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Purification and analysis of a 5 kDa component of enamel matrix derivative

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Abstract

High performance liquid chromatography (HPLC) methods were used to analyse a 5 kDa component purified from enamel matrix derivative (EMD), the active ingredient in Emdogain®, a commercial product for periodontal tissue regeneration. After initial purification by size-exclusion chromatography (SEC) on a 100 cm × 5 cm column (Bio-Gel P-30 Fine, 280 nm), collected fractions were analysed by size-exclusion HPLC (SE HPLC; TSK-Gel Super SW2000, 220 nm). The fractions containing only the 5 kDa component were analysed by reversed-phase high-pressure chromatography (RP HPLC; YMC-Pack ODS-A, 200 nm), revealing four peaks of the 5 kDa component. From 1200 mg of EMD (of which 9% is the 5 kDa component), approximately 65 mg of lyophilised 5 kDa component were obtained, corresponding to a recovery of 60%. The SE HPLC method was mainly suitable for qualitative analysis, whereas the RP HPLC method was appropriate for both qualitative and quantitative analysis.

Keywords: Size-exclusion HPLC; Reversed-phase HPLC; Protein purification; Qualitative analysis; Quantitative analysis; Method validation; Fractionation; Enamel matrix derivative; Emdogain

1. Introduction

Enamel matrix derivative (EMD) is the active ingredient in Emdogain® (BIORA AB, Straumann, Malmö, Sweden), a commercially used product for regeneration of periodontal tissue. The main component of the derivative is amelogenin, which is secreted by ameloblasts into the enamel compartment. Extraction of EMD for the Emdogain® preparation is carried out from the molar teeth of 6-month-old pigs. At this time, amelogenin corresponds to approximately 90% of total tissue protein, and the stages of secretion and early maturation in enamel formation have been reached.

The effects of EMD in the periodontal area on cementum, periodontal ligament, bone formation and wound healing have been studied quite extensively over the years [1–23]. Recently, researchers have started to look more at the bioactive parts of EMD (sometimes also referred to as EMP; Enamel Matrix Proteins) [20–22]. Even though EMD mainly consists of amelogenin (20 kDa) [11,16,24–26], it cannot be ruled out that other proteins besides amelogenin have biological activity. Therefore, in order to understand the overall biological activity of EMD, it is essential to investigate its components. Therefore, this paper focuses on one of the EMD components, namely the previously described 5 kDa fraction [24,27–32].

The EMD complex has previously been fractionated using, e.g. size-exclusion chromatography (SEC) [29,30,32], and reversed-phase chromatography (RPC) [32,33]. SDS-PAGE has been used for both fractionation [16,32,34], and analysis (e.g. quality control of purified fractions) [11,32,33], enabling direct determination of the molecular weights of the EMD components. In addition, immunoanalytical methods, such as Western Blotting, have been employed to confirm the identities of the separated EMD units [16,32,33].

In order to fully comprehend the mechanisms of EMD, prep-scale purification of its components is regarded as a main target. Purified proteins are crucial for *in vitro* screening studies to

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assess possible biological effects. Hence, it is a prerequisite that the components are purified under strictly controlled and reproducible processes, and that validated analytical tools are available for quality control of the obtained pure products.

To the best of our knowledge, nothing has previously been reported in the literature covering prep-scale purification and quality control of the EMD components. Therefore, in this paper, a contemporary three-method set-up is described for a 5 kDa component of EMD. The set-up comprises an initial purification step based on low pressure SEC, and two following complementary, validated high-pressure liquid chromatography (HPLC) methods for analysis and quality control. The HPLC methods are based on (1) size-exclusion HPLC (SE HPLC), and (2) reversed-phase HPLC (RP HPLC). This paper aims at purifying and characterising the 5 kDa fraction of EMD, as a first step towards the complete characterisation of the EMD components and their relevance in the biological healing processes.

2. Experimental

2.1. Chemicals and reagents

Formic acid (98–100%, analytical grade, art. no. 100264), sodium chloride (NaCl, analytical grade, art. no. 106405), and *o*-phosphoric acid (SupraPur 85%, art. no. 100552), were obtained from Merck, Darmstadt, Germany. Acetonitrile (ACN, HPLC Far UV grade, art. no. CO3C11X) was purchased from LabScan Ltd., Dublin, Ireland. Potassium hexafluorophosphate (KHFP, 99%, art. no. 191090010) was obtained from Acros Organics, New Jersey, USA. Glacial acetic acid (HAc, art. no. 329383) was purchased from Apoteket P&L, Gothenburg, Sweden. Water was obtained from a MQ system from Millipore Inc. EMD was internally supplied (In-house standard, enamel matrix derivative, BIORA AB, Straumann, Malmö, Sweden).

2.2. Methods

2.2.1. Purification by low pressure SEC

2.2.1.1. Sample preparation. The lyophilised in-house EMD standard was dissolved in 125 mM formic acid (1200 mg in 16 ml).

2.2.1.2. Column preparation. The BioGel P-30 Fine (45–90 μm hydrated beads, art. no. 150–4150, BioRad Laboratories, Hercules, CA, USA) was prepared according to the manufacturer's instructions (swelling, degassing), and packed in a 5 cm × 100 cm column (XK50/100, art. no. 56875300, Amersham Biosciences AB, Uppsala, Sweden). The bed volume was 1767 ml (bed height 90 cm).

2.2.1.3. Sample application, elution, and fraction collection. The sample (16 ml of dissolved EMD; corresponding to less than 1% of the total bed volume) was applied on the purification column equilibrated with 125 mM formic acid (elution buffer, degassed off-line). The column was connected to a 2510 UVICORD SD (LKB, Bromma, Sweden), equipped with a 280 nm UV filter (art. no. 80–1071–13, GE Healthcare, Uppsala, Sweden). The absorbance was registered by a chart recorder (Pharmacia, Uppsala, Sweden). Elution was obtained by using a peristaltic pump (Pharmacia, Uppsala, Sweden) set to 1.0 ml/min. Eluting fractions were collected automatically (12 min/tube) using a 2211 SUPERRAC (LKB, Bromma, Sweden).

2.2.2. MW determination by SDS-PAGE

All SDS-PAGE equipment and chemicals were obtained from BioRad Laboratories, Hercules, CA, USA. Running buffer was prepared from 10× TGS (art. no. 161–0772). Samples and Kaleidoscope Prestained Standards (art. no. 161–0324) were applied in the wells of Ready Gel 10–20% Tris–HCl (art. no. 161–1124). Gel electrophoresis was carried out at 200 V for 35 min. Staining was achieved with Bio-Safe Coomassie G250 Stain (art. no. 161–0786).

2.2.3. Quality control by high-pressure LC

2.2.3.1. Quality control by SE HPLC. The fractions collected in the low pressure SEC step were checked before pooling using a SE column coupled to an HPLC instrument (JASCO Corporation, Tokyo, Japan), equipped with an AS-1555 autosampler, a 880–30 solvent mixing module, a PU-1580 pump, a CO-2065 Plus column oven, a UV-1575 detector and a LC-NetII/ADC communication device. JASCO ChromPass Chromatography Data System was used for data acquisition (version 1.7.403.1). The SE column (TSK-Gel, SuperSW2000, 300 mm × 4.6 mm, art. no. 18674, Tosoh Bioscience GmbH, Stuttgart, Germany) was kept at 30 °C in the column oven. Samples were taken directly from the fraction collection tubes (diluted when necessary), and injected into the SE column (injection volume 5 μl, wavelength 220 nm, run time 15 min). Fractions were eluted isocratically at 0.3 ml/min using a mobile phase consisting of 30% ACN in 0.9% NaCl (degassed on-line). After preparation of the mobile phase, it was filtered using a 0.45 μm hydrophilic polypropylene membrane filter (GH Polypro, art. no. 66548, Pall Corporation, Ann Arbor, MI, USA). The SE HPLC parameters are summarised in Table 1.

2.2.3.2. Quality control by RP HPLC. In addition to the quality control by SE HPLC, fractions containing pure peaks of the 5 kDa component, as observed on the SE HPLC system, were also analysed using a RP column coupled to an HPLC instrument (JASCO Corporation, Tokyo, Japan). This was equipped with the following components: an AS-2057 Plus autosampler, two LV-2080-03 solvent selection units, a 880-31 solvent mixing module, two HPLC pumps, a CO-2065 Plus column oven, a MD-2010 Plus multiwavelength detector, and a LC-NetII/ADC communication device. JASCO ChromPass Chromatography Data System was used for data acquisition (version 1.7.403.1). The RP column (YMC-Pack ODS-A, S-5 μm, 30 nm, particle AP-303, 250 mm × 4.6 mm, art. no. AA30S05-2546WT, YMC Co. Ltd., Kyoto, Japan) was maintained at 40 °C in the column oven. Samples were taken from the fraction collection tubes, diluted with 0.1% acetic acid when necessary (i.e. in case of a too high concentration), filtered with 0.2 μm PVDF syringe filters (art. no. 6777–0402, Whatman Inc., Florham Park, NJ,

Table 1
Summary of parameters for the quality control steps based on SE HPLC and RP HPLC

Parameter	SE HPLC	RP HPLC
Column	TSK-Gel, SuperSW2000, 300 mm × 4.6 mm	YMC-Pack ODS-A, S-5 μm, 30 nm, particle AP-303, 250 mm × 4.6 mm
Elution type	Isocratic	Gradient; t = 0 min, 100% A; t = 1 min, 100% A; t = 30 min, 100% B; t = 32 min, 100% B; t = 35 min, 100% A; t = 45 min 100% A
Mobile phase	30% ACN in 0.9% NaCl	A = 65% KHFP/35% ACN, B = 35% KHFP/65% ACN
Injection volume	5 μl	20 μl
Flow rate	0.3 ml/min	1.5 ml/min
Run time	15 min	45 min
Autosampler temperature	Ambient	4 °C
Column oven temperature	30 °C	40 °C
Wavelength	220 nm	200 nm

USA), and injected into the RP system (injection volume 20 μl, wavelength 200 nm, run time 45 min). The 5 kDa component was eluted at 1.5 ml/min using a gradient of ACN (from 35% to 65%, by volume, premixed mobile phases, degassed on-line) in 50 mM pH 3.3 KHFP buffer. The KHFP buffer was filtered using a 0.45 μm hydrophilic polypropylene membrane filter (GH Polypro, art. no. 66548, Pall Corporation, Ann Arbor, MI, USA). The RP HPLC parameters are summarised in Table 1.

2.2.4. Pooling of fractions and lyophilisation

Samples containing the pure 5 kDa component (RP HPLC results: four main peaks) were pooled and analysed on both quality control systems (SE HPLC, RP HPLC) both before and after lyophilisation. Lyophilisation was done according to general procedures, i.e. shell-freezing by rotating the sample flask in a dry ice/ethanol mixture, followed by freeze-drying under vacuum (Freeze dryer unit Alpha I/6, Christ, Osterode am Harz, Germany).

2.2.5. MW determination by MALDI-TOF-MS

Lyophilised samples were sent to M-Scan Ltd., Berkshire, UK, for MW determination by MALDI-TOF-MS. Samples were dissolved in 0.1% TFA (aq.), and analysed by a Voyager STR Biospectrometry Research Station Laser–Desorption Mass Spectrometer coupled with Delayed Extraction.

2.2.6. Validation parameters

Both HPLC methods for analysis of the 5 kDa component were validated using common validation parameters, such as injection repeatability, precision (in standard preparation), carry over, linear range (LR), limit of detection (LOD), limit of quantification (LOQ), and accuracy [35,36]. Accuracy was evaluated by comparing the signals obtained for spiked samples with expected ones derived from the equation of the linearity curve, and calculating the recovery. Concerning the SE HPLC method, spiking was obtained by adding known amounts of the purified 5 kDa component to EMD samples. As for the RP HPLC method, spiking was obtained by adding known amounts of a 150% sample (5 kDa) to 5 kDa samples. Further, for the RP HPLC method, the first main peak of the 5 kDa component (t_R, 15.7 min with 100 mM buffer, 15.3 min with 50 mM buffer) was used in the evaluation of injection repeatability and precision. This peak was the largest (by area %), therefore the easiest one to integrate, and thus the most reliable results could be obtained. In the RP HPLC method validation, two different concentration ranges were used:

(1) low, where 100% of the working concentration = 50 μg/ml,
(2) high, where 100% of the working concentration = 500 μg/ml,
(3) thus covering different analytical applications, e.g. quality control and process control.

Additionally, for the RP HPLC method, sample stability and degradation pattern were investigated in a forced degradation study.

3. Results and discussion

3.1. Purification by low pressure SEC

The use of a polyacrylamide gel (Bio-Gel P-30 Fine), suitable for peptides and proteins in the range 2.5–40 kDa, resulted in fractionation of EMD into three major components. SDS-PAGE was used for approximate MW determinations of the components (20 kDa, |12 + 9| kDa, and 5 kDa). The SEC conditions were obtained after initial up-scaling experiments, where different column dimensions and sample loadings were applied.

Fig. 1 presents a typical chromatogram for the purification of the 5 kDa component from EMD using low pressure

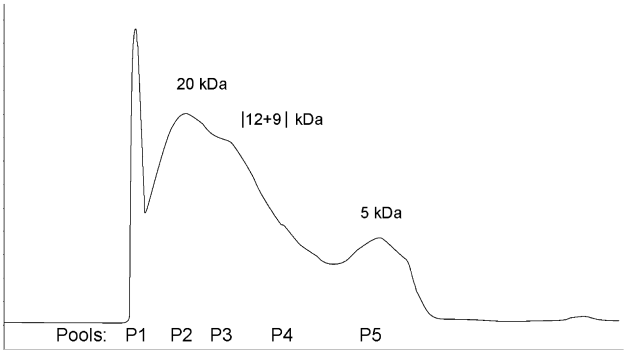

Fig. 1. Fractionation of EMD by low pressure SEC (BioGel P-30 Fine, 100 cm × 5.0 cm).

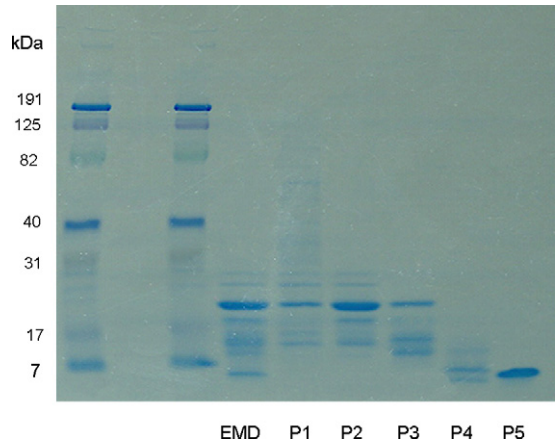

Fig. 2. SDS-PAGE of EMD and obtained pools (P1–P5; see Fig. 1) after fractionation by low pressure SEC.

SEC. The first peak (pool P1) was assumed to correspond to a mixture of unresolved proteins and aggregates, including components eluting with the void volume, i.e. having MW above the exclusion limit of the gel (Fig. 2). The second peak corresponded to the 20 kDa component, followed by the |12 + 9| kDa component, and finally, the 5 kDa component. The 20 kDa and |12 + 9| kDa components were not well resolved, hence the appearance of a 20 kDa band in the |12 + 9| kDa pool (P3 in Fig. 2). The 5 kDa component was completely resolved from the other ones; only one band on SDS-PAGE for the 5 kDa pool (P5 in Fig. 2).

The assumptions made concerning the "aggregate peak" (pool P1) are supported by reported data in the literature [24]. Fincham et al. compared the protein biochemistry of developing dental enamel matrix of five mammalian species [24]. Using SEC with BioGel P-30 for fractionation of amelogenin matrices, it was found that proteins eluting in the void volume peak (corresponding to pool P1 in this work) migrated close to 67 kDa in SDS gels. This fraction was assumed to contain some enamelin, and subsequent amino acid analysis also suggested a mixture of an amelogenin and enamelin protein. SDS-PAGE analysis of high-MW amelogenins by Limeback and Simic [32] also confirmed the presence of aggregates in the form of dimeric amelogenins.

A comparison of the herein presented fractionation results with previous results found in the literature is given in Table 2.

Judging from the SDS-PAGE results, it could be concluded that the "aggregate peak" (pool P1) in the present work corresponded to the combined A and B fractions described in Ref. [24]. Further, the purified 5 kDa component (pool P5) corresponded to the trailing peaks G–I in Ref. [24]. Considering the intermediate pools (P2–P4), conclusions were less straightforward due to the complexity of enamel matrix protein samples. However, the most abundant protein, the 20 kDa amelogenin, was clearly observed in both P2 and P3, confirming previous results presented in Table 2. In P3, smaller components at |12 + 9| kDa were seen, although they were not distinguishable in Ref. [24]. P4 was regarded as a "pre-5 kDa pool", probably arising from amino acid differences of the individual 5 kDa components.

Even though Limeback and Simic focused on high-MW amelogenin aggregates [32], using a BioGel P-100 matrix, results were presented also for the lower MW components of enamel matrix. Strong SDS-PAGE bands at 20 kDa, and also at <10 kDa for the trailing fractions of the SEC purification were observed. In complementary immunoblotting experiments, 5 kDa species (LRAP) were observed [32].

The herein presented results are thus in agreement with results found in the literature (here only briefly summarised).

Table 2
Fractionation of enamel matrix derivative

Reference	Sample for fractionation	SEC conditions	Fractions/pools	MW[a] (kDa)
Mumulidu et al. (present work)	EMD	5 cm × 100 cm, BioGel P-30 Fine, 125 mM formic acid, 280 nm, ambient temperature	P1 P2 P3 P4 P5	12–80 (20) 12–30 (20) 9–20 (20) 4–9 5
Fincham et al. [28]	Enamel matrix amelogenins from scrapings (porcine)[b]	1.5 cm × 190 cm, BioGel P-30, 0.1 M formic acid, 280 nm, 10 °C	A B C–E F G-I	35–70 30–35 14–25 (20) Up to 14 4–5
Limeback et al. [32]	Enamel matrix amelogenins from scrapings (porcine)	2.5 cm × 100 cm, BioGel P-100, 0.1 M formic acid, 280 nm	A B C D E–H	50–100 20–100 (20) 17–26, 40 (20) 16 <10
Maycock et al. [16]	(1) Emdogain[c] (2) Enamel matrix proteins (porcine)	NA NA	NA NA	3–65; 50% at 20 Similar pattern as for 1

[a] By SDS-PAGE (most abundant band).
[b] Also studied were amelogenins from cow, hamster, human and sheep.
[c] Emdogain without PGA vehicle, i.e. most likely the EMD in-house standard (BIORA AB, Straumann).

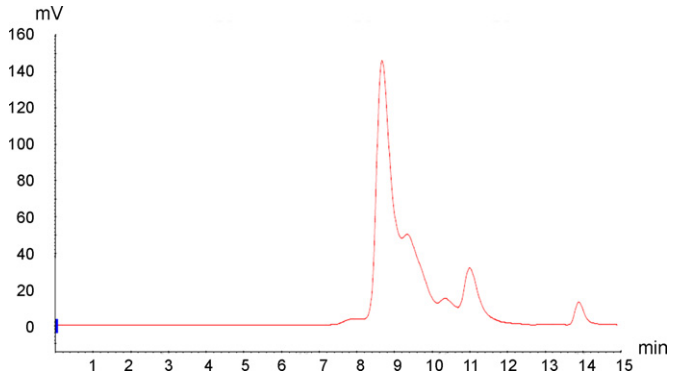

Fig. 3. Analysis of EMD by SE HPLC (SuperSW2000, 300 mm × 4.6 mm).

3.2. Analysis by SE HPLC—method validation

3.2.1. Analysis of EMD

Analysis of EMD by SE HPLC revealed three main components, i.e. the 20 kDa, the |12 + 9| kDa, and the 5 kDa components, respectively (Fig. 3). Although the 5 kDa component was relatively well separated (t_R, 11.0 min), the two other ones coeluted (at 8.7 and 9.4 min, respectively). In the validation of the SE HPLC method, EMD served as the reference material, and throughout the validation and also in coming quality control steps on the SE HPLC system, EMD was included as a standard for checking the system performance.

3.2.2. Analysis of the 5 kDa component

The 5 kDa component was successfully purified from EMD by the low pressure SEC method described above. Subsequent analysis by SE HPLC revealed one single peak at t_R 11.0 min (Fig. 4).

3.2.3. Validation results

The results of the SE HPLC method validation are summarised in Table 3. As seen, the developed method was found to be suitable for qualitative analysis of the 5 kDa component purified from EMD. For quantitative analysis, a precision in standard preparation of ≤2% RSD, and an injection repeatability of ≤1% RSD are usually targeted [35]. The accuracy was not acceptable at the lower sample concentration range (100–200 μg/ml; 117–118% recovery), probably due to the small amount of sample being introduced into the column (≤1.0 μg). Although acceptable accuracy was obtained in the concentration range 500–1000 μg/ml, the method was not recommended for quantitative analysis due to the relatively poor precision and repeatability.

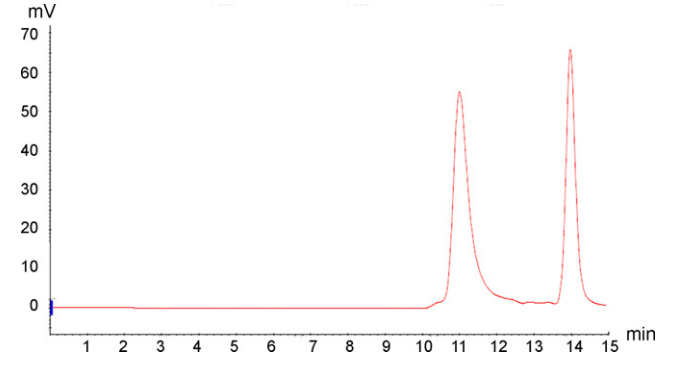

Fig. 4. Analysis of the purified 5 kDa component by SE HPLC (SuperSW2000, 300 mm × 4.6 mm).

3.3. Analysis by RP HPLC—method validation

3.3.1. Analysis of the 5 kDa component

Analysis of the purified 5 kDa component by RP HPLC revealed four main peaks (see top chromatogram in Fig. 5). Fractionation of the smaller (5 kDa) EMD components by RP HPLC has previously been reported by Fincham and Moradian-Oldak [30], where a C4 column was employed in RP HPLC, and four main peaks eluted using a 60% acetonitrile gradient in 0.1% trifluoroacetic acid at a flow rate of 0.5 ml/min. The obtained elution pattern differed slightly from the one shown in this work. This was most likely due to the different chromatographic conditions applied (e.g. column material, mobile phase). However, the observed four peaks were attributed to the presence of two TRAP (tyrosine-rich amelogenin polypeptide), and two LRAP (leucine-rich amelogenin polypeptide) species, respectively [30].

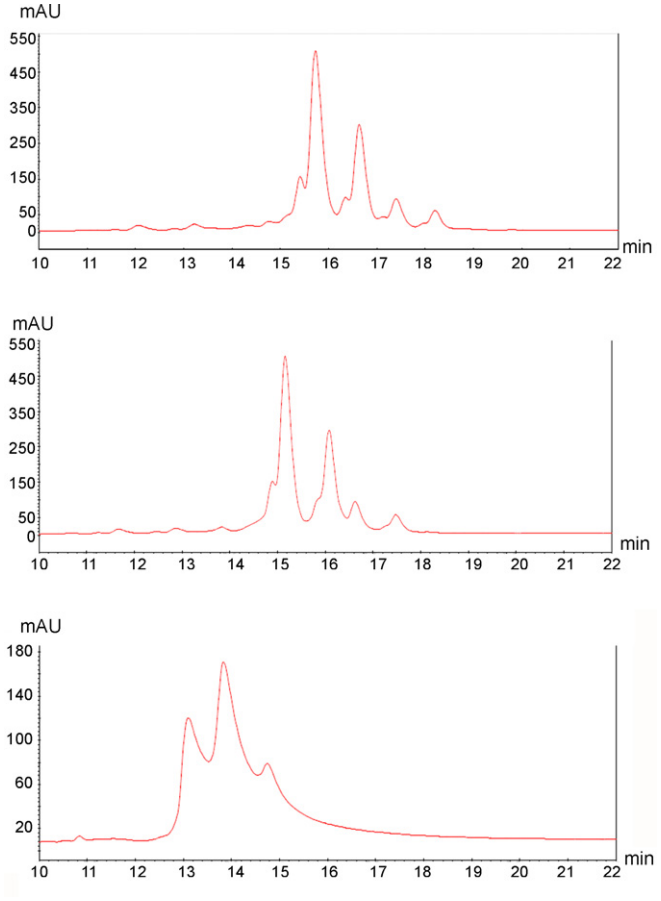

Fig. 5. RP HPLC analysis of the 5 kDa component using different buffer concentrations in the mobile phase (YMC-Pack ODS-A, 250 mm × 4.6 mm). Top: 100 mM, middle: 50 mM, and bottom: 10 mM.

Table 3
Summary of SE HPLC method validation results

Concentration (μg/ml)[a]	Repeatability (%RSD)[b]	Precision (%RSD)[c]	LOD (μg/ml)[d]	LOQ (μg/ml)[e]	Carry over
1000	≤3	5	1	2	No

Concentration (μg/ml)[a]	LR (%)[f]	LR (μg/ml)	Linear equation ($y = kx$)	Correlation coefficient (R^2)	Accuracy (%)[g]
1000	5–150	50–1500	$y = 2.6915x$	0.9984	102–104

[a] Sample concentration corresponding to 100% of the working concentration.
[b] The variability of six repetitive injections of a standard solution.
[c] The variability of the response factors of two standard solutions.
[d] Limit of detection (LOD).
[e] Limit of quantification (LOQ).
[f] Linear range (LR) as the concentration in % of the working concentration.
[g] Accuracy for spiked samples in the range 500–1000 μg/ml. EMD was spiked with the purified 5 kDa component.

In the herein presented work, a buffered acetonitrile gradient (from 35% to 65% ACN) was used, with pH ranging from 3.7 to 4.4 (Table 4). Initially, 100 mM KHFP was used in the mobile phase. Screening of lower salt concentrations was done in order to find suitable conditions for both separation efficiency (peak resolution) and column lifetime. A lower salt concentration would result in a longer column lifetime, and thus both economical and environmental benefits. However, a specific salt concentration is required to obtain sufficient peak resolution, and subsequently, reliable data for quality control. Fig. 5 presents obtained resolutions of the four main peaks of the 5 kDa component at different buffer concentrations.

As seen, lowering the salt concentration to 10 mM did not allow peak separation, but 50 mM resulted in clearly resolved peaks. Compared to using the initial buffer concentration, a lowered resolution was observed (R_S decreasing from 1.3 to 0.9 for main peaks 2 and 3), as well as a general shift in the retention times.

Since all four main peaks were still clearly distinguishable, and comparable to the peaks obtained using the 100 mM KHFP, the method was validated using (1) the higher buffer concentration (100 mM) to be used in cases where improved resolution is needed, and (2) at the lower buffer concentration (50 mM) for routine analysis on a daily basis, e.g. as a quality control step after purification of the 5 kDa component from EMD.

3.3.2. Validation parameters

3.3.2.1. Forced degradation study. Subjecting samples to forced degradation is generally done to demonstrate specificity when developing a stability-indicating method, i.e. a method that allows accurate measurement of active ingredient, degradation products, and other components in a drug product (GMP) [37]. Forced degradation can be obtained by exposing the samples to harsh conditions, e.g. acid, base, heat and light. Samples may then be spiked with the obtained degradation products, and specificity may be checked by comparing peaks of undegraded sample versus spiked sample. The peaks should not overlap, i.e. they should not have the same retention times. An alternative is to collect the analyte peak of the degraded sample, and analyse its composition by, e.g. LC–MS to check the peak purity.

Table 4
Comparison of mobile phase pH when using different KHFP concentrations

KHFP concentration (mM)	pH buffer	pH mobile phase A[a]	pH mobile phase B[b]
10	3.8	4.0	4.6
50	3.3	3.8	4.4
100[c]	3.1	3.7	4.4

[a] Mobile phase A = 65% KHFP/35% ACN (vol. %).
[b] Mobile phase B = 35% KHFP/65% ACN (vol. %).
[c] Concentration of the initially used buffer (100 mM).

In this case, the main purpose of the RP HPLC analytical method was to provide quality data of the purified 5 kDa component of EMD. Since the 5 kDa component was found to be stable (3 months when stored at 4 °C), and the RP HPLC quality analysis would be done shortly after the purification step, method specificity was of minor relevance. The objective behind the degradation study was to check the stability of the purified 5 kDa component, and to see if possible degradation products would be detected by the method, i.e. having retention times falling within the time window.

First, degradation of the 5 kDa component was investigated in different media, and at different temperatures (room temperature (RT) versus 95 °C). Moreover, light stability was studied (normal day light versus elevated UV light exposure in a UV cabinet). The higher buffer concentration of 100 mM was used in order to see possible degradation products more clearly, due to improved resolution. It was observed that the 5 kDa component was degraded in 0.1 M NaOH (pH 12.5), in 3% H_2O_2 (pH 5.0), and in UV light (6 × 8 W, 312 nm); all conditions at RT. At 95 °C, it was degraded in all tested media. All observable degradation products, i.e. those absorbing at 200 nm, had retention times between 11 min and 19 min under the described conditions. Further, the 5 kDa component was found to be stable in acidic media (pH 1.0–3.2) at RT, and more specifically, it was characterised by a 3-month stability when stored in acidic media at 4 °C.

The main part of forced degradation tests generally recommended, i.e. at acidic pH (in 0.1 M HCl), at basic pH (in 0.1 M NaOH), in an oxidative environment (in H_2O_2), and in a photolytic environment (in a UV cabinet) have thus been covered [35,36].

3.3.2.2. System performance and precision. The response factors (RF, i.e. the signal related to the concentration) of six

repetitive injections of a standard solution (S1) were compared in order to get a measure of the injection repeatability. The precision (in standard preparation) was evaluated by comparing the response factors of two standard solutions (S1, S2). Other parameters were also determined in order to get a measure of the system performance and precision (number of theoretical plates (NTP), and asymmetry factor (As.); both according to USP). The capacity factor (k'; also known as the retention factor, k) was determined by using the retention time of acetic acid (blank solution) as the t_0 value ($k' = (t_R - t_0)/t_0$).

Both injection repeatability and precision met general requirements, and no significant carry over was observed. See Table 5 in Section 3.3.2.5 (summary of RP HPLC method validation) for a summary of the results.

3.3.2.3. Linear range, LOD, and LOQ. The linearity was investigated between 25% and 150% of the working concentration at both concentration ranges (high, low). Two standards were used as controls, and the first main peak of the 5 kDa component was used in the evaluation of the results.

Generally, the linear curve should be forced through origo (0,0), i.e. $y = kx$. However, the linear fit can often be substantially improved by not doing so. If the intercept is low enough (a few % of the area obtained for the sample at 100% of the working concentration), it is generally regarded fully acceptable to use a linear equation $y = kx + m$. An acceptance level of 5% was used in this work (i.e. $m/y_{100\%} \leq 0.05$).

The method was found to be linear over a broad concentration range using either buffer concentration (50 mM, 100 mM KHFP). Using the higher buffer concentration, the method was successfully validated at both sample concentration levels (high, low). Using the lower buffer concentration, the method was validated only at the higher sample concentration range (where 100% = 500 μg/ml).

The limit of detection (LOD) is generally regarded as the concentration for which the signal is three times the noise level (S/N = 3) [35]. Similarly, the limit of quantification (LOQ) is generally the concentration for which S/N = 10. In the case of the 5 kDa component, the existence of the four main peaks made these "rules of thumb" difficult to follow. At lower concentrations, the process of peak integration was difficult, and unreliable results were thus obtained. An indication of this was that the normal relationships between the four main peaks of the 5 kDa component (peak ratios in %) were altered. At a higher concentration level (100% of working concentration), the peak ratios were approximately $15_{MP1}:9_{MP2}:3_{MP3}:1_{MP4}$ (corresponding to main peaks 1, 2, 3, and 4), while at a low concentration (10% of working concentration) they changed to approximately ($10_{MP1}:10_{MP2}:3_{MP3}:1_{MP4}$). The question was how large deviations could be accepted, hence, the LOD and LOQ should be defined as that specific concentration. Therefore, both LOD and LOQ were defined to be at a sample concentration of 13 μg/ml (corresponding to 25% of the working concentration), since at this level reliable peak ratios and integration were obtained.

3.3.2.4. Accuracy. Accuracy requirements depend on the type of method and its intended application. In this work, an acceptance level of ±5% was targeted, i.e. the resulting recoveries of spiked samples should be in the range 95–105%.

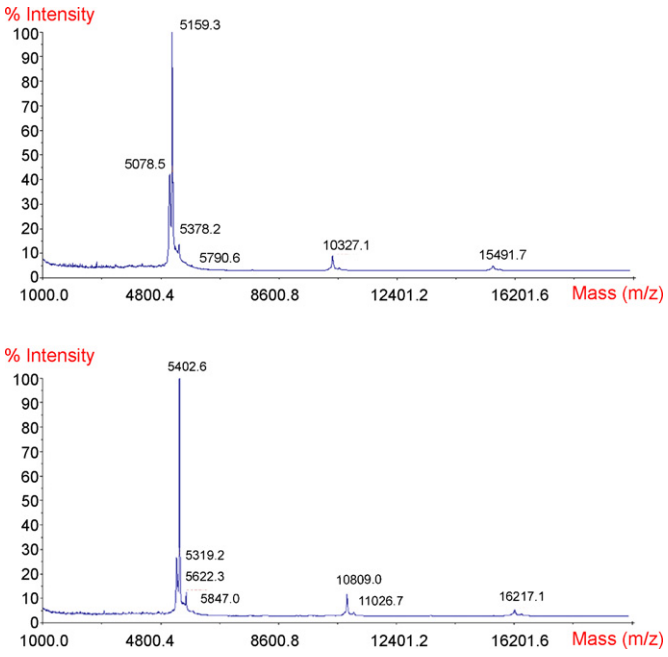

Fig. 6. MALDI-TOF-MS analysis of subfractions of the 5 kDa component. Top: main peak 1, MW 5.2 kDa. Bottom: main peak 2, MW 5.4 kDa.

The accuracy was not acceptable at the lower sample concentration range (82–93% recovery), but quite satisfactory at the higher range (93–105% recovery). The reason for the poor accuracy results in the lower concentration range was probably the small amount of sample being introduced into the column; only between 0.5 μg and 1.3 μg of the 5 kDa component was injected. However, checking the "recovery" of standards instead of using spiked samples (thus avoiding the introduction of analytical errors due to pipetting) resulted in recoveries in the range 95–105% even at the lower sample concentration range (see Table 5). Hence, the method was regarded as accurate.

3.3.2.5. Summary of RP HPLC method validation. Table 5 presents the obtained validation data using both buffer concentrations. As seen in the summary table (Table 5), the results obtained with the method using the lower buffer concentration were comparable to the ones obtained with the original method. Hence, due to the stability of the system, the method using the lower buffer concentration was expected to be valid also at the lower concentration range (where 100% = 50 μg/ml).

3.3.2.6. MW determination by MALDI-TOF-MS. To compare our results with previously reported ones [29,30], the two first and largest (by area %) subfractions of the 5 kDa component were isolated, lyophilised and sent for MW determination by MALDI-TOF-MS (M-Scan, UK). The obtained molecular weights were in agreement with reported data, i.e. approximately 5 kDa (see Fig. 6). This further signifies the suitability of the presented 3-method instrumental set-up for purification and analysis of the 5 kDa component of EMD.

Table 5
Summary of RP HPLC method validation

Buffer (mM)[a]	100% (μg/ml)[b]	Repeatability (%RSD)[c]	Precision (%RSD)[d]	Capacity factor (k')[e]	LOD & LOQ (μg/ml)[f]
50	500	<1.0	<2.0	5.1	13
100	500	<1.0	<2.0	5.3	13
100	50	<1.0	<2.0	5.3	13

Buffer (mM)	100% (μg/ml)	LR (%)[g]	LR (μg/ml)	Linear equation ($y = kx + m$)	R^2[h]	m/y (%)[i]
50	500	25–150	125–760	$y = 1.2133x - 5.4809$	0.9981	4.8
100	500	25–150	125–760	$y = 1.2105x - 1.1822$	0.9985	1.0
100	50	25–150	13–75	$y = 0.1134x + 0.1521$	0.9994	1.3

Buffer (mM)	100% (μg/ml)	Accuracy (%)[j]	Accuracy (%)[k]	NTP[l]	As.[l]	Carry over
50	500	NA	102–104	>17,000	1.4	No
100	500	93–105	102–105	>18,000	1.5	No
100	50	82–93	98–105	>15,000	1.4	No

[a] Concentration of the potassium hexafluorophosphate buffer (KHFP).
[b] The concentration corresponding to 100% of the working concentration.
[c] The variability of six repetitive injections of a standard solution.
[d] The variability of the response factors of two standard solutions.
[e] Capacity factor (k') determined by using the retention time of acetic acid (blank solution) as t_0 in the equation $k' = (t_R - t_0)/t_0$.
[f] Limit of detection (LOD) and limit of quantification (LOQ).
[g] Linear range (LR) as the concentration in % of the working concentration.
[h] Correlation coefficient (R^2) of data fitted to a straight line.
[i] Intercept (m) as % of the signal (peak area; y) obtained for sample at 100% of the working concentration. OK if ≤5%.
[j] Accuracy for spiked samples (analytical errors introduced).
[k] Accuracy for standard solutions.
[l] Number of theoretical plates (NTP) and asymmetry (As.) calculated according to USP.

4. Conclusions

The intense, worldwide activities around EMD and/or its constituents, concerning characterisation, sequencing, recombinants, bioactivity, etc., clearly indicate the need to develop reliable methods for both purification and analysis.

To the best of our knowledge, no combined purification/analysis set-up, suitable for lab-scale preparative purposes, has been reported in the literature for the 5 kDa component from EMD. The three-method set-up, as presented in this paper, was found to be highly suitable for purification and analysis of this 5 kDa component. The purification step, based on low pressure SEC, resulted in fractionation of EMD into a 20 kDa, a |12 + 9| kDa, and a 5 kDa component, respectively. Although the 5 kDa component was relatively well separated, easily enabling the collection of fractions with only this component, the pools containing the two other components, must be subjected to further purification in order to obtain pure 20 kDa and |12 + 9| kDa components, respectively. However, this was beyond the scope of the presented work.

The subsequent quality control steps were based on (1) SE HPLC, and (2) RP HPLC; the former suitable for the selection of fractions to be pooled, and the latter as a true quality control step of the purified 5 kDa component. The SE HPLC analysis resulted in a single peak of the 5 kDa component, whereas the RP HPLC analysis revealed four main peaks of the 5 kDa component. Both quality control steps were highly linear over broad concentration ranges (SE HPLC 50–1500 μg/ml, RP HPLC approximately 10–75 μg/ml and 125–760 μg/ml; evaluated at two concentration ranges). Concerning the injection repeatability and precision, general requirements (<2% RSD) were clearly met using the RP HPLC method, while somewhat poorer results were obtained with the SE HPLC method (<5% RSD). No significant carry over was observed for either system. For both methods, the accuracy was questioned at the lower concentration levels due to the very low amounts of the 5 kDa component being injected. However, at higher concentration levels, general requirements (95–105% recoveries) were met by both methods.

The herein presented work is considered to be an important step towards fully characterising the enamel matrix derivative, using a contemporary analytical three-system set-up, suitable for lab-scale preparative purposes.

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References

[1] L. Hammarstrom, L. Heijl, S. Gestrelius, J. Clin. Periodontol. 24 (1997) 669.
[2] L. Heijl, G. Heden, G. Svardstrom, A. Ostgren, J. Clin. Periodontol. 24 (1997) 705.
[3] J.T. Mellonig, Int. J. Periodont. Restor. Dent. 19 (1999) 8.
[4] Z. Schwartz, D.L. Carnes Jr., R. Pulliam, C.H. Lohmann, V.L. Sylvia, Y. Liu, D.D. Dean, D.L. Cochran, B.D. Boyan, J. Periodontol. 71 (2000) 1287.
[5] A.M. Hoang, T.W. Oates, D.L. Cochran, J. Periodontol. 71 (2000) 1270.
[6] T. Kawase, K. Okuda, H. Yoshie, D.M. Burns, J. Periodontal Res. 35 (2000) 291.

[7] S.P. Lyngstadaas, E. Lundberg, H. Ekdahl, C. Andersson, S. Gestrelius, J. Clin. Periodontol. 28 (2001) 181.
[8] Y. Nakamura, L. Hammarstrom, E. Lundberg, H. Ekdahl, K. Matsumoto, S. Gestrelius, S.P. Lyngstadaas, Adv. Dent. Res. 15 (2001) 105.
[9] M. Zeichner-David, Matrix Biol. 20 (2001) 307.
[10] H.R. Haase, P.M. Bartold, J. Periodontol. 72 (2001) 341.
[11] A.M. Hoang, R.J. Klebe, B. Steffensen, O.H. Ryu, J.P. Simmer, D.L. Cochran, J. Dent. Res. 81 (2002) 497.
[12] A. Sculean, P. Windisch, T. Keglevich, B. Fabi, E. Lundgren, P.S. Lyngstadaas, Clin. Oral Investig. 6 (2002) 183.
[13] Y. Nakamura, L. Hammarstrom, K. Matsumoto, S.P. Lyngstadaas, Int. Endod. J. 35 (2002) 407.
[14] A. Spahr, S.P. Lyngstadaas, C. Boeckh, C. Andersson, A. Podbielski, B. Haller, J. Clin. Periodontol. 29 (2002) 62.
[15] Y. Hamamoto, N. Kawasaki, F. Jarnbring, L. Hammarstrom, Dent. Traumatol. 18 (2002) 12.
[16] J. Maycock, S.R. Wood, S.J. Brookes, R.C. Shore, C. Robinson, J. Kirkham, Connect Tissue Res. 43 (2002) 472.
[17] K. Okubo, M. Kobayashi, T. Takiguchi, T. Takada, A. Ohazama, Y. Okamatsu, K. Hasegawa, J. Periodontal Res. 38 (2003) 1.
[18] U. Mirastschijski, D. Konrad, E. Lundberg, S.P. Lyngstadaas, L.N. Jorgensen, M.S. Agren, Wound Repair Regen. 12 (2004) 100.
[19] S. Keila, C.E. Nemcovsky, O. Moses, Z. Artzi, M. Weinreb, J. Dent. Res. 83 (2004) 134.
[20] J. He, J. Jiang, K.E. Safavi, L.S. Spangberg, Q. Zhu, Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod. 98 (2004) 370.
[21] T. Otsuka, H. Kasai, K. Yamaguchi, T. Nishihara, J. Dent. 33 (2005) 749.
[22] R.E. Grayson, Y. Yamakoshi, E.J. Wood, M.S. Agren, Biomaterials 27 (2006) 2926.
[23] K. Yuan, C.L. Chen, M.T. Lin, J. Clin. Periodontol. 30 (2003) 732.
[24] A.G. Fincham, A.B. Belcourt, D.M. Lyaruu, J.D. Termine, Calcif. Tissue Int. 34 (1982) 182.
[25] A. Veis, Cell. Mol. Life Sci. 60 (2003) 38.
[26] S.J. Brookes, C. Robinson, J. Kirkham, W.A. Bonass, Arch. Oral Biol. 40 (1995) 1.
[27] A.G. Fincham, A.B. Belcourt, J.D. Termine, W.T. Butler, W.C. Cothran, Biosci. Rep. 1 (1981) 771.
[28] A.G. Fincham, A.B. Belcourt, J.D. Termine, W.T. Butler, W.C. Cothran, Biochem. J. 211 (1983) 149.
[29] A.G. Fincham, Y.Y. Hu, Z. Pavlova, H.C. Slavkin, M.L. Snead, Calcif. Tissue Int. 45 (1989) 243.
[30] A.G. Fincham, J. Moradian-Oldak, Biochem. Biophys. Res. Commun. 197 (1993) 248.
[31] A.G. Fincham, J. Moradian-Oldak, P.E. Sarte, Calcif. Tissue Int. 55 (1994) 398.
[32] H. Limeback, A. Simic, Arch. Oral Biol. 35 (1990) 459.
[33] O.H. Ryu, C.C. Hu, J.P. Simmer, Adv. Dent. Res. 10 (1996) 150.
[34] J. Catalano-Sherman, A. Palmon, Y. Burstein, D. Deutsch, J. Dent. Res. 72 (1993) 1566.
[35] L.R. Snyder, J.J. Kirkland, J.L. Glajch, Practical HPLC Method Development, John Wiley & Sons Inc., New York, 1997.
[36] CDER, Reviewer Guidance, Validation of Chromatographic Methods, November 1994, http://www.fda.gov/CDER/GUIDANCE/cmc3.pdf.
[37] D. Reynolds, K. Facchine, J. Mullaney, K. Alsante, T. Hatajik, M. Motto, Pharm. Technol. (2002).