Quantitative Detection of Fibronectin in Porcine Small Intestinal Submucosa Extracellular Matrix

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Abstract Quantitative analysis of fibronectin in porcine small intestinal submucosa (SIS) acellular matrix was investigated using batch enzymatic digest and high performance liquid chromatography–mass spectrometry (HPLC-MS). The results indicated that batch enzymatic digest could improve the hydrolysis efficiency compared to static enzymatic hydrolysis. The hydrolysis time decreased to 6 hours from 96 hours and the enzymatic hydrolysis rate amounted to 95%. Marker peptide, SSPVVIDASTAIDAPSNLR, was detected from the tryptic digest of FN. Based on the peak areas of marker peptide from FN standards with different concentration, the FN content in VIDASISTM was determined as 0.43%. Amount of FN in BiodesignR was determined as 0.17%. The method precision (RSD, 1.31%) and the deviation were carried out in the methodology experiments. The results indicate the established quantitative method is effective for detecting FN content in acellular matrix.

Keywords porcine small intestinal submucosa; extracellular matrix; fibronectin; marker peptide; LC-MS

Introduction

Small Intestinal Submucosa (SIS, Small Intestinal Submucosa) is a membranous material prepared from the submucosa of the pig small intestine through decellularization, molding and sterilization processes. It is widely used in tendons, dura mater, abdominal wall and The repair or reconstruction of skin and other tissues has the advantages of high biocompatibility, biodegradability and absorbability [1,2]. The clinical application results show that SIS can effectively induce cell adhesion and growth, with fewer postoperative complications and infection rates. Low [3-5], confirming that SIS is an animal-derived implant material without immune rejection.

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As an acellular matrix, SIS is mainly composed of type I collagen and a small amount of other types of collagen; it also contains a small amount of fibronectin (FN), laminin, growth factors and other biologically active components [6]. FN is a glycoprotein in the form of a dimer (220 kDa) connected by disulfide bonds, which can be secreted by fibroblasts, muscle cells, endothelial cells, and many other cells. There are no significant differences in the molecular weight, three-dimensional structure and biological properties of FN from different animals [7]. FN exhibits adhesion and migration characteristics with a variety of cells including fibroblasts and endothelial cells, and has the reputation of extracellular adhesive [8]. FN plays a key role in embryogenesis, wound healing, and hemostasis. The abnormal expression and degradation process of FN are closely related to many diseases such as cardiovascular disease and tumor metastasis [9-10]. Therefore, the content of FN in acellular matrix The content directly affects the biological activity of SIS. Therefore, research and establishment of FN detection methods in acellular matrix have important practical value.

There are many FN detection methods reported in the literature [11-14], among which the most commonly used is the ELISA method, which is mainly used for the detection of plasma type FN. The FN in SIS belongs to the tissue type and exists in a bound form. If the ELISA method is used directly, it is difficult for the antibody to directly bind to the FN. Non-specific adsorption will also affect the accuracy of the result, resulting in a large deviation between the test result and the actual content. The protein detection method based on mass spectrometry for the quantitative analysis of low-abundance proteins has the advantages of high sensitivity and good repeatability. It can perform label-free quantitative detection based on the abundance of peptides in protein degradation products, but this method is directly used for FN in SIS There are difficulties in the detection of sequence-specific enzymes, such as difficulty in direct degradation, which leads to certain limitations in these methods.

In view of the problems in the detection of FN in the acellular matrix, this study intends to establish a quantitative detection method for FN based on enzymatic hydrolysis and mass spectrometry, which realizes that SIS is first degraded by collagenase, and FN is obtained by directed enzymatic hydrolysis of FN by trypsin. For characteristic peptides, HPLC-MS was used for content detection, and the linear range, precision, and stability of the method were verified.

1. Materials and methods

1.1 Materials and instruments

1.1.1 Materials

SIS (VIDASIS™) was provided by Beijing Biosis Healing Biological Technology Co., Ltd.; SIS (BiodesignR) was purchased from Cook Company in the United States; Type I collagenase (analytical purity) was purchased from Beijing Xinjingke Biotechnology Co., Ltd.; trypsin (sequence pure)) Purchased from Promega, USA Company; Fibronectin was purchased from CALBIOCHEM, USA.

1.1.2 Main instruments

HC-2518R high-speed refrigerated centrifuge, Anhui Zhongke Zhongjia Scientific Instrument Co., Ltd.; LGJ-100F vacuum freeze dryer, Beijing Songyuan Huaxing

Technology Development Co., Ltd.; High-performance liquid chromatography-mass spectrometry system (HPLC-MS) by The Agilent 1100 liquid chromatography and the Thermo Fisher LCQ DecaXP electrospray mass spectrometer, the data acquisition and processing software is Xcalibur 1.3; the liquid chromatography-triple quadrupole mass spectrometry system is composed of the Thermo Fisher U3000 liquid chromatography and TSQ Quantum ACCESS MAX consists of mass spectrometry, data acquisition and processing software is Xcalibur 1.3.

1.2 Experimental method

1.2.1 SIS degradation experiment

Static enzymolysis: Weigh 100 mg of SIS, add 100 mL of buffer (50 mM Tris-HCl, 0.36 mM CaCl2, pH 7.4), heat denaturate at 100 ° C for 10 min, cool to room temperature and add 1 mL of collagenase solution (1 mg/mL HBSS buffer), 37 ℃ water bath shake, centrifuge at 10,000 rpm for 3 min, and collect the supernatant.

Enzymatic digestion in batches: Weigh 100 mg of SIS, add 8 mL of buffer (50 mM Tris-HCl, 0.36 mM CaCl2, pH 7.4), heat denature at 100 ° C for 10 min, cool to room temperature and add 200 μ L of collagenase solution (1 mg/mL HBSS buffer), enzymatically digest at 37 ℃ for 1 h, centrifuge at 10000 rpm for 3 min, collect the supernatant, add 200 μ L collagenase solution and 8 mL buffer to the pellet, repeat the above operation 5 times, collect all the supernatants. Clear liquid.

1.2.2 Trypsin digestion experiment The collagenase degradation product of SIS was freeze-dried, and a 50 mM NH4HCO3 solution (pH 8.0) was used to prepare a 4 mg/mL solution, and 20 μ L Trypsin solution (50 μ g/ μ L) was added. mM NH4HCO3, pH 8.0), enzymatically hydrolyzed at 37 ° C for 12 h, and the hydrolyzed product was directly detected by HPLC-MS.

1.3 Analysis method

1.3.1 Analysis of amino acid composition

The analysis method of amino acid composition of SIS and its enzymatic hydrolysis products refers to the literature [15].

1.3.2 HPLC-MS analysis

Chromatographic column is Zorbax SB C18 [150 × 2.1 mm(ID), 5 μ m]; mobile phase A: water (containing 0.1% formic acid), B: acetonitrile (containing 0.1% formic acid); gradient 0~80 min, 5~50 % Acetonitrile; 80~90 min, 50~90% acetonitrile, injection volume 50 μ L; UV: 214 nm; flow rate 0.2 mL/min. Ion trap mass spectrometry conditions: ion source spray voltage 4.5 kV, capillary temperature 300 ° C, scanning range m/z 400-2000. Both the accurate mass scanning and the secondary mass spectrometry scanning are data-dependent scanning, and the collision energy is 35%.

Triple quadrupole mass spectrometry conditions: ion source spray voltage 3.5 kV, capillary temperature 300 ° C, evaporation temperature: 200 ° C, shell gas:
35 arb, auxiliary gas: 8 psi, scanning mode is selected ion monitoring, m/z 957.5.

2. Results and discussion

2.1 SIS degradation method study FN and collagen have high binding characteristics, and exist in the state of binding to collagen in the extracellular matrix [16]. Traditional methods are difficult to effectively detect FN in the acellular matrix. In order to make FN change from the bound state to the free state and be further enzymatically digested, the experiment first studied the release method of FN. Figure 1 is the dynamic change curve of SIS residues in the degradation process of different enzymatic hydrolysis methods. After adding collagenase by static enzymolysis, the residual amount of SIS in the solution gradually decreased. The degradation rate after enzymatic hydrolysis was only 60% after 24 hours. After 3 days of degradation, 10% of the SIS remained undegraded. The residual amount became stable after 4 days. Flocs that can't be degraded remain in it (Figure 1). The results of amino acid analysis showed that there was almost no hydroxyproline in the residue, indicating that the collagen component in SIS was effectively degraded. However, the process of using this method to degrade SIS is time-consuming, and there are problems such as easy contamination of bacteria, enzyme inactivation, and high random degradation rate. In order to improve the efficiency of enzymatic hydrolysis, the experiment tried the method of batch degradation, that is, after a certain period of degradation, solid-liquid separation was carried out, and the precipitate was re-enzymatically hydrolyzed. The residual amount of SIS changed significantly during the first three degradation processes. The residual amount was 70% in the first liquid change, 50% in the second liquid change, and 50% in the third liquid change.

The amount is about 30% (Figure 1). After the process is repeated five times, the SIS enzymatic hydrolysis is relatively complete. In order to minimize the loss of protein in the sample, the number of degradations in the experiment was increased to 6 times during the batch degradation process.

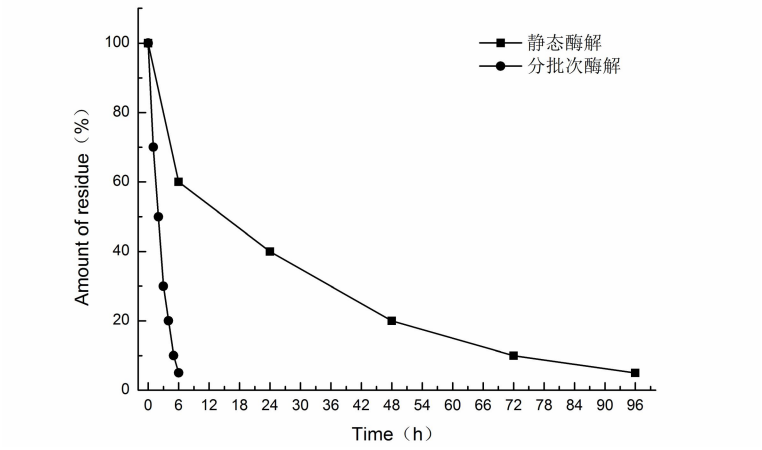

Fig 1 Dynamic changes of SIS residues in the process of static enzymatic hydrolysis and batch enzymatic hydrolysis

2.2 Detection method based on mass spectrometry

2.2.1 Screening of FN characteristic peptides

After FN is treated with pure trypsin, a large number of low-molecular-weight peptides are produced; the experiment has performed BLAST sequence alignment of the peptides, and the peptides detected only in pig-derived FN are characteristic peptides of pig-derived FN; peptide mass spectrometry information after enzymatic

hydrolysis Use SEQUEST software to search the database, and the peptide filter parameters are Xcorr>1.5 (single charge), Xcorr>2.0 (single charge), and Deltacn>0.1, the peptides generated are considered as positive results. Extract the ion chromatograms of characteristic peptides from the mass spectra of HPLC-MS analysis. Low-concentration samples can be extracted with higher abundance. At the same time, the signal-to-noise ratio S/N>10 is suitable for characteristic peptides.

The trypsin-digested porcine-derived FN was analyzed by ion trap mass spectrometry (LCQ) for full-scan analysis. Figure 2 shows the total ion chromatogram of the porcine-derived FN enzymatically hydrolyzed peptide. Using BLAST multiple sequence alignment, it was found that pig-derived FN had a certain number of characteristic peptides, and then Bioworks software was used to search the peptide mass spectrum information after pig-derived FN degradation, and the peptide fragments with positive results were screened. According to BLAST multiple sequence alignment and Bioworks software search The library results selected the polypeptide SSPVVIDASTAIDAPSNLR as the characteristic polypeptide of porcine FN, and its abundance and matching degree of secondary fragments were both high. Figures 3 and 4 are the extracted ion current diagram (including the primary mass spectrum) and the secondary mass spectrum of the polypeptide, respectively. The secondary fragment ions have a high degree of matching with the theoretical value. Therefore, the polypeptide can be selected as a characteristic polypeptide for type identification and quantitative detection of porcine FN.

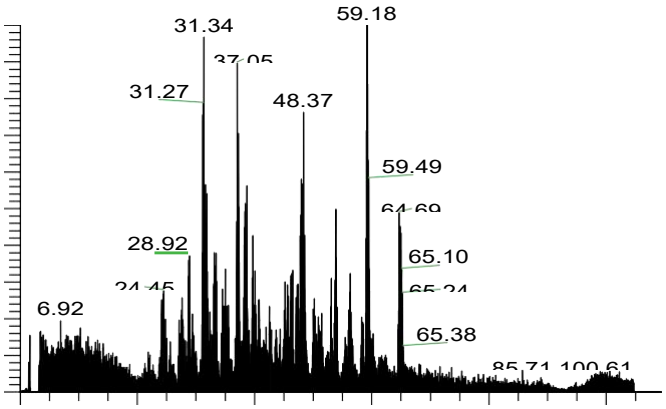

Fig 2 Total ion chromatogram of trypsin-digested pig FN

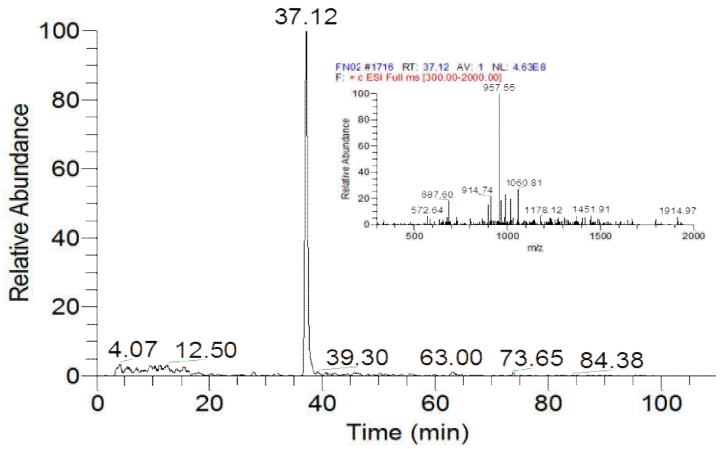

Fig 3 The extracted ion chromatogram and mass spectrum of marker peptide

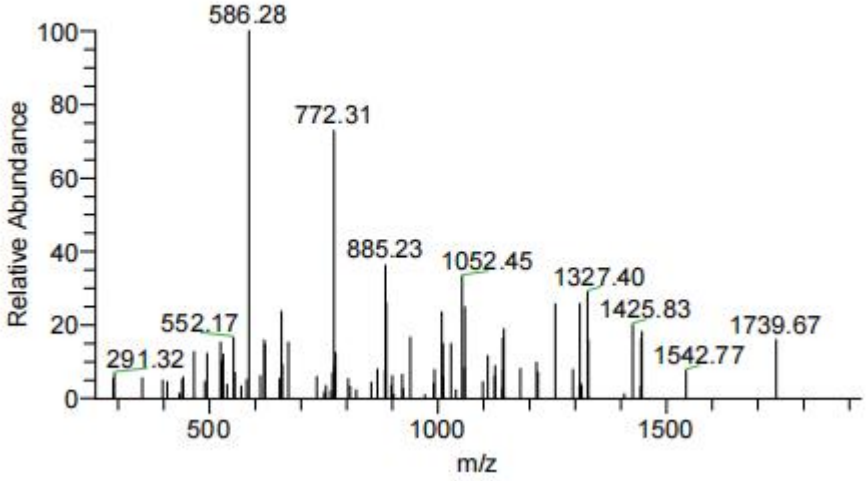

Fig 4 The MS/MS spectrum of marker peptide

2.2.2 The relationship between the concentration of pig-derived FN and the signal intensity of characteristic peptides

Take different concentrations of denatured enzymatically treated pig source FN standard solutions, and sequentially inject 50 μL samples from low concentration to high concentration. According to HPLC-MS quantitative detection conditions, monitor the target ion m/z=957.5 to obtain pigs with different concentrations. The extracted ion current diagram of the source FN characteristic peptide (Figure 5). According to the selected characteristic polypeptide extraction ion current diagram, the peak area is the ordinate and the concentration of pig source FN standard is the abscissa to perform linear regression (Figure 6). The regression equation is y =4109.5x-12.82 (R2=0.995), the linear relationship between the two is good in the concentration range of 0.0024~0.048 g/L.

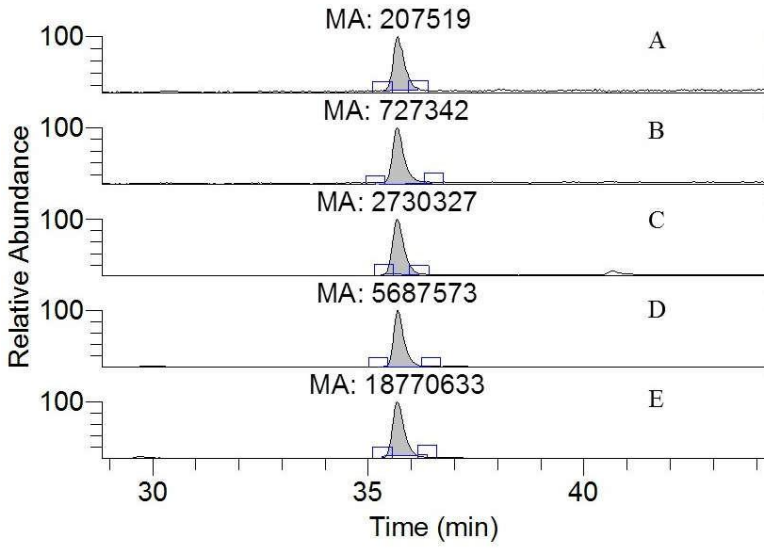

Fig 5 Extracted ion chromatograms of marker peptide concentration with marker peptide signal intensity of pig FN

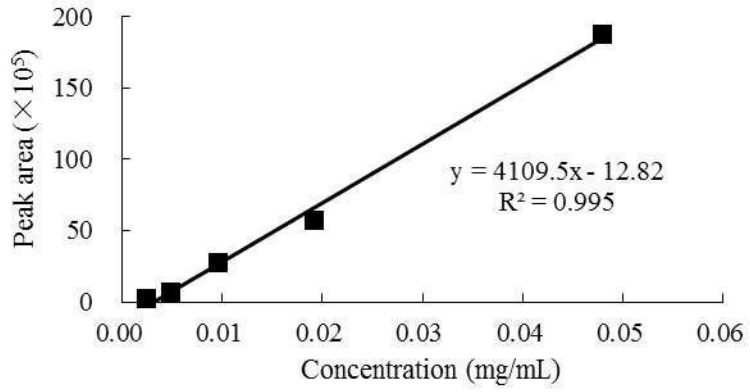

Fig 6 Correlation of pig standard FN concentration with marker peptide signal intensity

2.2.3 Test results of FN content in SIS

Weigh 50 mg of SIS (VIDASISTM) and add collagenase for 5 enzymatic hydrolysis, combine the supernatants of 5 enzymatic hydrolysis, and freeze-dry to obtain 123 mg of lyophilized product (containing the salt in the extract), take 4 mg The lyophilized product was reconstituted and digested with trypsin. The digested product was analyzed according to the HPLC-MS quantitative detection method. According to the peak area of the ion current chromatogram extracted from the FN characteristic peptide, the concentration of FN in the sample solution was 0.007 mg/ mL, the content of FN in SIS is 0.43%. Using the same method, the content of FN in SIS (Biodesign○R) was determined to be 0.17%.

2.3 Verification of precision and stability

2.3.1 Linear range

Dilute the same sample to different concentrations, respectively 2.4 μg/mL, 4.8 μg/mL, 9.6 μg/mL, 19.2 μg/mL, 48 μg/mL, 96 μg/mL, 192 μg/mL, and pass HPLC-MS Detect its fibronectin content, and determine the linear concentration range of the peak area according to the relationship between the concentration gradient and the peak area (Figure 7). The experimental results show that the method has a high degree of linearity.

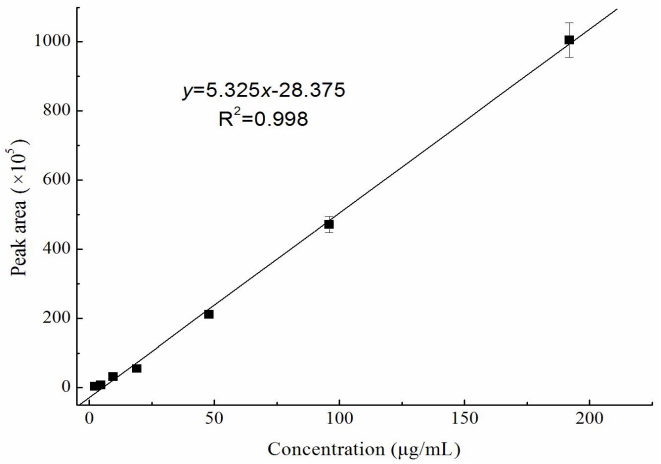

Fig 7 Linear scope of HPLC-MS detection method for fibronectin

2.3.2 Precision

Accurately measure 100 μL of the FN standard, analyze it under quantitative HPLC-MS conditions, and repeat the injection three times. The results are shown in Figure 8. The relative deviation RSD of the peak area of the characteristic peptide in the FN standard is 1.31%, indicating the precision of the method. good.

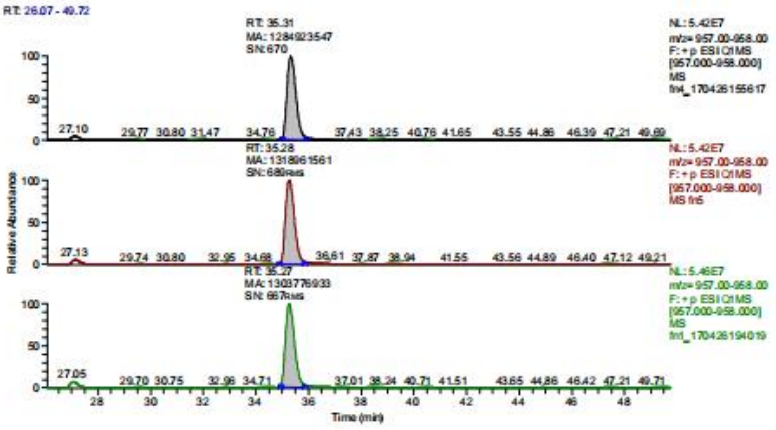

Fig 8 Precision analysis of HPLC-MS detection method for fibronectin

2.3.3 Stability

The samples were stored at -20℃, and the samples were measured by FULL SCAN three times at 10, 20, and 30 days. The peak areas of the samples were 3.82×108, 3.45×108, 3.16×108, respectively. The peak areas of the samples stored for 30 days were slightly reduced Small, indicating that the method has high stability.

3 Conclusion

As an important component of extracellular matrix, FN has many biologically active functions. Quantitative detection of FN content in SIS acellular matrix material was carried out by batch enzymatic hydrolysis and liquid-mass spectrometry technology. Optimize the collagenase hydrolysis scheme of SIS to eliminate the disadvantages of long time and low efficiency, and reduce the time of collagenase hydrolysis from 96 h to 6 h, which greatly improves the efficiency of enzymatic hydrolysis. In the experiment, the characteristic peptide obtained by the specific enzymatic hydrolysis of FN by Trypsin was SSPVVIDASTAIDAPSNLR. This characteristic peptide in FN was quantitatively detected by HPLC-MS, and the content of FN in SIS was accurately obtained. This method is simple to operate and is suitable for the detection of FN in tissues. The results showed that the content of FN in the two decellularized materials VIDASISTM and BiodesignR were 0.43% and 0.17%, respectively. Methodological experiments prove that the method has high accuracy and small error, and the linear relationship of the peak areas integrated by different concentrations is good. This study shows that the LC/MS technique can accurately determine the content of FN in the acellular matrix.

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