Application of online two-dimensional separation system using monolithic columns for proteome analysis of human cartilage

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Abstract In Shotgun proteome analysis where nano-flow is adopted to increase the sensitivity as well as extremely complicated samples such as proteolytic digest are inevitably confront monolithic capillary columns are widely used to improve the liquid chromatography separation performance. It is known that cartilage contains extensive amounts of extracellular matrix ECM in which collagens and aggrecans being the most abundant macromolecules. It is obvious that the high content of ECM components causes a challenge in the comprehensive proteome analysis of cartilage. In this study a 7 cm × 150 μm i. d. phosphate strong cation exchange SCX monolithic capillary column was coupled with an 85 cm × 75 μm i. d. C12 reversed-phase monolithic capillary column for online two-dimensional separation of 20 μg tryptic digest of proteins extracted from human cartilage. After 14 salt steps fractionation and following gradient separation coupled with tandem mass spectrometry detection finally 7434 unique peptides corresponding to 1901 distinct proteins were positively identified. Then the identified proteins were analyzed by Gene Ontology GO and it was found that most of the identified proteins were come from articular chondrocytes with low abundance which is important for the researches of articular diseases.

Key words monolithic column online two-dimensional separation proteomics cartilage tissue
“鸟枪法”(Shotgun)蛋白质组学是将蛋白质混合物先进行酶解,然后通过鉴定酶解后的肽段来鉴定蛋白质的一种“从下至上”(bottom up)的蛋白质组学研究策略。毛细管纳升液相色谱与串联质谱联用是“鸟枪法”蛋白质组学的技术核心,通过毛细管液相色谱对微量蛋白质酶解样品的高效分离,极大地提高了质谱对复杂样品进行蛋白质鉴定分析的能力。在蛋白质组学样品中具有重要生物学意义的蛋白质往往是低丰度蛋白质,进一步提高毛细管液相色谱的分离能力对低丰度蛋白质的质谱鉴定具有重要意义。多维液相色谱分离是最强大的液相色谱分离模式,而在线多维液相色谱分离又具有高效快速、样品用量小、样品损失少、无样品污染等诸多优点。在蛋白质组学研究中,对于微量的蛋白质样品,在线多维分离具有更大的优势。

23$45等在同一根毛细管中先后顺序分别填充了反相分离填料和强阳离子交换填料,将蛋白质的酶解物先上样到强阳离子交换部分,然后通过不同的盐梯度将富集在强阳离子交换部分的肽段分级冲洗到反相部分,再分别进行反相梯度分离和串联质谱的检测,从而实现了在线二维分离和串联质谱检测联用,对蛋白质样品具有强大的分离鉴定能力。这种方法也被称为“多维蛋白质鉴定技术”的方法,在蛋白质组学研究中得到了广泛的应用。但是由于23$45等人应用的是毛细管填充柱,分离柱的反压大,上样速度慢;而且为保证反相部分的分离能力,强阳离子交换填料填充部分不能太长,上样能力受到限制的同时也降低了第一维的分级分辨率。相对于填充柱而言,整体柱具有的多孔结构使其具有很高的渗透性和传质速率,从而实现高效快速分离。近年来,毛细管整体柱已经被广泛地应用到纳升毛细管液相色谱中,极大地推动了蛋白质组学的发展。

关节软骨包括软骨细胞(体积分数)和细胞外基质(主要组成部分包括胶原蛋白和蛋白聚糖。由于大量细胞外基质的存在,要对软骨细胞中的蛋白质进行充分的提取就必须对软骨组织进行充分的切片或研磨。但软骨组织被充分破坏后将导致大量的胶原和蛋白聚糖进入提取液,由于其丰度非常高,将极大地影响液相色谱/质谱联用对蛋白质样品中低丰度蛋白质的分析鉴定。现阶段人们对软骨组织蛋白质的组成了解不多,对软骨蛋白的大规模鉴定到现在为止还是一个难题。在已有的文献报道中,采用双向电泳方法的软骨蛋白质组分析只鉴定了不到20%的蛋白质。我们以带有磷酸基团的整体柱为富集柱,以带有羧基团的整体柱为反相分离柱组成二维分离系统,以串联质谱作为检测手段构建了一套自动在线多维分离/串联质谱联用系统。本文将该系统应用于软骨蛋白质组分分析,通过对软骨提取蛋白质的胰蛋白酶酶解物进行分析,在假阳性率(小于0.1%)的情况下共鉴定得到了200个独立肽段,对应于1非冗余蛋白质。
Bradford Assay was used to determine protein concentration. The assay is based on the absorbance of Coomassie brilliant blue dye complexed with protein in an alkaline medium. Protein samples were diluted to a concentration of 100 µg/mL in 1× Bradford Assay reagent and added to the reaction mixture, which consisted of 2× Bradford Assay reagent and a blank. The absorbance at 595 nm was measured using a spectrophotometer. The protein concentration was determined by comparison with a standard curve prepared with bovine serum albumin.

1.3 Overall Experimental Procedures

The overall procedure for preparing the peptide libraries is as follows: (1) Soften the polyethylene tube. (2) Assemble the reaction system. (3) Record the time and temperature of each step. (4) Use a water bath to react the solution. (5) Collect the reaction mixture. (6) Wash the solid material. (7) Wash the solid material with a water bath. (8) Record the time and temperature of each step. (9) Wash the solid material. (10) Use a water bath to react the solution. (11) Collect the reaction mixture. (12) Wash the solid material. (13) Wash the solid material with a water bath. (14) Record the time and temperature of each step. (15) Wash the solid material. (16) Use a water bath to react the solution. (17) Collect the reaction mixture. (18) Wash the solid material. (19) Wash the solid material with a water bath. (20) Record the time and temperature of each step. (21) Wash the solid material. (22) Use a water bath to react the solution. (23) Collect the reaction mixture. (24) Wash the solid material.

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LTQ mass spectrometer was used for further analysis. The mass spectrometer was operated in positive ion mode. The analyzer was set to a resolution of 7000 at m/z 500. The ion source was set to a temperature of 200 °C. The capillary voltage was set to 1.8 kV. The tube lens voltage was set to 35%. The ion trap was set to 30% MS/MS data dependent analysis. The collision energy was set to 6. Xcalibur Version 1.4 was used to process the data. The software was run on a desktop computer.
Fig. 2 Base peak chromatograms of a 14-cycle online two-dimensional analysis of 20 μg cartilage proteins tryptic digest

Conditions\[1\] The three buffer solutions used for the chromatography were 0.1% formic acid aqueous solution\[1\] buffer A, 0.1% formic acid acetonitrile solution\[2\] buffer B and 1 mol/L ammonium acetate at pH 3. buffer C. Cycle 1 consisted of a 2-min gradient from 0 to 8% buffer B, a 90-min gradient from 8% to 30% buffer B, and after a 2-min gradient from 30% to 0% buffer B the system was equilibrated with 100% buffer A for 25 min. Each of the next 12 cycles was 250 min with the following procedures: 5 min of X% buffer C in the mixture of buffer A and buffer C, 10 min of 100% buffer A, then the separation gradient was set from 0 to 8% buffer B for 5 min, then from 8% to 30% buffer B for 205 min and after a 5-min gradient from 30% to 0% buffer B the system was equilibrated with 100% buffer A for 20 min. The 5-min buffer C in cycle 2–13 was as follows: cycle 2: 3%, cycle 3: 10%, cycle 4: 15%, cycle 5: 20%, cycle 6: 25%, cycle 7: 30%, cycle 8: 35%, cycle 9: 40%, cycle 10: 45%, cycle 11: 50%, cycle 12: 60%, cycle 13: 80%. Cycle 14 consisted of a 10-min 100% buffer C wash followed by a 10-min 100% buffer A wash and the separation gradient was set from 0 to 8% buffer B for 5 min, then from 8% to 30% buffer B for 205 min and from 30% to 80% buffer B for 10 min and after the a 10-min holding at 80% buffer B the system was equilibrated with 100% buffer A for 25 min.
2.1 SCX 1 mol/L SEQUEST 7 434 1 901 606 7 487 2 014 1129 56.1% 5 229 1 458 70.3% 76.7%

2.2 RP 7 cm SCX 85 cm C12 1 901

Fig. 3 Distribution of the newly identified a unique peptides and b distinct proteins by the 14 salt steps in online two-dimensional separation
成功地鉴定得到了蛋白质按其来源进行分类,结果见表1。据我们的文献调研,这是迄今有关软骨蛋白质分类的最好结果。根据生物信息学的分析,大部分鉴定到的蛋白质都是来自于软骨细胞内部,只有少部分来自于细胞外基质。该表充分说明了虽然高丰度的蛋白质大量存在于细胞外基质的有几种而只有几种来自于细胞内部,只有几种来自于细胞外基质的有几种而只有几种来自于细胞内部。可以预见,这些发现将对许多关节类疾病的治疗有帮助。表1

<table>
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<tr>
<td>Protein complex</td>
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<td>271</td>
</tr>
</tbody>
</table>

a. GO categorization of the 30 most abundant proteins based on cellular components. b. GO categorization of all the identified proteins based on cellular components.

## Reference

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