

The Salivary Lipocalin Von Ebner's Gland Protein Is a Cysteine Proteinase Inhibitor*

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The lipocalins make up a heterogeneous superfamily of proteins. Although showing almost no sequence homology, they share very similar secondary and tertiary structures. Their ability to bind hydrophobic ligands is well established, but the physiological function of most lipocalins remains unclear. The lipocalin from the human Von Ebner's Gland of the tongue (VEGh) contains three sequence motifs corresponding with the papain-binding domains of cystatins, a family of naturally occurring cysteine proteinase inhibitors. We found that VEGh inhibited papain activity to a similar extent as salivary cystatin S. Furthermore, synthetic peptides derived from VEGh and cystatin C, comprising these three motifs, inhibited papain, too. We conclude that VEGh is a physiological inhibitor of cysteine proteinases and therefore can play a role in the control of inflammatory processes in oral and ocular tissues.

VEGh¹ is a salivary protein secreted by the Von Ebner's glands located around the circumvallate and foliate papilla of the human tongue that belongs to the lipocalin superfamily (1). The members of the lipocalin protein family possess very similar structural features despite a nearly complete absence of sequence homology. They have a similar subunit molecular mass (approximately 20 kDa), and membership of the lipocalin family is predominantly based on secondary and tertiary structure homology. Generally, in the secondary structure of lipocalins nine β -strands and a single α -helix linked by β -turns and short flexible fragments can be recognized (see Fig. 1A). The β -sheets are bundled to form a calyx-shaped molecule with a hydrophobic pocket, which provides a binding site for various small hydrophobic molecules (2). Because of the ability to bind hydrophobic ligands, it has been suggested that the primary function of lipocalins is to accommodate lipophilic molecules in a hydrophilic environment, allowing transport of these molecules through body fluids toward their physiological receptors. To what extent this property is relevant for the *in vivo* function is largely unclear. The hypothesis has been put forth that the salivary VEGh protein is involved in perception of bitter taste by binding lipophilic bitter compounds and transporting them

to the taste buds (3). On the other hand, the VEGh protein in tears, designated as tear-specific prealbumin (TSP) (4) or tear lipocalin (5), has been implicated in binding and transport of retinol to the corneal epithelium, similar to another lipocalin called retinol-binding protein. Others have suggested that VEGh has a protective function and that the retinol-carrier function may be justified by the bacteriostatic activity of retinol (3, 5, 6).

The only amino acid sequence shared by almost all lipocalins is the GNWY motif at the N terminus of the first β -strand. The tryptophan residue in this motif is strictly conserved throughout the lipocalin superfamily and is essential for the binding of lipophilic ligands (2). Closer inspection of the amino acid sequence learns that in the VEGh protein this sequence is part of a larger motif, QDVSGTWY, which includes the motif QNVNG. This latter motif is a well conserved domain in the family 2 cystatins (see Fig. 2) that is essential for their biological activity as physiological inhibitors of cysteine proteinases. Cystatins are ubiquitously present in human tissues and secretions (7). They have antibacterial (8) and antiviral (9, 10) properties and, by inhibiting bacterial and host proteinases, are involved in the control of inflammatory processes (7, 11). In cystatins three domains have been identified that are crucial for their cysteine proteinase inhibitory activity. The first domain (see Figs. 1 and 2) is located closely to the N terminus of the proteins and consists of four lipophilic residues, of which the third is a highly conserved glycine (12, 13). The second domain is located in the first hairpin loop and consists of the QNVNG motif. The third domain is located in the second hairpin loop and contains a highly conserved tryptophan residue. Although these three domains are distant in the amino acid sequence, three-dimensionally they are closely arranged. Strikingly, in VEGh three analogous domains can be recognized, which in this case are close together in the N-terminal amino acid sequence (Figs. 1 and 2). Apart from these three domains VEGh shares no sequence homology with cystatins. The presence of these analogous domains tempted us to investigate whether VEGh can exert cystatin-like effects, *e.g.* inhibit cysteine proteinases.

EXPERIMENTAL PROCEDURES

TSP was obtained as a gift from Dr. P. T. Janssen and Dr. O. P. van Bijsterveld (14). Polyclonal antibodies against TSP were obtained as a gift from Dr. A. M. F. Gachon. The recombinant cystatin S was obtained as a gift from Dr. E. Saitoh and Dr. S. Isemura (15), and the recombinant cystatin C was a gift from Dr. A. Grubb and co-workers (16). Dimethyl sulfoxide was obtained from Merck, the other chemicals were obtained from Sigma.

VEGh was isolated from human tears. Tear secretion was stimulated by aiming a stream of air at the eye, and the fluid was collected from the corner of the eye with a soft silicone tubing. Samples from six donors were pooled.

Size exclusion chromatography was performed on a Sephacryl S-100 HR column (1.5 $\times$ 90 cm, Pharmacia Biotech Inc.). Tear samples (1.5 ml, 6.5 mg/ml) were applied and eluted with 0.1 M ammonium acetate buffer, pH 7.0, at a flow rate of 12 ml/h with spectrophotometrical

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¹ The abbreviations used are: VEGh, human Von Ebner's gland protein; Fmoc, 9-fluorenylmethoxycarbonyl; TSP, tear-specific prealbumin, identical with VEGh; PAGE, polyacrylamide gel electrophoresis.

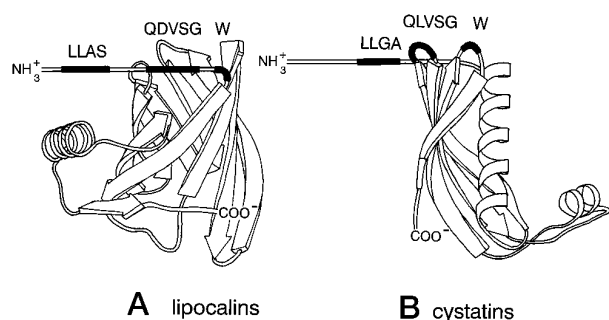


FIG. 1. **Three-dimensional structure drawings of lipocalins and cystatins.** Both N-terminal sequences have little conformational constraints and are drawn as linear stretches. In the family 2 cystatins (B) (28), three domains have been identified that are responsible for binding to cysteine proteinases. These domains are represented by *dark blocks* and the corresponding sequences in the figure. Domain 1, with the sequence LLGA (chicken egg white cystatin taken as example) is located near the N terminus; domain 2, with the sequence QLVSG, is located in the first hairpin loop between the first and second β -strands (represented in the figure by *arrows*); and domain 3, containing a highly conserved tryptophan residue (W), is located in the second hairpin loop between the third and the fourth β -strands (12). These domains are located far apart in the amino acid sequence, separated by 40 residues (Fig. 2) but spatially close together in the tertiary structure. VEGH has a lipocalin organization (A) (29), with eight antiparallel β -strands that form a calyx-shaped molecule with a hydrophobic cavity in which small hydrophobic molecules can be bound, completely different from the cystatin three-dimensional structure. In VEGH three analogous cysteine proteinase-binding domains are also found, sequentially contiguous and located near the N terminus. They appear in the same order as in synthetic peptides, derived from cystatins, with cysteine proteinase-inhibiting properties (24), suggesting that VEGH exhibits cystatin activity.

detection at 280 nm. Fractions were collected (2 ml) and checked for purity by SDS-PAGE. Peak fractions were pooled, lyophilized, and reconstituted in water. All peaks were tested in the papain inhibition assay.

Ion exchange chromatography was performed on fast protein liquid chromatography with a Resource Q-column (1-ml bed volume, Pharmacia). The VEGH-containing fractions (± 20 ml, ± 5 mg) from five size exclusion chromatography runs were pooled and dialyzed against 0.05 M Tris buffer, pH 8.4, applied to the column, and eluted with a linear 0–0.8 M NaCl gradient in 15 min (1 ml/min). Peak fractions were pooled, dialyzed against water, and tested in the papain inhibition assay.

Peptides were synthesized using the T-bag method adapted for Fmoc-strategy as described by Beck-Sickinger *et al.* (17) and Van 't Hof *et al.* (18). Fmoc-protected amino acids were obtained from Novabiochem (Läufelfingen, Switzerland).

Papain activity was determined with α -N-benzoyl-DL-arginine- β -naphthylamide as substrate (19) in a protocol adapted for use in a microtiterplate assay as described in Henskens *et al.* (20).

SDS-PAGE and isoelectrofocusing analysis, followed by silver staining, were performed with the PhastSystem (Pharmacia) following the standard procedures described in the PhastSystem manual.

Amino acid analysis was performed by hydrolysis in 6 M HCl (24 h, 110 °C), followed by precolumn derivatization with fluorenylmethyl chloroformate (Fmoc-Cl). After separation by reversed-phase high pressure liquid chromatography the amino acid derivatives were determined spectrofluorometrically (excitation wavelength, 254 nm; detection wavelength, 630 nm).

For amino acid sequence analysis, after SDS-PAGE followed by blotting to Immobilon polyvinylidene difluoride membrane (Millipore, Bedford, MA) and staining with Coomassie Brilliant Blue to localize the VEGH protein, the band was excised and directly subjected to automatic Edman degradation in a gas-phase protein sequencer (Applied Biosystems, model 473A) for 11 or 12 degradation cycles according to the manufacturer's instructions.

RESULTS

To verify our hypothesis we tested the papain inhibitory activity of VEGH, purified from human tears by size exclusion

and ion exchange chromatography, similar to the method of Selsted and Martinez (6) and compared it with the activity of the sample obtained from Janssen and Van Bijsterveld (14). Inhibition assays showed that the inhibitory activity comigrated with the protein throughout the purification process (Figs. 3A and 4A). After size exclusion chromatography of a human tear sample, a number of peaks were obtained. After pooling of the corresponding fractions, in each of these peaks the cystatin activity was determined. Only three pools displayed papain inhibitory activity (Fig. 3A). These pools were analyzed in SDS-PAGE and immunoblotting (data not shown). Pool I contained a mixture of high molecular weight proteins, most likely including kininogens, members of the cystatin family 3. Pool II contained VEGH. Pool III contained the low molecular weight family 2 cystatins, as deduced from the apparent M_r of 14,000 on SDS-PAGE. The relatively higher inhibitory activity of the latter can probably be explained by the presence of cystatin species, *e.g.* cystatins C and D, that display much higher affinity toward papain (7). After further purification by ion exchange chromatography, the VEGH-containing fraction inhibited papain activity as strongly as cystatin S did (Fig. 4A).

The purity and identity of the VEGH preparation was verified to ascertain that the inhibitory effect was not caused by any minor contamination of cystatins or other papain inhibiting proteins but by VEGH itself. For both VEGH preparations one single band was observed on SDS-PAGE at the M_r of 18,000, no cystatin-like bands were observed at the M_r of 14,000 (Fig. 3C). Western blot analysis showed that crude tear fluid, the VEGH-containing fractions collected after size exclusion chromatography, the purified preparation, and the Janssen and Van Bijsterveld preparation, reacted with polyclonal antibodies against TSP, whereas no cross-reaction with a monoclonal antibody against cystatin S (21), the most prominent cystatin in human tears, was observed (data not shown). Furthermore, the purity of the sample was demonstrated by isoelectrofocusing analysis, in which in the preparation of Janssen and Van Bijsterveld only one small extra band was observed when a large amount of VEGH was applied (Fig. 3D). Amino acid analysis was in good agreement with the amino acid sequence of VEGH (22) for both preparations (data not shown). Sequence analysis of the 18-kDa band showed that our VEGH preparation contained a single protein with the N-terminal sequence HHLLASDEEIQD, identical with the N-terminal sequence of VEGH deduced from cDNA sequence analysis (1). The Janssen and Van Bijsterveld preparation contained a mixture of three proteins in a 1:4:2 molar ratio. The first N-terminal sequence, HHLLASDEEIQ, matched the VEGH sequence; the second N-terminal sequence, ASDEEIQDVSG, has not been reported yet and may correspond to one of the not yet fully characterized isoforms of this protein; and the third N-terminal sequence, SDEEIQDVSGT, corresponded to the TSP isoform tear lipocalin 17/4.9 (23). All four N-terminal sequences corresponded to the sequence of VEGH, and none corresponded to the sequence of any of the known cystatins. Two of these sequences contained the QDVSG motif (and most probably the tryptophan residue), two contained the LLAS motif (and most probably the QDVSG motif and the tryptophan residue), explaining the papain inhibitory capacity of this mixture.

The involvement of these domains in the inhibition of papain activity by VEGH was corroborated by inhibition studies with chemically synthesized peptides. By organic chemical synthesis two synthetic peptides were prepared, one comprising residues 3–21 of VEGH with the sequence LLASDEEIQDVSGTWYLKA, including the LLAS and QDVSG and TWY motifs, and one with the sequence KLVGGAQIVAGAPWLK, containing the LVGG,

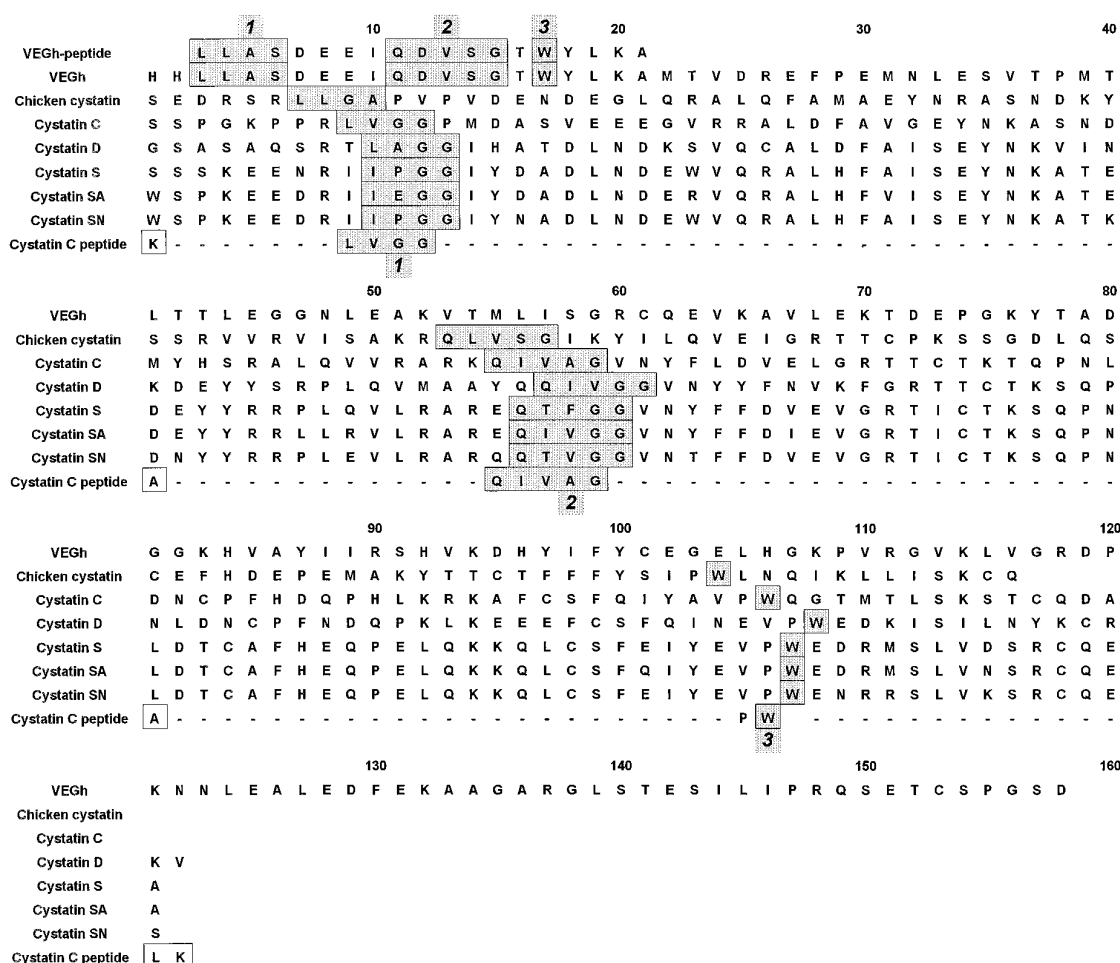


FIG. 2. Amino acid sequences of human Von Ebner's gland protein, chicken egg white cystatin, and the family 2 salivary cystatins. The papain-binding domains of cystatins (7) are shaded and numbered (bottom), as well as the homologous sequence motifs of VEGH (1) (top). Sequence motif 1 of cystatins, LVGG for cystatin C and LLGA for chicken egg white cystatin, corresponds to the LLAS sequence of VEGH, motif 2, QIVAG for cystatin C and QLVSG for chicken egg white cystatin, corresponds to the QDVSG sequence of VEGH, and the crucial residue of motif 3, tryptophan 106 of cystatin C and tryptophan 104 of chicken egg white cystatin, corresponds to tryptophan 17 of VEGH. The VEGH peptide LLASDEEIQDVSGTWYLKA consisted of the contiguous stretch of residues 3–21 and the cystatin C peptide KLVGGAQIVAGAPWLK contained the three papain-binding domains that have been identified for cystatins (24) in the same order of appearance as in the parent protein (the flanking residues and spacers are boxed in the figure). These domains occur at large intervals in the amino acid sequence, whereas the three homologous sequence motifs of VEGH occur in close sequential proximity and in the same order. Apart from these three motifs, negligible sequence homology is present between VEGH and the family 2 salivary cystatins.

QIVAG, and W sequences of cystatin C, separated by a one-residue spacer and appearing in the same order as in the parent protein (Fig. 2). Both peptides inhibited papain at similar concentrations (Fig. 4B). In line with results of earlier studies these peptides inhibited papain at much higher concentrations than the respective parent proteins (24).

DISCUSSION

Based on our results we conclude that VEGH is a cysteine proteinase inhibitor with a potency comparable with that of the members of the family 2 cystatins. The somewhat lower affinity of VEGH toward papain may be caused by minor sequential differences between the analogous domains of VEGH and cystatins. In the LLAS motif of VEGH the two leucine residues probably contribute substantially to the binding energy of the enzyme-inhibitor complex. This is based on the finding that in the LLGA motif of chicken egg white cystatin they contribute about two-thirds to the binding energy of the complete motif (25). The alanine residue in the VEGH motif may to some extent sterically hinder binding to the active site of papain. Introduction of alanine in recombinant substitution analogs of cystatin C, obtained by site-directed mutagenesis, has led to a

20-fold increased K_i value (26). The serine is probably a suitable residue, because recombinant substitution analogs of cystatin C with a small residue without a pronounced charge in this position, such as alanine in chicken egg white cystatin, exhibit similar inhibitory properties (26). In the QDVSG motif of VEGH, the glutamine, valine, and glycine residues, highly conserved in the cystatins and essential for papain inhibition, are all present. The second and fourth residues in this motif show tolerance for substitution (27). Chicken egg white cystatin contains a serine in the fourth position, as does VEGH; the effect of charged residues in the second position, such as the aspartic acid residue of VEGH, still has to be established. It is remarkable that proteins from two such totally different protein families have a similar biological activity. In view of the well established antimicrobial (8) and antiviral (9, 10) properties of these cystatins, we postulate that VEGH, similar to cystatins, plays a role in the nonimmunological defense against microorganisms and viruses and in the regulation of inflammatory processes in the oral cavity and ocular tissues. This function is compatible with the occurrence of VEGH in secretions from a wide variety of epithelial glands, such as saliva,

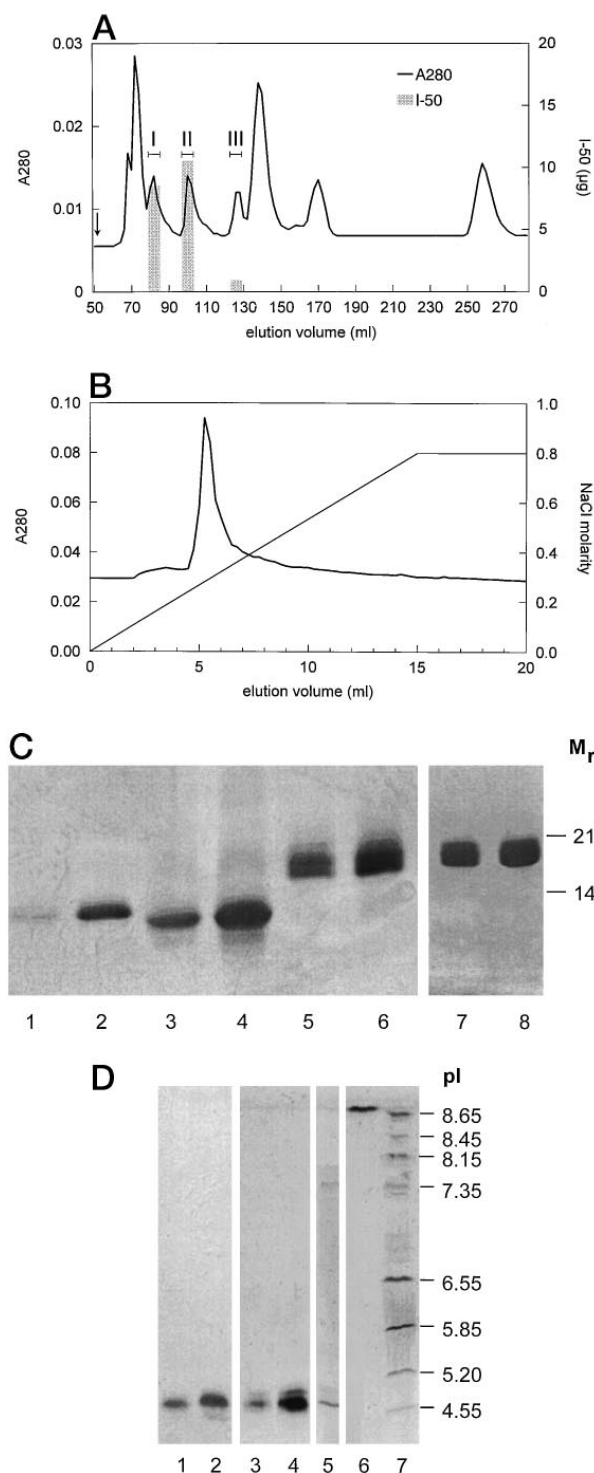


FIG. 3. Purification and characterization of the VEGh preparation. A, size exclusion chromatography on a Sephacryl S-100 HR column (1.5 × 90 cm, flow rate 12 ml/h). Tear samples (1.5 ml, 6.5 mg/ml) were applied and eluted with 0.1 M ammonium acetate buffer, pH 7.0, with spectrophotometrical detection at 280 nm (left-hand axis). The void volume is indicated by an arrow. For those peak fractions that inhibited papain activity, the amount that gave 50% inhibition is represented by bars (right-hand axis). The papain inhibitory activity of pool I can be explained by the occurrence of, among other high molecular weight proteins, family 3 cystatins, as detected on SDS-PAGE (data not shown). Pool III gave one single band on SDS-PAGE, with an apparent M_r of 14,000, corresponding with that of the family 2 cystatins, thus explaining its papain inhibitory activity (data not shown). Pool II also gave one single band on SDS-PAGE, with an apparent M_r of 18,000, corresponding with that of VEGh. After blotting to nitrocellulose, this band reacted with monoclonal antibodies against TSP (VEGH). B, ion exchange chromatography on fast protein liquid chromatography

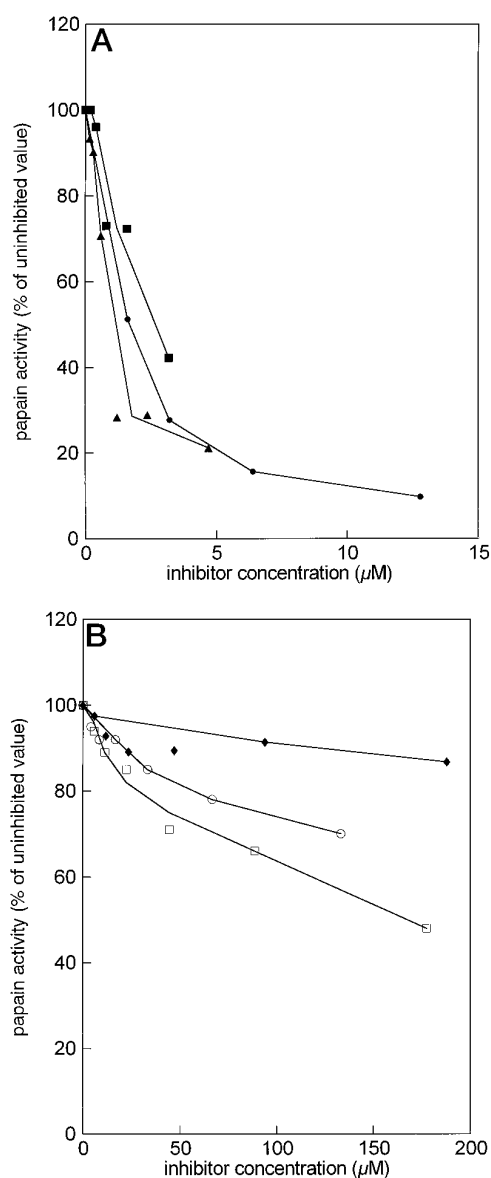


FIG. 4. Inhibition of papain activity. A, papain inhibition by VEGh (●) compared with inhibition by the Janssen and Van Bijsterveld preparation (■) and by recombinant cystatin S (▲). B, papain inhibition by the VEGh peptide 3-21 (○) compared with inhibition by the cystatin C peptide (□). As negative control for peptide inhibition synthetic histatin 5 (◆) was used, a salivary peptide that inhibits several other cysteine proteinases but not papain (30).

with a Resource Q-column (1-ml bed volume; flow rate, 1 ml/min). The VEGh-containing fractions (± 20 ml, ± 5 mg) from five size exclusion chromatography runs were pooled and dialyzed against 0.05 M Tris-buffer, pH 8.4, applied to the column, and eluted with a linear 0–0.8 M NaCl gradient (right-hand axis) in 15 min with spectrophotometrical detection at 280 nm (left-hand axis). C, SDS-PAGE, 20% SDS, under reducing conditions, showed a single band at 18 kDa for our VEGh preparation (lane 7, 0.25 μg; lane 8, 0.50 μg) and for the Janssen and Van Bijsterveld preparation (lane 5, 0.25 μg; lane 6, 0.50 μg). No cystatin contamination was observed by comparison with the 14-kDa recombinant cystatin S (lane 3, 0.25 μg; lane 4, 0.50 μg) or with the recombinant cystatin C (lane 1, 0.03 μg; lane 2, 0.25 μg). D, isoelectrofocusing analysis showed a single band near pI = 5 (lane 1, 0.1 μg; lane 2, 0.2 μg) for our VEGh preparation, the Janssen and Van Bijsterveld preparation (lane 3, 0.1 μg) gave one major band; however, by overloading the gel a second minor band with a similar pI was observed (lane 4, 0.2 μg). As references salivary cystatin S (lane 5, 0.1 μg), recombinant cystatin C (lane 6, 0.1 μg) and a set of standards, pI 3–10 (lane 7, Pharmacia), were used.

tears, and possibly sweat (22), and may shed a new light on the functions of other members of the lipocalin superfamily.

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