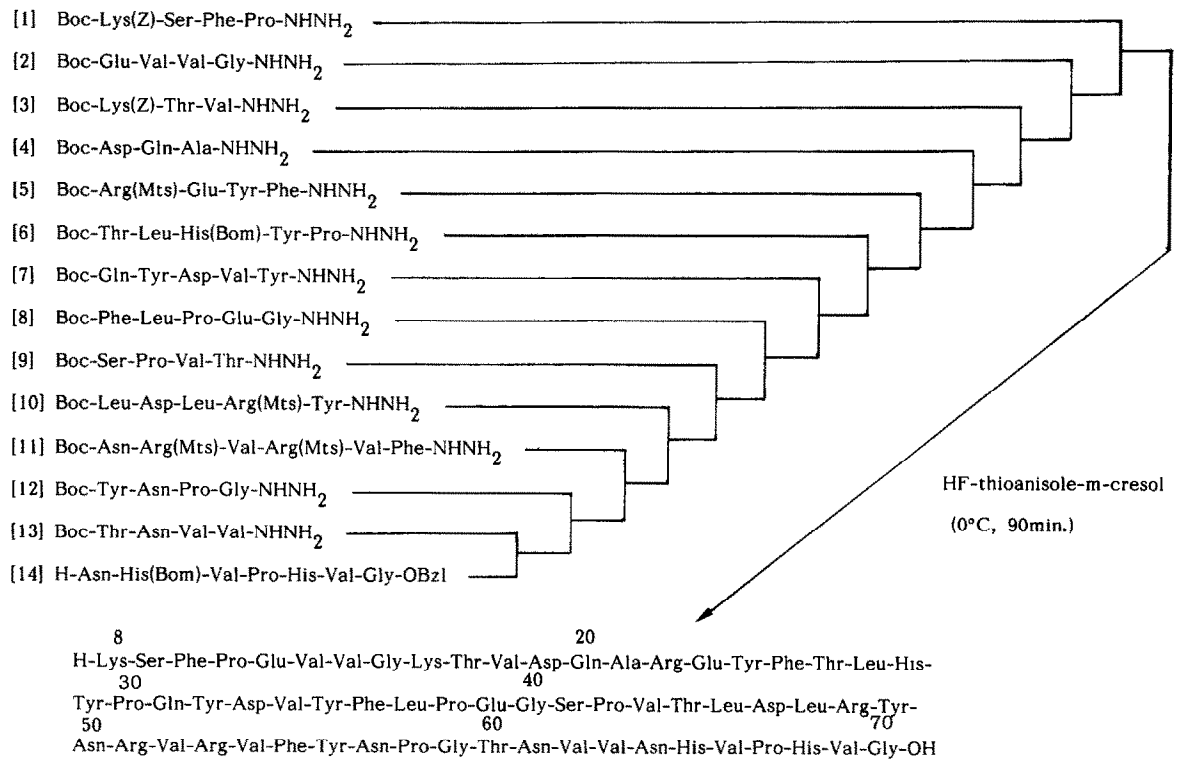


Fig. 1. Synthetic scheme for eglin c (8–70).

Human leukocyte elastase [10] and cathepsin G [11] were prepared in our laboratory by newly developed affinity chromatography. α-Chymotrypsin was purchased from Miles Co. Ltd., Elkhart.

Assay of leukocyte elastase, cathepsin G and α-chymotrypsin: Enzymatic activities were determined by the method described previously [10] using Suc-Ala-Tyr-Leu-Val-*p*NA [12] for leukocyte elastase and Suc-Ile-Pro-Phe-*p*NA [13] for cathepsin G and α-chymotrypsin.

Effects of synthetic peptides on the enzymes: Enzymatic activity was assayed in the presence or absence of the peptide examined. K_i values for the trihexacontapeptide were calculated according to the method previously described [14] and for the other peptides according to the method previously described [15].

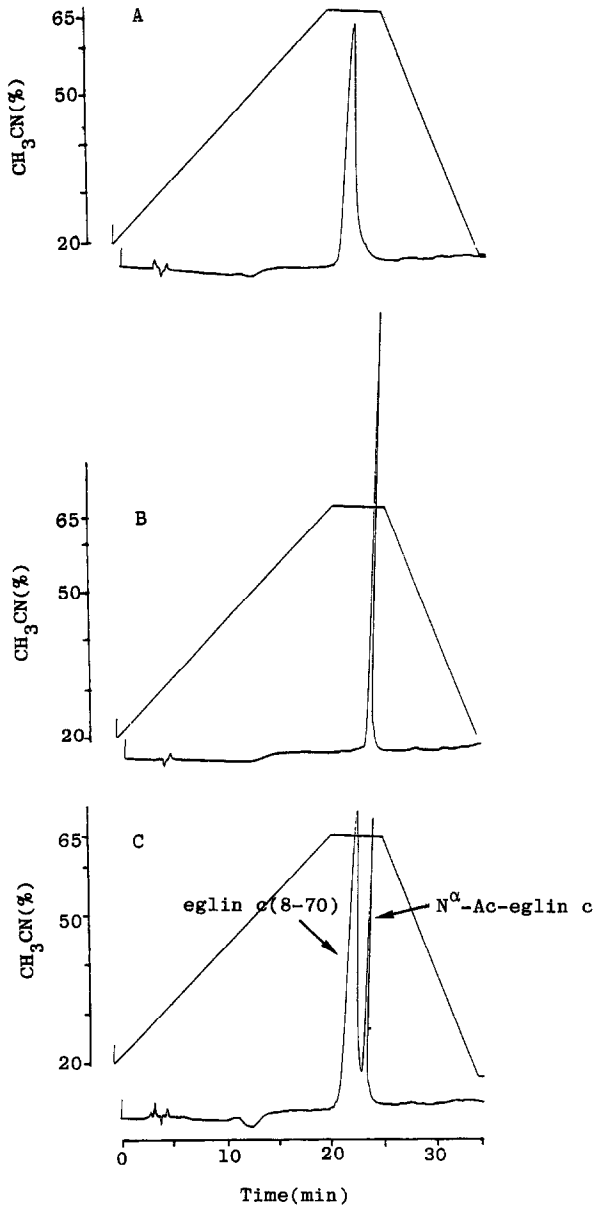
3. RESULTS AND DISCUSSION

The synthetic trihexacontapeptide exhibited a symmetrical single peak on HPLC as shown in Fig. 2A. The amino acid ratios in acid hydrolysates (6 M HCl, 110°C, 18 h; the Val-Val bond resisted acid hydrolysis; therefore the value for the Val residue was corrected) of the peptides obtained were in agreement with the expected values (data not shown).

As reported previously [16,17], a synthetic nonapeptide corresponding to the sequence 41–49 of eglin c (eglin c (41–49)), which is a binding loop of eglin c with subtilisin [4], inhibited cathepsin G and α-chymotrypsin, of which the enzymatic similarity was previously reported [18], ($K_i = 4.0 \times 10^{-5}$ M and 2.0×10^{-5} M, respectively) but not leukocyte elastase. Meanwhile, a synthetic eglin c (60–63) inhibited leukocyte elastase with a K_i value of 1.6×10^{-4} M, but not cathepsin G and α-chymotrypsin [16]. These results indicated that the interacting site of eglin c with leukocyte elastase and cathepsin G and α-chymotrypsin might be different, although eglin c itself potently inhibited the above three enzymes. It is of interest to clarify which part of eglin c is required to inhibit the above three enzymes.

As shown in Fig. 3, eglin c (60–70) inhibited only leukocyte elastase, with a K_i value of 1.7×10^{-3} M, which is higher than that of eglin c (60–63). Eglin c (50–70) and eglin c (45–70) inhibited leukocyte elastase and α-chymotrypsin but not cathepsin G, implying some differences between cathepsin G and α-chymotrypsin. Eglin c (41–70) could inhibit leukocyte elastase, cathepsin G and α-chymotrypsin ($K_i = 1.2 \times 10^{-4}$ M, 2.1×10^{-4} M and 7.0×10^{-6} M, respectively). Besides the reactive site of eglin c, Leu^{45}-Asp^{46} [1,4], the peptide part corresponding to the sequence 41–44 might be important for the manifestation of inhibitory activity against cathepsin G. This phenomenon is compatible with that in the case of eglin c (41–49) [16,17].

K_i values for the inhibition of eglin c (41–49) against cathepsin G and α-chymotrypsin are $10^{5\text{-}6}$-times larger than those for eglin c [16,17]. In the eglin c molecule, Thr^{44}, Asp^{46} and Arg^{48} form electrostatic and hydrogen bonds with Arg^{53}, Arg^{51} and Gly^{70}, respectively, to stabilize the reactive site [4]. However, the eglin c (41–49) does not have such electrostatic and hydrogen bonds needed to maintain the comfortable and rigid conformation necessary for interaction with enzymes. This is a possible reason as to why the K_i values of eglin c (41–49) are much larger than those of eglin c. The K_i



values of eglin c (41–70) for cathepsin G and α-chymotrypsin are also similar to those of eglin c (41–49), indicating that this peptide fragment cannot form a proper conformation with electrostatic and hydrogen bonds.

Eglin c (31–70) and eglin c (22–70) inhibited the above three enzymes, but the K_i values were similar to those of eglin c (41–70). Even in eglin c (22–70), electrostatic and hydrogen bonds cannot be formed to maintain a three-dimensional structure suitable for interaction with the enzymes.

It is of interest that the synthetic eglin c (8–70) inhibited the above enzymes as potently as N^{α}-acetyleglin c, as shown in Fig. 3, supporting the result of the X-ray analysis of the eglin c-subtilisin complex [4].

Previously, it was reported that incubation of native eglin c with chymotrypsin for 90 min gave three main components, inactive complex (molecular mass: about 30 000), native eglin c and a peptide which was identified as the C-terminal fragment of eglin c comprising position 46–70 [1]. After preincubation of N^{α}-acetyleglin c with α-chymotrypsin or cathepsin G for 90 min, K_i values towards the above enzymes decreased significantly, indicating that a covalent complex formed between N-terminal fragment [N^{α}-acetyleglin c (1–44)] and enzyme was stable. However, in the case of eglin c (41–49) with cathepsin G or α-chymotrypsin, enzyme activity regenerated in proportion to the increment of preincubation time, suggesting that a covalent complex formed was not so stable and hydrolyzed rather soon

Fig. 2. HPLC of eglin c (8–70) and N^{α}-Ac-eglin c. (A) eglin c (8–70); (B) N^{α}-Ac-eglin c; (C) eglin c (8–70) + N^{α}-Ac-eglin C. Column, YMC-PACK R-C8-5 (4.6 × 250 mm); solvent, A = H_2O (0.05% TFA) B = CH_3CN (0.05% TFA) gradient (A/B 80/20 – 20 min→35/65 – 5 min→35/65 – 15 min→80/20); flow rate, 1.0 ml/min; absorbance measurement, 220 nm.

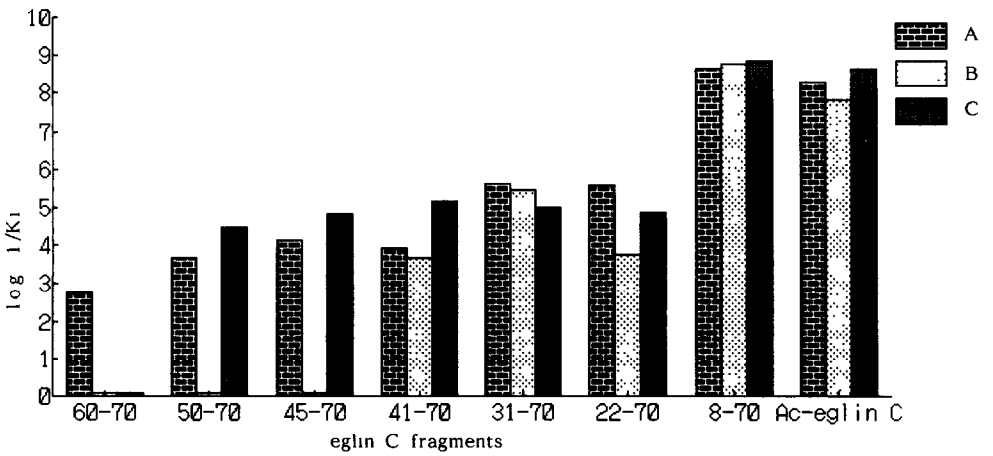


Fig. 3. Inhibitory effects of eglin c fragments and N^{α}-Ac-eglin c on leukocyte elastase, cathepsin G and α-chymotrypsin. (A) Leukocyte elastase; (B) cathepsin G; (C) α-chymotrypsin.

(unpublished data in our laboratory). From these results, it was deduced that eglin c derivatives prepared above were also substrates for enzymes, although in the short assay used above for determination of K_i values of synthetic peptides, the split of the reactive site, Leu^{45}-Asp^{46}, was negligible.

In conclusion, it was suggested that besides the reactive site in proteinase inhibitor, some amino acid sequences are required to form a three-dimensional structure for manifestation of full inhibitory activity by means of the increase of the facility of formation of enzyme-inhibitor complex and the increase of the stability of a covalent complex.

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