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Journal of Chromatography B, 818 (2005) 67–73
JOURNAL OF CHROMATOGRAPHY B
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Isolation of fibronectin type III like peptide from human placental extract used as wound healer

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Received 14 June 2004; accepted 15 September 2004
Available online 11 November 2004

Abstract

A peptide of around 7.4 kDa has been purified from the aqueous extract of human placenta used as wound healer. Derived partial amino acid sequence from mass spectrometric analysis showed its homology with human fibronectin type III. Under nondenaturing condition, it formed aggregate, the elution pattern of which from reverse-phase HPLC was identical with that of fibronectin type III. Immuno-blot of the peptide with reference fibronectin type III-C showed strong cross reactivity. Since fibronectin type III plays important roles in wound healing, similar peptide in the extract is likely to take part in curing process.

Keywords: Human placental extract; Fibronectin type III; HPLC; Amino acid sequence; Dot blot

1. Introduction

Use of aqueous extract of human placenta in wound healing, ophthalmology, infertility, apoplexy, epilepsy etc has evolved from folk knowledge. Supported by scientific validation, it is now in practice worldwide under different trade names. This evolution was rational because placenta being the protective shield of the fetus, is a rich source of damage repairing agents like hormones, proteins, glycosaminoglycans, nucleic acids etc. Active components present in aqueous placental extracts vary depending on the method of its preparation [1]. In India, such extract is used as a wound healer in burn injuries, chronic wounds and surgical dressings [2]. Applications of similar extracts are also reported in cases of chronic nonhealing wound, arthritis and melanogenesis [3–5]. Though the therapeutic potency of the extract is confirmed by practicing physicians, neither the components are well characterized nor the mechanism of wound healing has been predicted with confidence.

Recently, we have initiated evaluation of this extract, which is used as a licensed drug. Though the extract is made from natural source, analysis of its different batches using a variety of chromatographic and spectroscopic techniques revealed remarkable consistency of their constituents [6]. This is likely to be a reflection of the standardization of the manufacturing process and also invariability of placental composition within limits irrespective of the nutritional status of the mothers. Further, NADPH, a component that takes part in wound healing, was found to be consistently present in this extract [2]. Antimicrobial property of the extract against different pathological and drug resistant strains has also been reported [7]. Towards characterization of protein/peptide components of this extract, it was observed that a peptide of approximately 7 kDa occurred in high abundance. Here we report identity of the peptide and discuss its possible biological significance.

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doi:10.1016/j.jchromb.2004.09.059

2. Materials and methods

2.1. Reagents

Human placental extract was supplied by the drug house M/s Albert David Ltd., Calcutta, India as sold under the trade name 'Placentrex'. Method of preparation of the extract holding manufacturers proprietary protocols has been described recently [2]. To ensure biological safety, each fresh placentae used for extraction was tested for HIV antibody and hepatitis B surface antigen. Further, sterilization of the product was done before and after filling in ampoules under conditions leaving sufficient margins to destroy most resistant spore-producing species like *Clostridium tetani*. Benzoylated dialysis membrane (cut off range 2 kDa), human fibronectin type III-C, peroxidase-conjugate goat anti mouse IgG, Tween-20, 3,3′-diaminobenzedine tetrahydrochloride, Freund's complete and incomplete adjuvants and dansyl chloride were from Sigma, USA. Centricon YM-10 (cut off range 10 kDa) filtration units were from Amicon (Bioseparations, Millipore). Tris-base, hydrogen peroxide and sodium phosphate were of analytical grade.

2.2. Purification of peptide

Pooled placental extract in 20 ml volume as supplied, was dried in wide mouth glass beaker in vacuum desiccators over sodium hydroxide pellets at ambient temperature. The dried mass was reconstituted with 200 μl of water after slowly stirring with a glass rod and collected. Recovery from the beaker was repeated twice using 25 μl of water and the pooled fraction was centrifuged in 1.5 ml eppendorff tubes at 10K rpm for 10 min in a bench top centrifuge. The yellowish liquid that was separated between the precipitate and the floating fat layer was carefully collected as far as possible. It was centrifuged again. The supernatant was diluted with water to 1 ml and filtered under centrifugal force (avg. 2000 × *g*) by Centricon Nylon membrane (YM-10, cut off limit 10 kDa). The filtrate was dialyzed by benzoylated dialysis membrane against water with several changes to remove salts and very small size peptides, if any. Finally, 100 μl aliquots of the dialyzed fractions were passed through 1ml 'spin column' of Sephadex G-15 equilibrated with water for removal of a yellowish tinge [8]. A colorless solution was recovered. The dialysis membrane and the Centricon nylon filters were submerged in a 20-fold diluted placental extract overnight to avoid nonspecific binding of purified placental components.

2.3. Electrophoretic analysis

SDS–polyacrylamide gel electrophoresis (20%) was done at pH 8.8 using mini gel apparatus of Genei (Bangalore, India). Crude placental extract and purified peptide (15 μg each) were loaded in the gel along with aprotinin (6.5 kDa) serving as molecular weight marker. Electrograms were viewed after silver staining. SDS–PAGE was also done with dansylated derivatives of proteins and viewed under UV transilluminator. Nondenaturing PAGE were done at pH 8.8 and viewed after silver staining.

2.4. HPLC

Crude extract and purified peptide were analyzed with a Protein Pak 60 size exclusion (SE)-HPLC column (Waters, fractionation range 1–20 kDa, size 7.8 mm × 30 cm). It was equilibrated and eluted isocratically in presence of 10 mM Na-phosphate, pH 7.5 containing 0.2 M NaCl at a flow rate of 1 ml/min and followed at 280 nm. The column was precalibrated with the following markers: bovine serum albumin (BSA, 66 kDa, for determination of void volume), myoglobin (17.9 kDa), lysozyme (14.2 kDa), cytochrome C (12.5 kDa) and aprotinin (6.5 kDa). A linear dependence of log (molecular mass) versus retention time was observed ($R^2 = 0.988$ where R is regression coefficient). Sample size was 15 μl containing 2 μg of protein.

The purified peptide (2 μg in 5 μl aqueous solution) was analyzed with C_{18} μ-bondapak reverse phase (RP)-HPLC column (Waters, 7.8 mm × 300 mm; 125 Å, 10 μm). The column was equilibrated and eluted isocratically with 10 mM Na-phosphate, pH 7.5 at a flow rate of 1 ml/min and followed at 220 nm. Human fibronectin type III-C (2 μg), dissolved in water, was applied under identical conditions.

2.5. Mass spectrometry

The molecular mass of the peptide was determined after exhaustive dialysis against water and using a Q-Tof micro (Micromass) instrument. For partial sequence determination, the dialyzed peptide (100 μg) was lyophilized. Its disulphide links were reduced under denaturing conditions and all cysteine residues were carboxymethylated to prevent oxidation. In short, the peptide was reconstituted with 25 μl of 8 M urea in 0.4 M ammonium bicarbonate containing 5 μl of aqueous solution of 45 mM dithiothreitol and was kept at 50 °C for 15 min. This was followed by addition of 5 μl of aqueous solution of 100 mM iodoacetamide. Derivatization was done at 25 °C for 2 h in the dark. Trypsin digestion of the sample was done by adding 2 μg (in 2 μl) of the enzyme in 50 mM ammonium bicarbonate containing 50% dimethylformamide. The digestion was continued at 37 °C overnight. Finally, 10 μl of 2% acetonitrile and 0.1% formic acid were added into the sample to dissociate peptide aggregates, if any. Approximately 15% of the digest was loaded onto a 75 μm × 10 cm POROS 10R2 capillary column by infusion from a 10 μl syringe. A solvent gradient from 2 to 90% acetonitrile in 0.1% formic acid was programmed in 80 min with an approximate flow rate of 200 μl/min. The eluted peaks were analyzed by LC–MS/MS. Data dependant MS/MS analysis was performed with 4-product ion scans following each full MS scan. The MS/MS data was analyzed with ThermoFinnigan Sequest BioWorks Browser, searching the data against the nr database from NCBI.

2.6. Dot blot

The immunological cross reactivity between proteins/peptides were checked by dot-blot experiments performed at room temperature. In short, nitro-cellulose membrane were cut into strips and soaked in 25 mM Tris buffer saline (TBS), pH 7.6 and semidried by paper towels. Smallest possible volume (1–2 μl) of normal mouse sera (negative control), purified peptide (as positive control) and human fibronectin type III-C (as reference) were applied into the strip and allowed to dry for at most 30 min. The nonspecific sites of the nitrocellulose strips were then blocked by incubating in blocking buffer (100 mM TBS containing 1% Tween-20) for 1 h with constant shaking. Washed once with washing buffer (0.05% Tween-20 in TBS to help in reduction of nonspecific background), the membrane was dried thoroughly. Strips were then incubated for 1 h with mouse antisera of purified peptide diluted 1:100 in washing buffer with shaking. The blots were then washed thrice for 20 min each and incubated for 1 h with 1:1000 dilution of peroxidase-conjugate goat anti mouse IgG (Sigma) again with shaking. This was followed by washing twice by TBS containing Tween-20 for 5 min each. Finally the strips were washed with only 0.1% Tween-20. Enzymatic activity was revealed from color development of 3,3′-diaminobenzedine tetrahydrochloride (Sigma Immunochemicals, 15 mg in 30 ml of TBS containing 15 μl of 30% H_2O_2) placed dropwise over the spots [9].

2.7. Other methods

Protein estimation was done after modified method of Lowry with BSA as reference [10]. Crude placental extract or purified peptide in 100 μl of 50 mM K-phosphate, pH 9.0, was incubated with 5 μl of DNS-Cl (10 mg/ml in acetone) overnight at ambient temperature in the dark [11]. The product was dialyzed against water before SDS–PAGE.

Antibody against the purified peptide was raised in BALB/c mice by injecting subcutaneous 25 μg in 100 μl saline after emulsifying with equal volume of Freund's complete adjuvant. The process was repeated five times after every 15 days using Freund's incomplete adjuvant. Blood was drawn after 15 days from the last application of the antigen from tail vain of the animals and serum was collected [12]. The animals were sacrificed by cervical dislocation and destroyed in incinerator.

2.8. Instrumentation

A transilluminator (model CHROMATO-VUE cabinet transilluminator TM-40, UVP Inc., San Gabriel, California, USA) was used for viewing dansylated peptides. Optical absorbance was measured with a digital UV–vis spectrophotometer (Digispec-200 GL, SICO, India). HPLC system used was of Shimadzu Corp. (Japan) and was equipped with two pumps (LC-10AT), a PDA detector (SPD-M10 Avp) and a controller (CBM-10A) to control mobile phases. The molecular mass of the peptide was determined using a Q-Tof micro (Micromass) instrument (housed at this institute) under positive ionization electrospray mode at a desolvation temperature of 200 °C. Argon as a collision gas at 2 kg/cm^2 having a collision energy of 10 eV was applied. Micro channel plate detectors were used. LC-MS/MS was performed with a Thermo Finnigan LCQ mass spectrometer (housed at University of Massachussets Medical School) using positive mode.

3. Results

3.1. Purification of the peptide

The supplied placental extract being low in protein content, (0.55 ± 0.05 mg/ml, $n = 4$), its SDS–PAGE did not show any distinct band after silver staining. Concentrating the extract 25-fold followed by SDS–PAGE resulted in appearance of three to four bands between 50–100 kDa together with peptide/s of 6–7 kDa of relatively high abundance. Standardization of the purification procedure revealed that starting from a 100-fold concentrated sample from 20 ml extract using a wide mouth glass beaker resulted in best recovery of the peptide. Under the conditions applied, majority of the high molecular weight proteins were removed either from co-precipitated with the insoluble materials or remained adhered with the floating fat layer during centrifugation. Remaining proteins were separated during Amicon filtration. Recovery and yield of the peptide in purification steps are summarized in Table 1.

Since no biological assay has been attributed to the recovered peptide, there was some inherent inaccuracy in its estimation. Further disadvantage in its quantification was that gel staining dyes and protein estimation reagents are usually less sensitive towards peptides compared to proteins [13]. In addition, peptides of 6–7 kDa in the original extract existed almost completely as higher molecular weight mass as a result its abundance could not be predicted from SE-HPLC profile (described later, Fig. 2). The peptide in impure form was estimated from densitometric scanning of SDS–PAGE gels while the pure form by modified Lowery's method [10]. It's recovery was limited to 18–20% as it is suspected that a majority

Table 1
Purification of fibronectin type III like 7 kDa peptide

Fraction	Total protein/ peptide (mg)	7 kDa peptide (mg)	Recovery (%)
Supplied placental extract (20 ml)	10.0	6.0	100
100-fold concentration and centrifugation	2.0	1.8	30
Centricon YM-10 filtration	1.5	1.5	25
Dialysis	1.2	1.2	20
Spin column	1.2[a]	1.2[a]	20[a]

[a] Recovery in 'spin column' was nearly 100%.

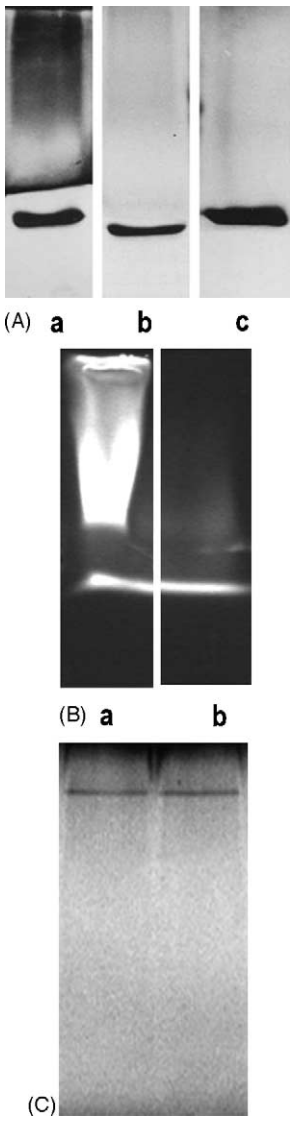


Fig. 1. (A) 20% SDS–PAGE of: (a) crude placental extract, (b) aprotinin, and (c) purified peptide. (B) 20% SDS–PAGE of dansylated: (a) crude placental extract, and (b) purified peptide. (C) 20% PAGE of the purified peptide from two different batches.

of the peptide co-precipitate during concentrating the extract and/or remains associated with the lipid layer (Table 1).

3.2. Purity and molecular weight

The 20% SDS–PAGE profiles of 25-fold concentrated placental extract, purified peptide along with aprotinin has been shown in Fig. 1A. The peptide appeared as homogeneous having comparable mass to that of aprotinin. To detect presence of other peptides that remained poorly detected after silver staining, dansylated crude extract and purified peptide (10 μg each) were electrophoresed under identical conditions (Fig. 1B). No contamination in the peptide preparation could be detected. The freshly prepared peptide in 20% PAGE at pH 8.8, once again showed a single band (Fig. 1C). The occurrence of multiple peaks in SE-HPLC and RP-HPLC, as

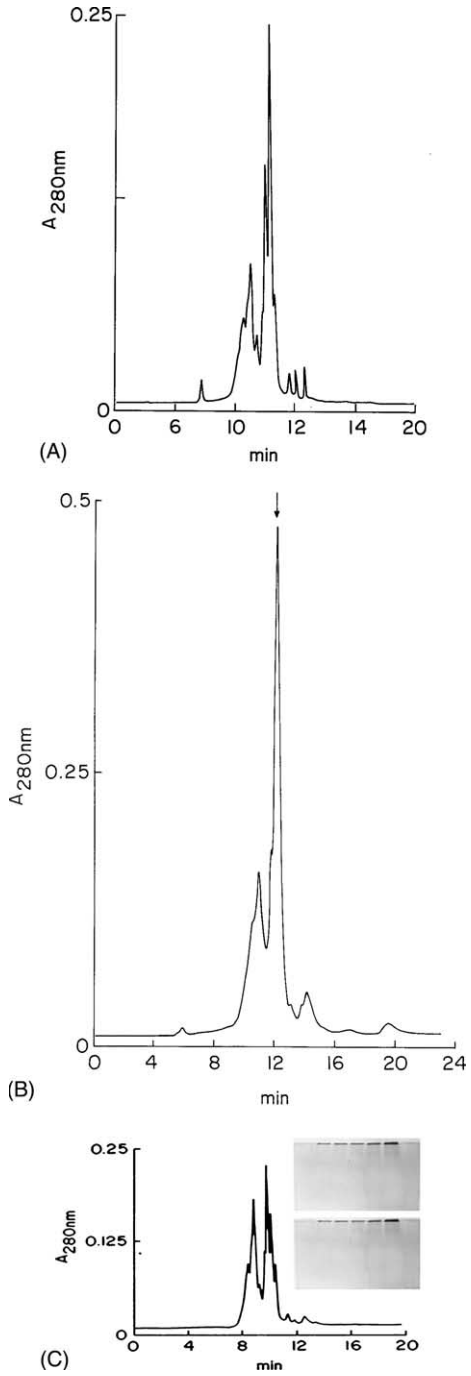
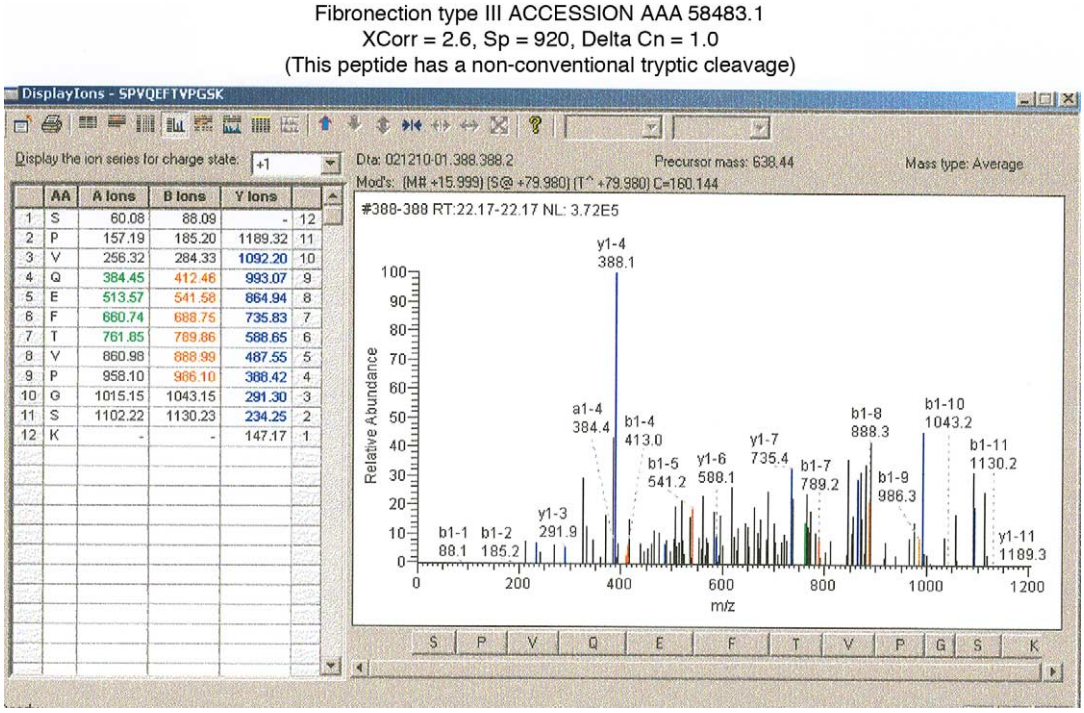


Fig. 2. SE-HPLC profiles of: (A) crude extract, (B) purified peptide after 24 h of preparation where the elution position of aprotinin, 6.5 kDa has been marked by an arrow and (C) after storage for 7 days at 4 °C. (Inset) upper panel, 20%, and lower panel, 7.5% PAGE profile of the stored sample.

described below, were apparently related to inherent tendency of peptides and small proteins towards aggregate formation.

3.3. SE-HPLC

SE-HPLC profiles under defined conditions for the crude extract and the purified peptide after storage for



	AA	A Ions	B Ions	Y Ions	
1	S	60.08	88.09	-	12
2	P	157.19	185.20	1189.32	11
3	V	256.32	284.33	1092.20	10
4	Q	384.45	412.46	993.07	9
5	E	513.57	541.58	864.94	8
6	F	660.74	688.75	735.83	7
7	T	761.85	789.86	588.65	6
8	V	860.98	888.99	487.55	5
9	P	958.10	986.10	388.42	4
10	G	1015.15	1043.15	291.30	3
11	S	1102.22	1130.23	234.25	2
12	K	-	-	147.17	1

Fig. 3. Mass spectrometric analysis of a tryptic fragment of the purified peptide showing derivation of partial amino acid sequence.

24 h at 4 °C have been shown in Fig. 2A and B respectively. The extract showed three major peaks of retention time $R_t = 10.5 \pm 0.05$; 11.0 ± 0.02; 11.2 ± 0.02 min together with several minor peaks of $R_t = 7.2 \pm 0.07$, 11.9 ± 0.03, 12.0 ± 0.02, 12.3 ± 0.05 min ($n = 3$). The purified peptide showed one major and one minor peak of $R_t = 12.0 \pm 0.02$ and 10.5 ± 0.05 min, respectively. Its major component closely corresponded to aprotinin ($R_t = 12.0 \pm 0.05$). From these profiles it is evident that about 33% of the purified peptide formed dimer while in the original extract it existed almost exclusively as multimer.

3.4. Aggregation on storage

The SE-HPLC profile of the purified peptide after storage for 7 days at 4 °C in water has been shown in Fig. 2C. The peak corresponding to 6.5–7 kDa disappeared completely and several multimers were formed. This pattern was comparable to the SE-HPLC profile of the crude extract. The PAGE profiles of the stored peptide (Fig. 2C, Insets) showed their resistance into entering the gel — an indication of their tendency to aggregate.

3.5. Mass spectrometric analysis

The peptide showed the following MS data (FAB): *m/z* $[(M + H)^+]$ of 7381 and 14,762 together with other fragmented products. This indicated that the peptide has molecular mass of around 7.4 kDa and the ability to form a strong noncovalent dimer. Higher multimers were not detected. Partial sequence of one of its tryptic digest was found to be 'SPVQEFTVPGSK' (Fig. 3). BLAST search of the sequence corresponded to fibronectin type III (Accession No. AAA58483.1).

3.6. Reversed phase (RP)-HPLC

The elution profile of the purified peptide was compared with standard fibronectin type III in terms of retention time from a RP-HPLC column monitored at 220 nm under defined condition (Fig. 4). In either case, overlapping multiple peaks appeared between retention time (R_t) = 6–12 min. The R_t of the major components of the two sets were very similar, e.g., for purified peptide 6.97 ± 0.05, 7.52 ± 0.03 and 8.22 ± 0.01 min while that of fibronectin type III, 6.90 ± 0.05, 7.52 ± 0.05, 8.30 ± 0.05 and 9.56 ± 0.01 min respectively ($n = 3$). Relative abundance of the peaks were, however different. Occurrence of multiple peaks in RP-HPLC from pure peptides which are prone to aggregation is a general phenomenon.

3.7. Dot blots

In the dot-blot experiments, strong immunological cross reactivity of anti-mouse sera, raised against purified peptide was observed with the purified peptide as positive control. No cross-reactivity was observed with normal mouse sera as negative control but significant reaction was observed with human fibronectin type III-C (Sigma) ($n = 3$) (Fig. 5). This further indicated fibronectin type III like character of the pep-

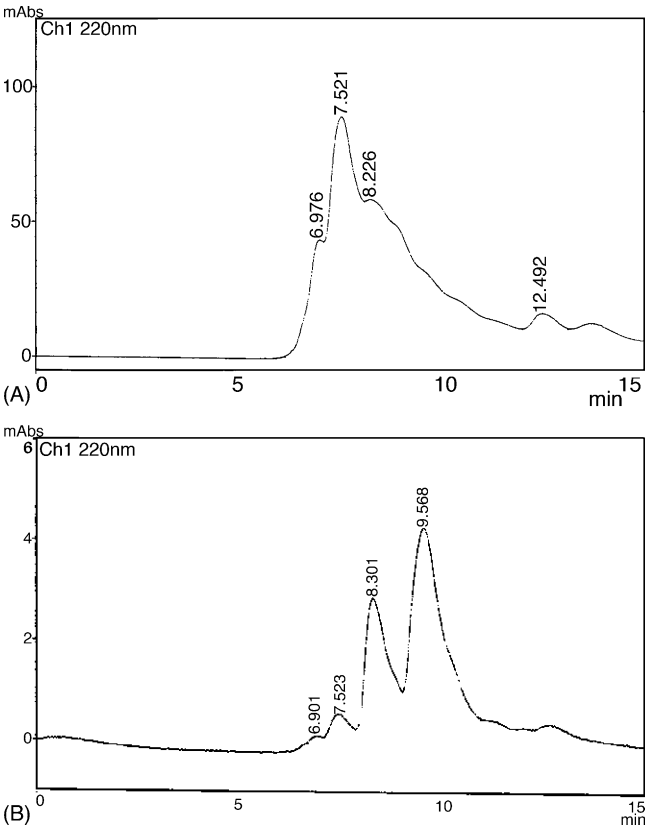


Fig. 4. Reversed phase HPLC profile of: (A) purified peptide and (B) fibronectin type III.

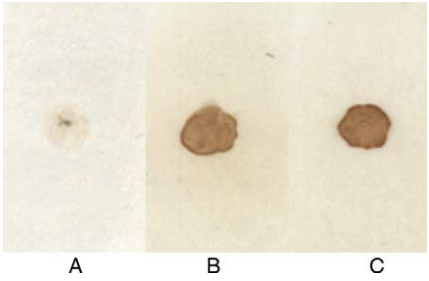


Fig. 5. Immunological cross reactivity between anti-sera of the purified peptide and (A) mouse serum, (B) purified peptide as analyte, and (C) human fibronectin type III-C (Sigma).

tide supporting the previous observations. This protocol is, however, semi quantitative.

4. Discussion

While characterizing the aqueous extract of human placenta used as wound healer, a peptide of 7.4 kDa of high abundance has been purified to apparent homogeneity. Its size and partial amino acid sequence indicated its similarity to human fibronectin type III. The purity of the freshly prepared peptide was demonstrated by SDS–PAGE, PAGE, SE-HPLC and mass spectrometric analysis. The recovery of the peptide was about 20% as a majority of it was co-precipitated during purification. It has tendency to aggregate, which is a general property of peptides. Its immunological cross reactivity with reference fibronectin type III was demonstrated by dot-blot experiment. The presence of fibronectin type III or like peptide in placental extract in high abundance might be significant to understand its mode of action in wound healing.

Fibronectins are adhesive mosaic glycoproteins comprised of three types of homologous repeating modules. These modular units are used as basic building blocks to form domains that develop distinct functions such as establishment and maintenance of normal cell morphology, cell migration, homeostasis and thrombosis, wound healing and oncogenic transformation [14]. Though fibronectin is encoded by single gene, this protein exists in a number of variant isoforms due to alternative splicing and/or posttranslational modifications such as glycosylations and splicing at three general regions of the precursor mRNA; IIICS, ED-A and ED-B [15]. The plasma fibronectin isoform lacks the alternatively spliced domains, whereas the cellular fibronectin isoform contains the ED-A domain in their protein [16]. Fibronectin isoforms containing the domains III-A (ED-A), III-B (ED-B) and III-V (II-ICS) are expressed to a higher degree in transformed human cells and in tumor tissues than in their normal counterparts [17,18]. Especially, fibronectin containing the III-B sequence is detectable almost exclusively in healing wounds, in fetal tissues and in tumor tissues [19,20]. The fibronectin isoforms, synthesized by tumors and fetal tissue including placenta, are called oncofetal fibronectin.

In human placenta, fibronectin molecule is detectable in various sites such as amnion, basal plate, chorionic plate, decidua, placental fibrinoid, umbilical cord and villi. These molecules especially oncofetal isoforms play roles in the differentiation of trophoblast cells. It has been reported that human amnion epithelial cells secrete cellular (III-A domain containing) and oncofetal (III-B containing) fibronectin isoforms that are assembled in the extracellular matrix [21]. Fibronectins in the amniotic basal lamina, particularly oncofetal isoform, is a part of healing process in this tissue due to the rapidly growing fetus causing partly shears in the amnion [22].

Type III module contains the Arg-Gly-Asp (RGD) sequence that binds with other integrins and heparin resulting in cell adhesion [23]. Trophouteronectin, one of the oncofetal isoform of fibronectin, mediates attachment of the placenta to the uterus [24]. The importance of fibronectin in cell migration events during embryogenesis is well known [25]. It has also been reported that the oncofetal fibronectin is involved in endothelial cell proliferation, developing tissue and wound healing more efficiently than normal fibronectin. The integrin binding RDG sequence present in type III module is critical in integrin-extracellular matrix signaling. Interaction of cell's fibronectin receptors, a member of integrin family, with fibronectin results in adhesion and spreading of cells [26]. These events collectively favor wound repairing and is likely to be supported by the extract analyzed.

Acknowledgements

A grant from M/s Albert David Ltd., awarded to D.B. supported the research. P.D. was a senior research fellow of the same source. Ms. S. Pal, Mr. S. Saha (Division of Infectious Disease, IICB) and Mr. C. Sarkar (Division of Cellular Biochemistry, IICB) helped us at different stages. We are deeply indebted to Ms. Karin Green (Dept. of Biochemistry and Molecular Pharmacology, University of Massachussets Medical School, USA) and Mr. Kalyan Sarkar (IICB) for performing mass spectrometric analysis.

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