Purification from human plasma of a tetrapeptide that potentiates insulin-like growth factor-I activity in chick embryo cartilage

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Abstract Human plasma has been shown to contain a low molecular weight factor that potentiates human IGF-I stimulation of glycosaminoglycan synthesis in chick embryo cartilage. The peptide was purified and characterized by Edman degradation and electrospray mass spectrometry. The primary structure determined was: Trp-Gly-His-Glu. A homologous synthetic peptide similarly promoted matrix biosynthesis in cartilage exposed to IGF-I.

Key words: Glycosaminoglycan synthesis; Insulin-like growth factor I; Peptide purification; Wound repair; Human plasma; Chick embryo chondrocyte

1. Introduction

Insulin-like growth factors (IGF-I and IGF-II) are single-chain polypeptides that have structural homology with proinsulin [1–3]. They have been shown to regulate proliferation and differentiation in numerous cell types (review in [4]). Unlike insulin, they are produced in most tissues and are abundant in the bloodstream, thus acting via endocrine, as well as autocrine and/or paracrine, mechanisms [5].

One of the initially recognized biological activities of the IGFs was their ability to stimulate sulfated mucopolysaccharide synthesis in articular cartilage [6]. They were found not only to promote sulfation of the glycosyl residues in proteoglycans, the final step in glycosaminoglycan biosynthesis, but also to enhance synthesis of the core protein [7] and elongation of existing chontroitin sulfate chains [8,9].

Early purification protocols for IGF-I and IGF-II were montored by measuring the stimulation of [^{35}S]sulfate uptake into rat [10], chick embryo [11] and pig [12] cartilage explants. However, Froesch et al. [6] demonstrated that highly purified IGFs per se failed to stimulate [^{35}S]sulfate incorporation into chick embryo cartilage, but that, when added to serum from hypothyroid, hypophysectomized rats, which itself exerted no stimulation, they became potent sulfation factors.

Heulin et al. [13] in our laboratory found that human serum contained factors with molecular weights (MWs) below 1000 Daltons (Da), that were necessary for the sulfation activity of IGF-I in chick embryo pelvic leaflets. In view of their size and biological properties, they were named low molecular weight growth promoting factors (LMW-GFs). The bioassay [14] was used in the purification of several molecular forms of the IGFs from human COHN paste IV [15] or fetal ovine serum [16].

We now describe the complete isolation and characterization of a tetrapeptide from normal human plasma, which modulates the effects of recombinant human (rh) IGF-I on proteoglycan synthesis in cultured chick embryo cartilage. Its identity was confirmed using a synthetic peptide with identical electrophoretic migration and biological activity.

2. Materials and methods

2.1. Isolation

2.1.1. Partial purification; Gel permeation chromatography. LMW-GF was partially purified from normal human plasma as previously described [16]. Briefly, 15 liters human plasma were ultrafiltered through a PTAC membrane (1000-Da cut-off; Millipore, Bedford, MA, USA) at 4°C. Ultrafiltration was stopped when retentate volume reached 1/3 of the original volume. The ultrafiltrate (10 liters) was extracted (v/v) with chloroform for 24 h at 4°C so as to remove hydrophobic molecules such as steroid hormones. The lyophilized aqueous phase (50 μg peptide/ml ultrafiltrate, as determined according to Sargent [18]) was applied to a 100 × 10-cm column packed with 200–400-mesh Biogel P2 (BioRad, Hercules, CA, USA). Elution was carried out using distilled water at a flow rate of 150 ml/h. Fractions were collected over a period of 20 min and their biological activities measured as described below.

2.1.2. Reverse phase HPLC purification of the tetrapeptide. Biogel P2-active fractions eluted between K_{av} 0.45–0.65 (370–700 Da) from four successive runs were pooled for further purification.

Lyophilized samples were dissolved in 100 ml 0.1% (v/v) trifluoroacetic acid (TFA)/water and pumped (20 ml) at a flow rate of 2 ml/min onto a 250 × 10-mm Vydac C_{18} reverse-phase (RP) HPLC column (Separations Group, Hesperia, CA, USA) equilibrated with the same buffer (0.1% TFA in water: Solvent A). After 10 min washing of the column with Solvent A, the acetonitrile concentration in the elution solvent was raised over 35 min to 10.5% (v/v) then over 30 min to 17.5% (total time: 65 min) using linear gradients. Absorbance was read at 214 nm; 2-ml fractions were collected. Bioactive factions, which eluted between 39–44 min (Fig. 1A), from five successive runs were pooled, evaporated to dryness, re-dissolved in 500 μl 0.1% TFA and concentrated by RP HPLC on an analytical Vydac C18 column (250 × 4.6 mm, 300 Å pore, 5 μm bead), using 0.1% TFA (Solvent A) and 0.08% TFA in acetonitrile (Solvent B). 50-μl aliquots were loaded onto the column equilibrated with Solvent A and the peptides eluted using the same gradient as above at a flow rate of 0.6 m/min.

The peak, which eluted at 42.5–43.5 min, was collected manually (Peak 2, Fig. 1B) and further purified to apparent homogeneity by additional runs on the same column. The acetonitrile concentration was raised to 15% through a flatter linear gradient. Samples of approximately 250–400 pmol peptide (purified Peak 2) were then subjected to sequencing and mass analysis.

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Abbreviations: IGF, insulin-like growth factor; Da, Daltons; rh, recombinant human; MW, molecular weight; RP HPLC, reverse phase high-performance liquid chromatography; Boc, *t*-Butyloxycarbonyl; BOP, Castro's reagent; TFA, trifluoroacetic acid.

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2.2. Characterization

2.2.1. Peptide sequencing. Automated Edman degradation was carried out on an Applied Biosystem (Foster City, CA, USA) 470 A protein sequencer. Phenylthiohydantoin (PTH) derivatives were identified by HPLC after automatic injection into an Applied Biosystem 120 A analyser coupled to the sequencer. The methods used have been described elsewhere [16].

2.2.2. Analytical capillary electrophoresis (CE). The CE apparatus consisted of a Beckman (Palo Alto, CA, USA) Model P/ACE 2100 automated system with autosampler and a Beckman Gold data collection system. UV detection was at 200 nm and the data collection rate, 5 Hz. A 50-μm-i.d. deactivated silica capillary (Beckman) with a separation distance of 70 cm (total length 77 cm) was maintained at 25°C. Experimental conditions are described in the legend to Fig. 4.

2.2.3. Electrospray mass spectrometry (ESI-MS). A lyophilized sample of purified Peak 2 (Fig. 1B) was made up to 30 μl in an aqueous methanol solution (50:50 v/v) containing 1% acetic acid. ESI-MS analysis was carried out by injecting the sample at a flow rate of 2 μl/min into an HP 5989 mass spectrometer (Hewlett Packard, San Fernando, CA, USA) equipped with an electrospray ion source (Analytica of Brandford type) and scanning over a mass range of 200–1100. The sum of several scans was used to obtain the final ESI-MS spectrum.

For mass scale calibration, the multiply-charged molecular ions obtained from a separately introduced sample of heart myoglobin (average molecular mass: 16,951.5 kDa) were employed.

2.3. Synthetic peptide assembly

Solid phase synthesis of the WGHE tetrapeptide was performed manually using chloromethylated resin containing 0.7 mmol/g Glu (OcHx). Boc-Trp, Boc-Gly and Boc-His (Dnp) were incorporated using BOP, as described elsewhere [19]. The peptide-resin compound was treated with thiophenol (50% in DMF) for 30 min to remove the Dnp group prior to final HF cleavage (1 h at 0°C in the presence of anisol). After trituration in ether, the crude compound was recovered in a water/acetic acid mixture (10:90) and lyophilized.

Purification of the synthetic peptide was achieved by HPLC: the crude product was injected onto a semi-preparative column (Merck Hibar, 25 × 250 mm, 10 μm bead) and eluted through a gradient using an acetonitrile/water mixture containing 0.1% TFA. Fractions of > 95% purity as monitored by analytical HPLC were lyophilized as the purified compound.

2.4. Bioassay

All purification steps were monitored by measuring the ability of samples to promote IGF-I-induced [^{35}S]sulphate incorporation into glycosaminoglycans (GAGs) in chick embryo leaflets, as described in detail elsewhere [14,20,21].

Recombinant human (rh) IGF-I was tested, either alone at 20 ng/ml, or combined with either a reference human serum ultrafiltrate (1000-Da cut-off) or eluted fractions at appropriate dilutions.

One arbitrary unit is defined as the amount of peptide with the same ability as 10% (v/v) serum ultrafiltrate to stimulate GAG synthesis in the presence of 20 ng/ml rhIGF-I over a 20-h incubation period.

2.5. Statistical analysis

Bioassays were repeated 3–5 times. Results are expressed as means ± S.E.M. Differences between means were assessed by variance analysis (ANOVA and Bonferroni *t*-test) using SIGMASTAT software (Jansen Scientific Software, Erkrath, Germany).

3. Results

3.1. Purification of the LMW-GF tetrapeptide with growth promoting activity

Pure IGFs are known to be weak sulfation factors in chick embryo cartilage when added alone to synthetic medium [6]. We previously showed that normal human serum contains at least one LMW-GF that potentiates IGF-I's stimulation of GAG biosynthesis in this tissue. Partial purification from human plasma was attempted at the time, using ultrafiltration and chromatography on molecular sieving gels [17].

In this study, semi-preparative RP HPLC was used to purify the single major peak with bioactivity recovered from Biogel P2 gel permeation chromatography [20] (Fig. 1A). Growth promoting activity (represented by the bar graph) emerged in two peaks between 11.6% and 13.5% acetonitrile.

Analytical HPLC of the fractions with the highest activities revealed LMW-GF in two well-defined peaks (Peak 1 and Peak 2, indicated by the arrows in Fig. 1B). Successive runs of Peak 2, eluted at 43.0 min (12.4% acetonitrile), led to purification to apparent homogeneity of LMW-GF peptide (not shown) which was then used for further characterization.

3.2. Peptide characterization

3.2.1. Sequence analysis. The primary structure of the human LMW-GF isolated was established as: Trp (242) – Gly (163) – His (105) – Glu (estimated at 47), or WGHE (international one-letter amino acid abbreviations). The values in brackets are the yields of the PTH-derivatives in pmol. The

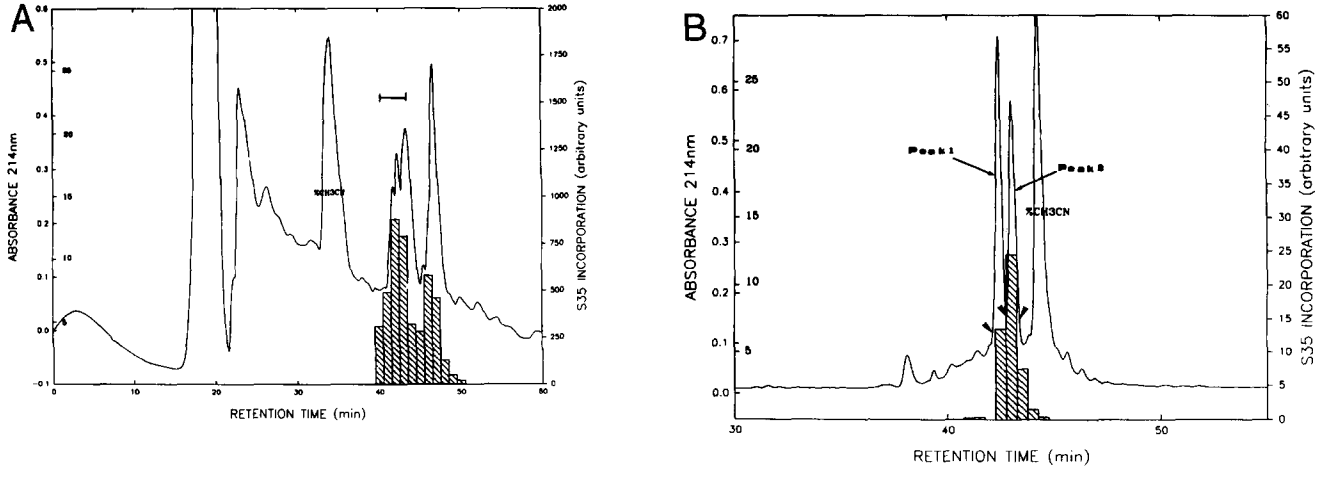

Fig. 1. (A) Semi-preparative RP HPLC purification of LMW-GF. Bioactive Biogel P2 fractions were pooled and concentrated, then chromatographed on semi-preparative Vydac C18 columns using linear gradients of acetonitrile in 0.1% TFA. Fractions collected between 39 and 44 min, indicated by the horizontal line above the peak, were then purified further. (B) Analytical RP HPLC purification. The fractions from (A) were applied to analytical Vydac C_{18} columns and eluted using a flatter acetonitrile gradient. The arrows show where manual collection began and ended. Purified Peak 2 was used for sequencing, CE and ESI-MS analysis. The dotted lines indicate acetonitrile concentrations in the elution solvents. The inset bar graphs represent [^{35}S]sulfate incorporation (arbitrary units per fraction).

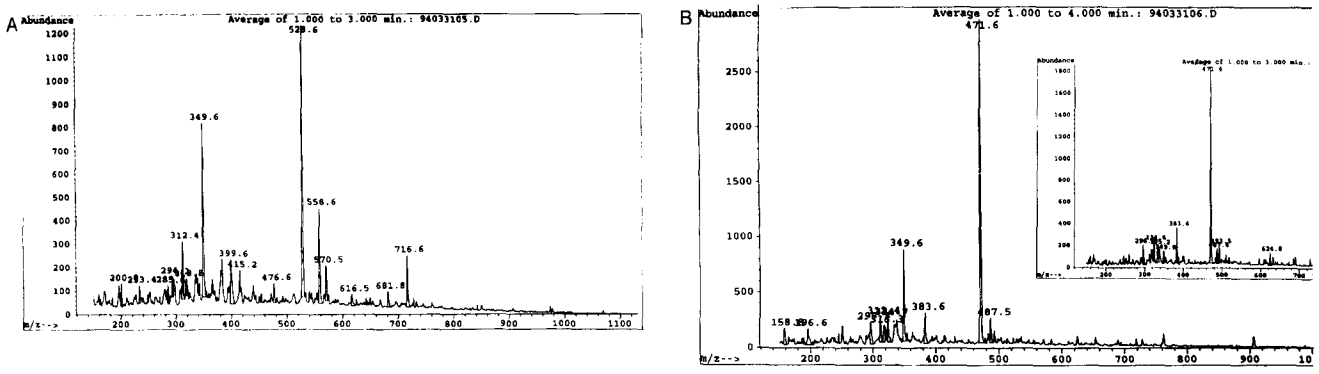

Fig. 2. (A) Electrospray mass spectrometry (ESI-MS) analysis of purified Peak 2 (Fig. 1B) (capillary exit voltage: 220 V). (B) ESI-MS analysis of Peak 3 in Fig. 1B (capillary exit voltage: 190 V). Inset shows the same ESI-MS analysis with capillary exit voltage, 240 V.

strong absorbance of the purified peptide at 280 nm confirmed the presence of a tryptophan residue (spectrophotometric measurement not shown).

A minor sequence was also found, which proved to be a contaminant from Peak 1 (Fig. 1B). Its sequence was: His (28) – X (?) – Glu (estimated at 51) – Ser (12). It was suspected that X may be a tryptophan residue. It may equally have reflected build-up of the lag of PTH-Trp, the first residue of the major component, but it was confirmed as a tryptophan by sequence data and mass spectrometry analysis of Peak 1 (Fig. 1B) (manuscript in preparation).

Precise quantification of PTH-Glu proved impossible, owing to the presence of the minor sequence.

3.2.2. Mass spectrometry analysis. Purified Peak 2 was subjected to electrospray mass spectrometry. As shown in Fig. 2A, several peaks were obtained. The mass averages for the two major peaks were 528.6 and 349.6, respectively. The value for the highest peak, 528.6 Da ($M + H^+$), agreed with that predicted on the basis of the amino acid sequence of the purified peptide accurate mass: 527.2 Da).

The unidentified component with a mass average value of 349.6 corresponded a contaminant from Peak 3 in Fig. 2B and was therefore probably not biologically active. Furthermore, the inset (Fig. 2B) shows that this component was absent with a capillary exit voltage of 240 V.

The two other peaks of 558.6 and 716.6 were shown to be contaminants from Peak 1 (manuscript in preparation).

3.3. Biological activity

On the basis of these findings, we prepared a synthetic WGHE tetrapeptide and compared its biological activity with that of the native peptide, purified Peak 2, in the 11-day-old chick embryo pelvic cartilage bioassay.

As shown in Fig. 3:

(1) neither the WGHE tetrapeptide at 30 ng/ml, nor rhIGF-I at 20 ng/ml affected basal [^{35}S]sulfate uptake (104% and 117%, respectively: not statistically significant);

(2) a combination of 20 ng/ml rhIGF-I and WGHE at five different concentrations (20–50 ng/ml) synergistically increased incorporation of the tracer, indicating enhanced GAG synthesis (and corroborating the results obtained with native peptide);

(3) stimulation was dose-dependent and statistically signifi-

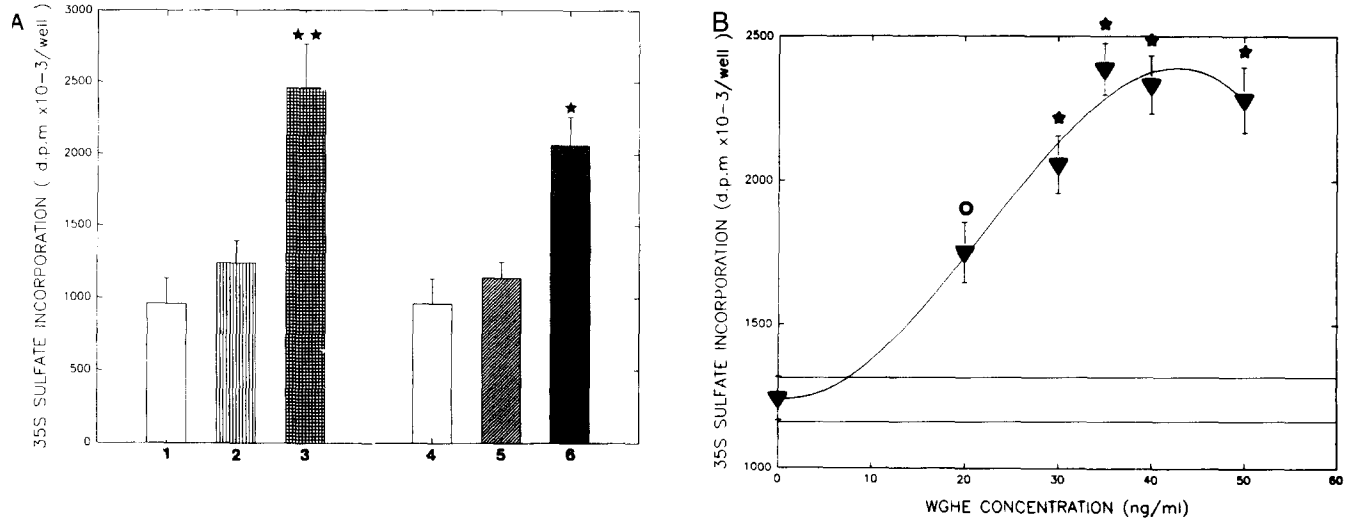

Fig. 3. [^{35}S]Sulfate incorporation into glycosaminoglycans in chick embryo pelvic leaflets. (A) Cartilages were incubated with: control culture medium (Bars 1 and 4); or 20 ng/ml rhIGF-I (Bar 2); or 30 ng/ml pure synthetic WGHE peptide (Bar 5); or rh IGF-I plus 10% (v/v) reference human serum ultrafiltrate (Bar 3); or rh IGF-I plus WGHE (Bar 6). Results are expressed as means ± S.E.M. for five separate incubations. (B) Cartilages were incubated with 20 ng/ml rhIGF-I alone or in combination with synthetic WGHE peptide (at five concentrations). Results are expressed as the means ± S.E.M. for 8 cartilages. * = $P < 0.05$; ** = $P < 0.01$ compared with controls (ANOVA).

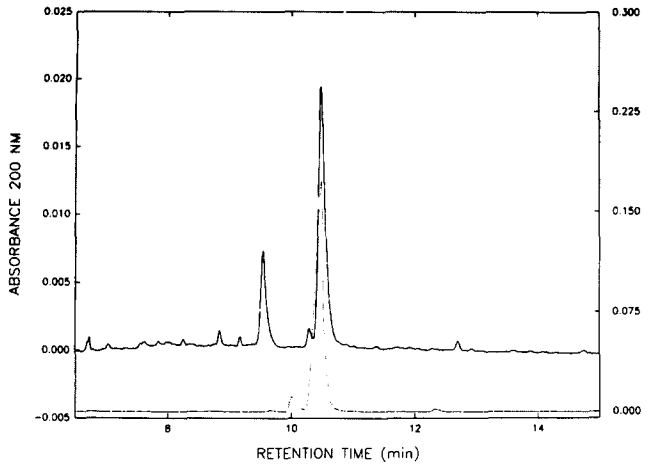

Fig. 4. Analytical capillary electrophoresis of purified Peak 2 (Fig. 1B) and of synthetic WGHE. Experimental conditions: buffer: 25 mM sodium borate, pH 8.3; pressure injection: 15 s; voltage: 25 kV; running temperature: 25°C; UV detection: 200 nm, 0.025 AUFS. Solid line, Peak 2; dashed line, Synthetic WGHE.

cant at WGHE concentrations between 10^{-9} and 10^{-7} M. The effect was biphasic, with maximal stimulation (186%) at 6.6×10^{-8} M. Stimulation varied from one batch of cartilages to another, ranging from 165% to 205% above the (control) effect of rhIGF-I alone.

4. Discussion

This is the first report of isolation from human plasma and structural characterization of the tetrapeptide, WGHE, which modulates the effects of IGF-I on proteoglycan synthesis in chick embryo cartilage in culture.

Mass spectrometry analysis yielded a molecular mass which agreed with that deduced from the primary sequence.

Peptides from Peak 1 (Fig. 1B) were recovered in Peak 2 (Fig. 1B), but contamination was less than 15% on the basis of the area under the peak in analytical capillary electrophoresis (Fig. 4).

Confirmation of the nature and activity of the tetrapeptide was obtained using a synthetic WGHE peptide which had the identical migration time in borate buffer capillary electrophoresis and, in combination with rhIGF-I, exhibited the same biphasic, dose-dependent stimulation of [^{35}S]sulfate uptake in pelvic cartilage explants. This classical biphasic biological effect is also observed in GAG synthesis stimulated by vitamin D3 [22] or the tripeptide, GHK [23].

It remains to be determined whether or not the effects of the WGHE tetrapeptide are species-specific, or peculiar to a particular stage of development, or limited to a single biological activity, such as GAG sulfation. Its presence in normal human plasma indicates that it may play a role in the regulation of growth and differentiation or it may likewise be involved in cartilage growth disorders. Jacob et al. [24] recently demonstrated that LMW-GF bioactivity ([^{35}S]sulfate incorporation) was drastically reduced in patients with chronic renal failure and was restored to normal levels after successful kidney transplantation. This would suggest that the human kidney may be involved in LMW-GF biosynthesis and/or processing. The ability of IGF-I to enhance matrix synthesis in normal cartilage is well established, both in vivo [25] and in vitro [26,27]. However, both IGFs are produced ubiquitously, together with specific, high-affinity binding proteins (IGFBPs), and their biological activities are mediated by the Type 1 IGF receptor [28,29]. In so complex a system, WGHE tetrapeptides would constitute an abundant source of bioactive probe in investigating structure/function relationships in the IGF-I signaling pathway.

The WGHE motif is present in several human and mammalian cell proteins, like the carboxy-terminal domain of human actin-binding protein (ABP-280) at amino acid position, 2632–2635 [30], or the human high MW kininogen sequence, at position 431–434 [31,32]. Kininogens are potent inhibitors of thiol-proteases and have been implicated in the pathogenesis of inflammation. Recent findings suggest that the renal vasodilatory effect of IGF-I is mediated by kinins [33]. We suspect that our WGHE tetrapeptide may be produced by a variety of cell types and/or be generated by proteolysis of these high MW proteins.

The extracellular matrix (ECM) is an important template in the regulation of cell responses to growth factors [34–36], although the mechanisms involved remain to be elucidated. Sulfated GAGs are major constituents of the ECM and, through enhanced synthesis, are predominant in the late post-inflammatory phase of wound repair [37]. Negative or positive regulation of matrix production would depend on the proportions of specific proteases present during tissue regeneration. Here, the WGHE tetrapeptide may be a minimum sequence of an unidentified polypeptide involved in the maturation of the ECM, possibly by inhibiting ECM degrading enzymes, and in promoting the completion of wound repair.

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