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Analysis in gingival crevicular fluid of two oligopeptides derived from human hemoglobin β-chain

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HPLC on a reversed phase column, amino acid sequencing and mass spectrometry were used to determine the structure of two human gingival crevicular exudate oligopeptides (Leu-Thr-Pro-Glu-Glu-Lys-Ser-Ala-Val-Thr-Ala-Leu and Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe) which were shown to have been derived from the β-chain of hemoglobin. These sequences may simply represent two degradation products of the β-chain. However, their preservation in an exudate characterized by active peptidolysis may also prompt the question about their possible more specific role.

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The human gingival crevicular fluid (CF) is an exudate that contains a variety of components derived from tissues, plasma and the bacteria that inhabit the periodontal pocket. Previous literature (1–3) has discussed the protein and enzyme composition of CF, whereas the presence of tissue-derived oligopeptides in CF has not been studied extensively. However, peptides derived from collagen, fibronectin and fibrin, and which are generally larger than the oligopeptides studied in this paper, have been demonstrated in CF (4, 5). Knowledge of the chemical nature of smaller oligopeptides could throw light on the pathogenesis of periodontal diseases. Tissue-derived peptides may also act as inflammatory mediators, or serve as sources of energy of the periodontal flora. The CF can be expected to contain several oligopeptides. Their analysis can become quite difficult, however, owing to the small volumes of CF available and to the short lifetime and low concentrations of several such peptides. We have demonstrated in human CF the presence of a decapeptide and a dodecapeptide derived from the hemoglobin (Hb) β-chain. It may be worth contemplating the significance of the preservation of these two sequences in an inflammatory exudate known to be a rich source of peptidolytic enzymes (1–3) derived from the host and the subgingival dental plaque.

Material and methods

The gingival inflammatory exudate was collected from 25 periodontally diseased sites of six adult subjects (2 women and 4 men, age 35–66 years) who visited the Department of Periodontology of the University of Turku Institute of Dentistry. The exudate was collected from 5- to 8-mm deep gingival pockets using Periopaper® strips. The exudate-containing ends of 30 strips were cut off, pooled and treated for 90 min in 2 ml of 0.01% trifluoroacetic acid (TFA) at 4°C, shaking the mixture for 10 s with a Vortex mixer at about 10-min intervals. The extract was recovered. The paper strips were rinsed with 1 ml of TFA and the wash was combined with the extract. The extract was passed through an Amicon Centricon-3 membrane (nominal cut-off 3,000). The filtrate (C3) was evaporated in a SpeedVac evaporator to 0.1 ml. One 10-μl sample of the C3 was used for SDS-PAGE, after which the filtrate was evaporated to dryness. Thirty unused Periopaper® strips were treated as above, and the resulting filtrate was used as a control. After the above studies, a further filtrate ob-

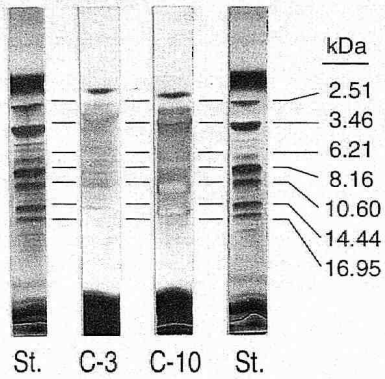

Fig. 1. SDS-PAGE of the Amicon Centricon-3 filtrate (C-3) used in the study. PhastGel high density gel (Pharmacia) and silver staining were used as described in text. C-10 represents a filtrate (not used in study) passed through a Centricon-10 membrane. Some peptides may have washed out of the gel before fixing and staining and several small peptides may have remained unstainable. The band representing a mol. mass of <2.51 kDa (topmost band in lane C-3) may contain most of the smaller peptides separated by microbore HPLC (Fig. 2), including the two human Hb β-chain fragments.

tained from 34 used Periopaper strips (from one woman and two men, aged 73, 42 and 46) representing 24 tooth surfaces, was used to verify the results obtained in the first effort.

The dry residues were brought up in 0.1 ml of 0.01% TFA and the resulting solutions were subjected to mass spectrometry for the determination of the molecular mass of peptides, and to reversed phase HPLC for the separation of peptides for subsequent amino acid sequencing and identification. Mass spectrometry was based on the Matrix-Assisted Laser Desorption/Ionization Time-of-Flight technique (MALDI-TOFMS) which has a sensitivity in the picomole range, and allows the determination of the molecular weight range of analytes from several hundred Da to more than a hundred kDa with an accuracy of 0.02-0.05% (6, 7). Fragmentation of molecules is rarely seen with this method. The instrument used was a Vestec Model 2000 MALDI-TOFMS. Molecular weights of peptides were calculated from established sequences using PROCOMP (6, 7).

The molecular weight determination error, using an external calibrant, was estimated to be 0.05%. Mass calibration was performed prior to and following analyses of C3 using apomyoglobin (horse heart; molecular weight=16,950.7). In HPLC, a Vydac 218TP52 C_{18}-column (0.21×25 cm) was used. Amino acid sequencing was performed using the Applied Biosystems (Foster City, Calif.) Sequenator 470A which provides automated Edman degradation and phenylthiohydantoin-amino acid analysis.

The SDS-PAGE was carried out with Pharmacia High-Density Gels for low-molecular weight peptides as described in the manufacturer's Separation Technique File No. 112. The low-molecular weight standard (MW-SDS-175 Kit for molecular weights 2,500–17,000) was purchased from Sigma. Silver staining was used. The development was performed using sodium thiosulfate as the background reducer, and also without thiosulfate. The purpose of SDS-PAGE was only to check the performance of the Centricon filters and to ascertain that C3 did contain stainable peptides with a mol. wt.< 3 kDa. Fig. 1 (lane C-3) shows that the concentrated C3 also contained traces of some other low-molecular (<16.95 kDa) stainable substances which contributed to the relatively heavy background stain of the gel, generally characteristic to this particular SDS-PAGE procedure. Some of these substances may have leaked through the membrane during the concentration which typically lasted several hours, or they may have derived from the membrane material itself. However, the SDS-PAGE showed that the C3 also contained a peptide fraction (topmost dark band in lane C3) that represented most of the stainable oligopeptides (with masses <2.51 kDa) which had not been washed out of the gel before fixing and staining,

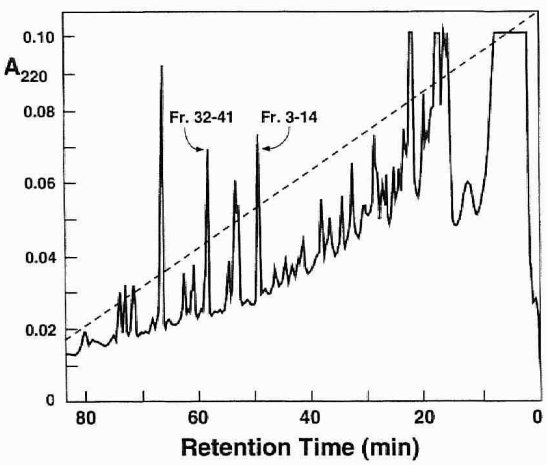

Fig. 2. Separation by microbore HPLC of substances based on their absorption at 220 nm and which were present in C3 after passing the CF sample through an Amicon Centricon-3 membrane. Buffer A was 0. 1% TFA in water and buffer B (0.08% TFA in water) contained an acetonitrile gradient from zero to 70% (dashed line). Several peptides with a blocked amino terminus and non-peptide substances were obviously also separated. The full scale (A_{220}) is 0.1 absorption units. The two human Hb β-chain fractions that were identified, are shown (arrows).

Table 1. Oligopeptides identified in the 3-kDa filtrate of human CF[a]

Amino acid sequence detected	Observed mol. wt.[b]	Calculated mol. wt.[c]	Identification
LTPEEKSAVTAL (Leu-Thr-Pro-Glu-Glu-Lys-Ser-Ala-Val-Thr-Ala-Leu)	1,237.4	1,239.4	Human Hb β-chain fragment 3–14 (dodecapeptide)
LVVYPWTQRF (Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe)	1,309.0	1,307.0	Human Hb β-chain fragment 32–41 (decapeptide)

[a] Pooled CF from six subjects with periodontal disease. The exudate was prefiltered to give a fraction with substances with a mol. wt. of approximately 3 kDa and less. The filtrate was chromatographed by means of microbore HPLC. Peptides with unblocked α-amino terminus were subjected to sequencing and MALDI-TOFMS.
[b] Based on MALDI-TOFMS.
[c] Based on amino acid composition.

and which obviously included the two Hb degradation products discovered in CF.

Results

SDS-PAGE separation of molecules present in C3 revealed that the filters had not leaked any high-molecular weight tissue proteins such as Hb (Fig. 1). The subsequent microbore HPLC of the C3 separated several substances that were detectable by their absorption at 220 nm (Fig. 2). Repeated attempts to study the chemical nature of molecules associated with these peaks showed that two could be positively identified, one representing a decapeptide (LVVYPWTQRF) and the other representing a dodecapeptide (LTPEEKSAVTAL) (Table 1). The MALDI-TOFMS-based determination of the molecular masses of the peptides is shown in Fig. 3. Only these unblocked peptides were present at a sufficiently high level for reliable identification. The present amino acid sequencing procedure analyzed only peptides with an unblocked α-amino function. It is possible that peptides with a blocked α-amino terminus were present, but their analysis could not be carried out. Both sequences indicated above matched up completely

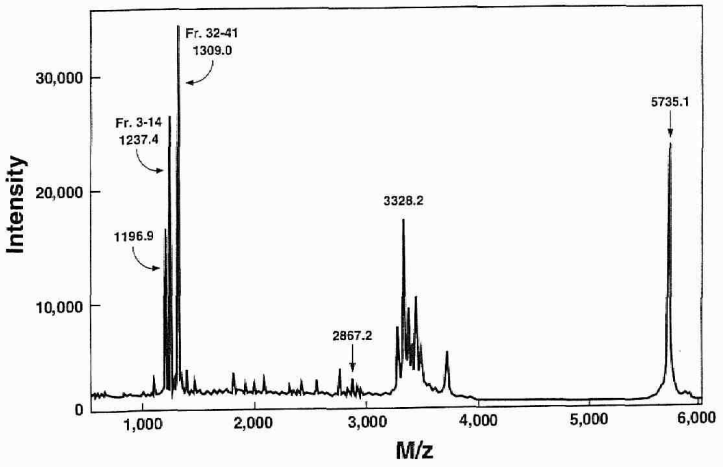

Fig. 3. Mass spectrum of C3 obtained from human CF (shown as counts versus the mass to charge ratio (M/z). The use of two internal standards (vertical arrows) is indicated. The molecular masses of the standards and selected peaks are shown.

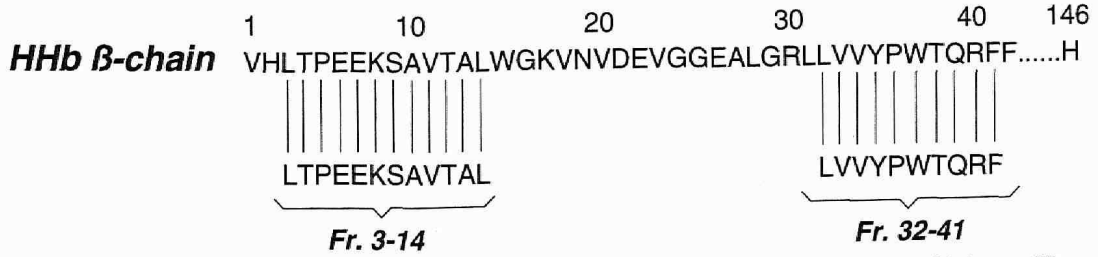

Fig. 4. Partial sequence of human Hb β-chain and sequences of Hb β-chain fragments 3–14 and 32–41 observed in human CF.

with all vertebrate Hbs tested, and were shown to have 100% identity with the human Hb β-chain sequences 32–41 and 3–14, respectively (Fig. 4). It must be emphasized that the peaks shown in Fig. 2 do not all represent peptides because several organic molecules within this mass range also absorb light at 220 nm.

Discussion

It is not surprising to detect fragments of tissue proteins in an inflammatory exudate. The difficulties in demonstrating the presence of such peptides may rather be related to their extremely small concentrations and possible short lifetimes in the dynamic, changing chemical conditions of CF (it was estimated that the 0.1-ml TFA sample, representing six patients, contained 0.1–0.3 nmoles of both peptides). The human CF can be expected to contain several peptidases and proteinases that could accomplish an active breakdown of Hb into smaller fragments and eventually into constituent amino acids. It may, therefore, be somewhat surprising to observe that two particular Hb β-chain sequences were preserved in CF at concentrations higher than those of several other oligopeptides present in CF (judged from the relative heights of peaks shown in Fig. 2). It is possible that these two sequences represent enzymatically more stable fragments. The CF was obtained from clinically advanced cases. It is possible that most "degradable" subtrates had already been degraded before CF sampling.

The following characteristics of these peptides and their formation from Hb deserve attention. Both peptides display considerably hydrophobic termini and especially the liberation of the sequence 32–41 from the Hb β-chain must involve proteases that specialize in the cleavage of peptide bonds surrounded by quite hydrophobic, bulky amino acid residues. It is premature to speculate about the possible role of these peptides in the restricted biological niche where they were discovered. It is interesting, however, that peptides of the present size (or a more generally octa- through dodecapeptides) are frequently pharmacologically and physiologically active, and act as inflammatory mediators and in other important capacities (8, 9). Both peptides may also act as substrates of proline-specific peptidases studied in treponemes isolated from the human subgingival dental plaque (9, 10).

The present study suffered from the weakness that the inflammatory exudate from several subjects was combined. This was deliberately done to ascertain that enough material was available for analyses. Based on the sensitivity of MALDI-TOFMS, HPLC, and peptide sequencing, it is possible that the concentration of some stable CF peptides could be determined in individual samples, particularly if the same diseased site can be sampled repeatedly. The present analyses focused on crevicular fluid from diseased subjects. It is possible that successful periodontal therapy will reduce the levels of these peptides below the detection limits of the present procedures.

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