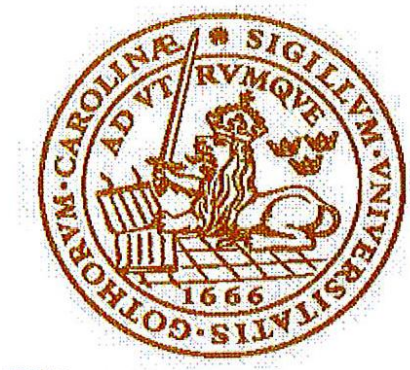

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Master thesis

MMP-13 substrate specificity in cartilage breakdown

Prepared by Atef Mannaa

Supervisor: Patrik Önnerfjord

Clinical Sciences Lund

Skeletal Molecular Biology section, BMC

Faculty of Medicine, Lund University

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Abstract

MMP13 (Collagenase 3) is a member of the matrix metalloproteinase (MMP) family. In pathology it is overexpressed in rheumatoid arthritis **(RA),** osteoarthritis **(OA)** and human carcinomas. It is secreted in its inactive proforms from which it can be activated. The project studies how MMP13 can degrade/digest the normal femoral head human articular cartilage. Peptides were separated using reversed phase chromatography coupled on-line with various mass spectrometry techniques including ion trap, quadropole Time-Of-Flight (Q-TOF) and triple quadropole (QQQ) instruments. Guanidine hydrochloride (GuHCl) was used to extract proteins from the cartilage tissue. Sodium dodecyl sulfate poly acrylamide gel electrophoresis was used also to give visualize similarities and differences between the control and the MMP13 treated sample. MMP13 showed an effect on both media (released proteins from the cartilage tissue via the buffer solution) and a little effect on the cartilage tissue (pellet). The main result showed that the tissue sample preparation was critical in order to obtain sufficient release of proteins. The powderisation of tissue was much better in releasing proteins than intact tissue plugs probably due to larger contact area and shorter diffusion distance.

Introduction

Aim of the project

MMP13 was studied before and in the laboratory it was shown to play a role in cartilage degradation (fibromodulin and type IX collagen) **[1]** so, the aim in this work is to use MMP13 to release multiple proteins from the tissue and study their fragmentation, "including the cleavage sites by MMP-13" using mass spectrometry and tandem MS experiments.

Background about the cartilage

The cartilage is a connective tissue found in different places in the body of human and animals such as the joints between bones, the knee and intervertebral discs. The normal articular cartilage is classified (Fig. 1) into the superficial zone (upper zone), intermediate zone (middle zone, deep zone) and calcified zone (subchondral bone and cancellous bone), (see Fig. 1).

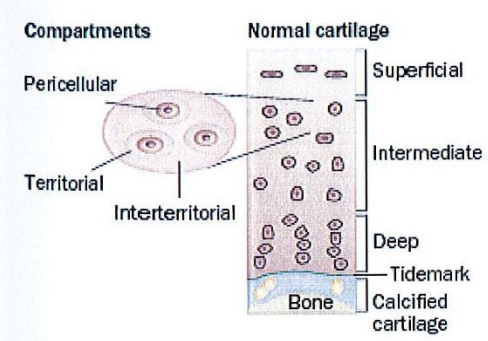

Fig. 1 shows the different layers in cartilage, reprinted from [1].

Cartilage extracellular matrix (ECM) contains more than 200 extracellular proteins **[1].** The main component for the tissue function is aggrecan (stand load/ attracts water and resist to load) and collagen II (support skeletal muscles). It is also shown in Fig. 2, that the cartilage

matrix surrounding chondrocytes in the normal articular cartilage is well ordered into different zones (layers) are defined by their distance from the chondrocytes cells (pericellular, territorial and interterritorial). The pericellular matrix presents in the region around the cell where the molecules interact with the cell surface molecules are located (such as hyaluronan interact with receptor CD44). The next zone is territorial which is not far from the cell. The last and the largest distance away from the cell is the interterritorial matrix.
Each zone contains specific collagen (type I, II, IX collagens) and collagen binding proteins (COMP, PRELP, CILP-1, etc).

Osteoarthritis disease (OA)

It is difficult to define the start of the OA disease and also to diagnose different disease stages due to lack of diagnostic tools. As a result of this, the disease is normally only diagnosed at rather late stages, where most of potential therapies are too late. The final outcome of the disease is normally joint replacement, so most of therapy options are limited. Some mutations in the extracellular matrix proteins (such as collagen II, collagen IX or cartilage oligomeric matrix protein (see Fig. 2) drive early development and high incident of OA **[2, 3].**

In the early alterations stage of cartilage in joint disease (OA), it is still difficult to know what the steps are and in what sequence they occur. To try to solve this matter, the identification of the early events in the joint damage is critical. The advantages from knowing the early molecular events lead to the progression of the OA disease can drive to opportunities to develop suitable diagnostic tools (ex, biomarkers) **[1].**

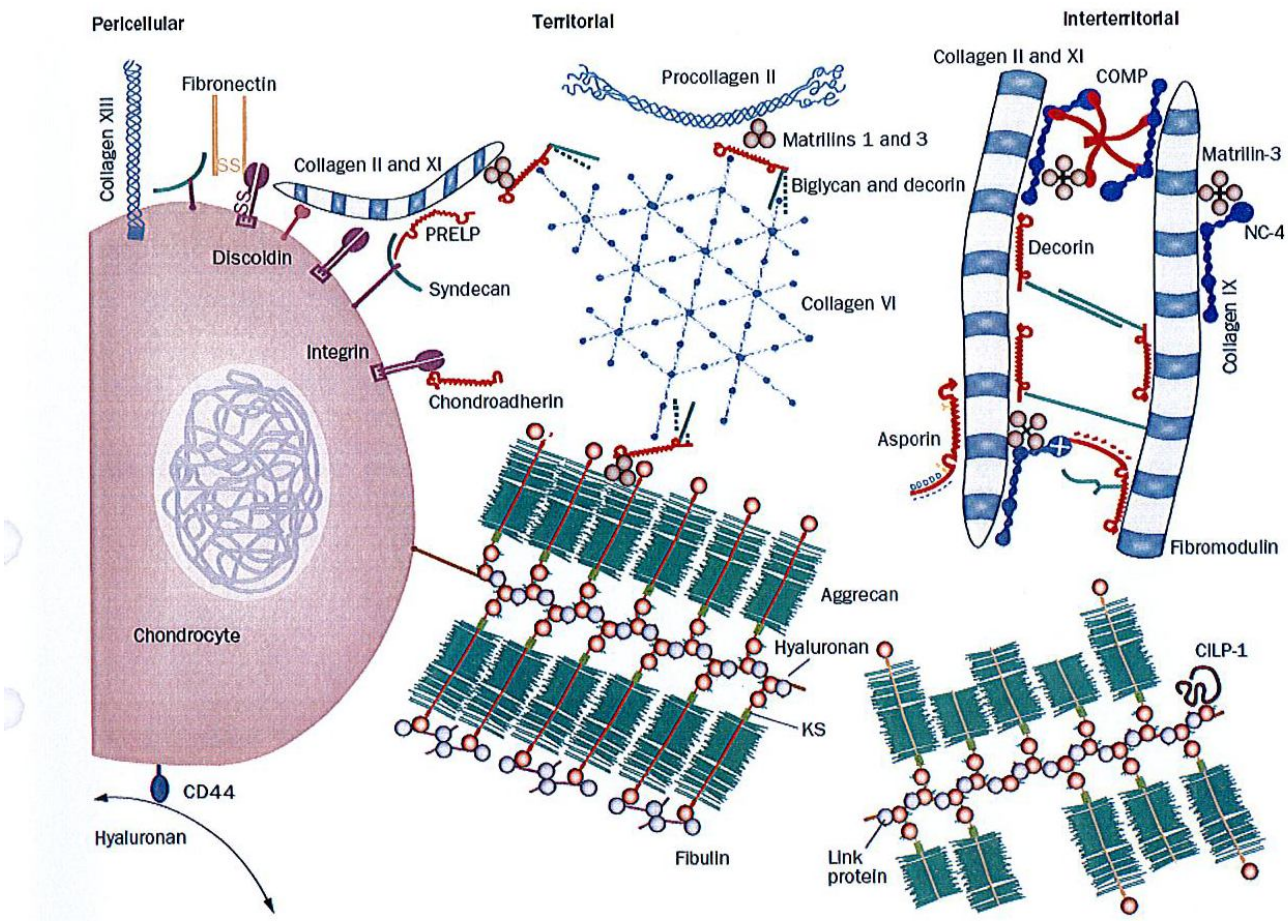

Fig. 2 shows the molecular organization of normal articular cartilage, reprinted from [1].

The studied enzyme in this project (MMP13) is also playing a role in cartilage breakdown. It was studied before **[1]** that the active MMP13 was found in the cleavage of Collagen IX and fibromodulin (FMOD) in the tissue break down models as the sulfate domain of (FMOD) that binds collagen IX was not found in the normal cartilage tissue **[4].**

Another type of protein molecules, aggrecan (see Fig. 2, the green color), are the proteoglycans, which mainly is represented by aggrecan (see Fig. 2). Degradation of aggrecan is early stage in development of OA and a considerable amount of research has been performed to identify the enzymes that are responsible for that **[5]**. Some reports in the literature elucidate that aggrecan is a substrate for ADAMTS-4 in the early stage joint disease that can lead to OA**,** as four cleavage sites of aggrecan were defined that were good substrates to ADAMTS-4 **[6]** and these cleavages release the glycosaminoglycan (GAGs) chains that fix the highly negative charge coming from the chondroitin sulfates (CS) chains on the aggrecan.

GAGs play an important role in cartilage, GAGs attract water and in the place in which the resistant to load comes from **[7]**. Glycosaminoglycan (GAGs) are defined as long unbranched polysaccharides. These polysaccharides consist of repeating disaccharide units and each unit consists of hexose and hexosamine (each disaccharide unit has a negative charge due to SO_4^{2-} groups).

The role of MMP-13 in OA

Earlier studies explained that MMP-13 seems to be a key factor in cartilage break down (degradation) that appears in osteoarthritis OA **[8, 9].** MMP-13 has been found in articular cartilage and synovial fluid from rheumatoid arthritis patients **[10].** Many components were susceptible to be degraded by MMP-13 in vitro such as collagen type IX, type X and type II **[11, 12].** The non-collagenous protein such as fibromodulin has been cleaved by MMP-13 as well **[13].** Studies on transgenic mice have shown that the collagenolytic metalloproteinase MMP-13 plays an important role in collagen degradation **[14].**

Mass spectrometry (Qualitative and Quantitative analysis)

Different mass spectrometric methods were used in the project work, ion trap MS/MS, quadruple time of flight **(Q-TOF)** and multiple reactions monitoring **(MRM). T**he ESI-Ion trap **[15]** mass spectrometer is used for identification of proteins. In the front of the MS there is a liquid chromatography system connected on-line, where an autosampler injects the sample into a sample loop. The sample in the loop is transported to a small pre-column, where peptides are bound and enriched, while unbound materials (salts) are washed away. The sample is then separated on a reversed phase C18 column using a gradient from 5% B to 95% B in 80 min, where B is (80% ACN in 0.2% F.A). The compounds in the samples will be separated depending on the polarity, so the polar or more polar compounds will elute first then

the less polar and non-polar (hydrophobic compounds) will elute later. Between each injection a blank (0.2% F.A) is run to minimize cross-contamination.

Q-TOF is the method that can be used for both identification (qualitative analysis) and semi quantification of proteins. It has an advantage that it gives a better mass resolution than the Ion trap, but they have similar sensitivity. The Q-TOF consists of two quadruples and a V-shape TOF analyzer (see Fig. 4), where the first quadrupole (Q1) acts as a mass filter and the second one (Q2) acts as a collision energy cell, where an inert gas (Argon) is used as a collision gas. The protein sample is ionized then energized with an electrospray ionization (normally positively charged ions), the protein sample goes through (flies) to the ion guide (quadrupole ion guiding cell, Q0). At Q0, the ions are focused and cooled, the ion (peptide) of interest is filtered (selected) in the first mass filter quadrupole (Q1) then goes to the second quadropole (Q2) that acts as a collision energy cell. Each ion (peptide) has a certain mass/charge (m/z) in the TOF, and the time of flight of each fragmented ion (peptide) can be measured as the TOF is directly proportional to the m/z. Ions of m/z will move towards the reflectron then they are reflected to the opposite direction (V-shape), towards the detector. Small ions will have shorter flight times than large ions.

Q-TOF can also be used as a quantification method especially if iTRAQ (isobaric tags and relative absolute quantification) is used to label the amino groups of the N-terminous and\lysine (C-terminous) residues **[16, 17].** In the MS/MS experiments, the Q1 will isolate the desired m/z ion and only this mass will enter the collision cell, where it is fragmented. The resulting fragment ions are separated in the TOF analyzer.

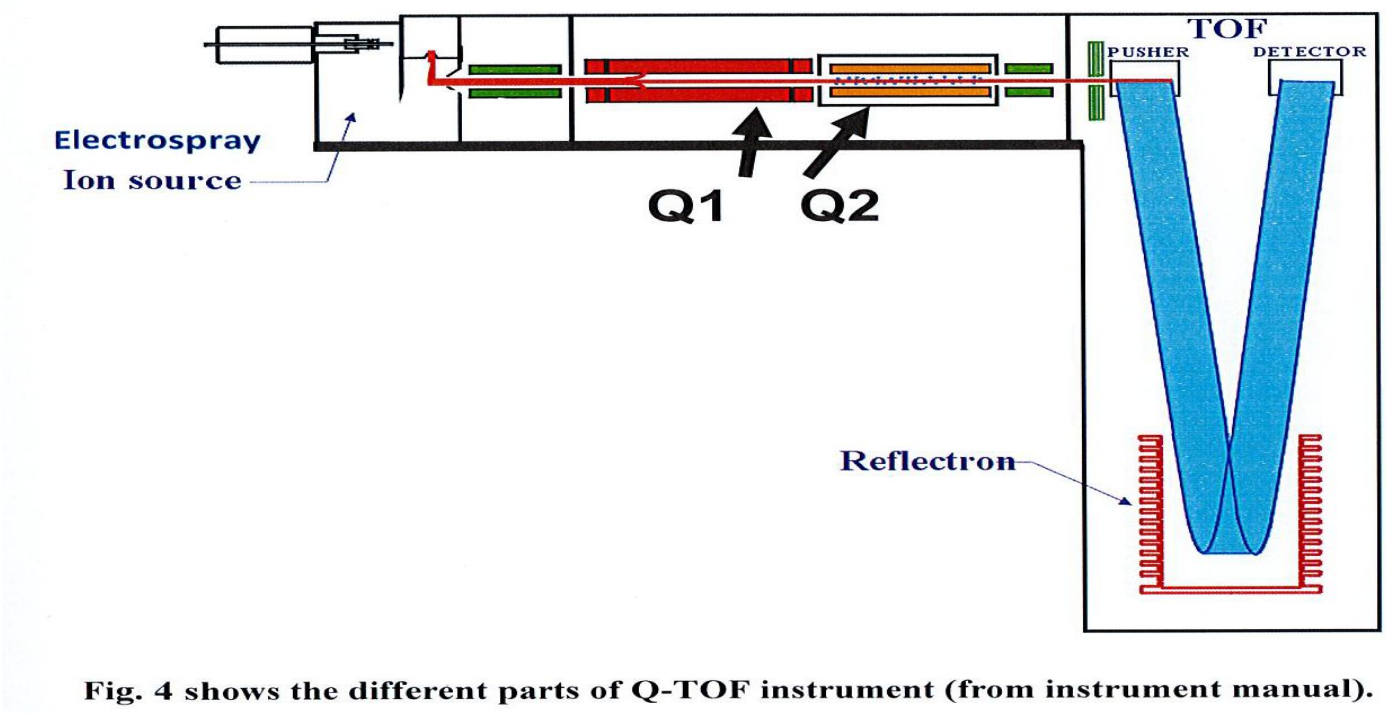

Fig. 4 shows the different parts of Q-TOF instrument (from instrument manual).

The triple quadropole instrument can be used for MRM (multiple reaction monitoring). MRM, is used as a quantification method, it is a highly sensitive and selective method for the quantified selected (targeted) peptides.

In MRM experiments, the Q1 and Q3 act as a mass filter and the Q2 acts as a collision energy cell that fragment the selected ion (peptide) from the first quadruple into small fragment ions. The combination of Q1/Q3 filter settings results in very selective measurement of a specific peptide ion and its fragment ion, know as single reaction monitoring. When multiple peptides are targeted in the same run it is called multiple reaction monitoring.

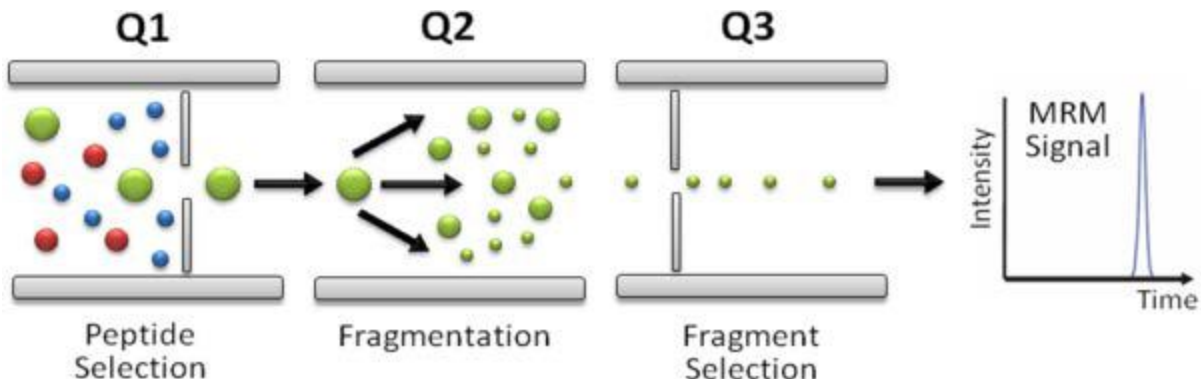

Fig. 5 shows the different parts in the QQQ instrument in case of the MRM technique, reprinted from **[18].**

Material methods

Sample preparation

Experiment 1

Large cartilage tissue plugs (5 mm each) of normal human femoral head cartilage (dead tissue) from femoral head was removed with a large punch tool. The articular cartilage was removed from subchondral bone using a scalpel. Small plugs (1 mm) were taken with a needle (homemade punch tool). Two samples were prepared, both of them contained ca 5 mg of cartilage tissue. The first one is a control sample that contains 5 mg of cartilage tissue, 150 µl of MMP-buffer (200 mM NaCl, 50 mM tris-HCl, 5 mM $CaCl_2$, 0.02% NaN_3 ***(toxic)***, pH 7.6). The second sample is the sample with MMP13, that contains 4.8 mg of cartilage tissue, 144 µl of MMP-buffer and 6.8 µl of (**pro-MMP13** (0.59 µg/ µl), 1 µl of aminophenyl mercuric acetate (20 mM APMA stock (7 mg of APMA ***(toxic)***, 4.18 µl of liquid NaOH, then complete till 1 mL DD H_2O) and 14 µl of MMP-buffer). The samples were incubated for 19 h at 37 ºC, 350 rpm for digestion. A small aliquot (20 µl) was saved for SDS-PAGE and stored at -20 ºC. The supernatants (50 µl) of the two samples were transferred to new 0.5 mL safelock Eppendorf microtubes. Samples were reduced using 2 mM DDT (1 µl of 100 mM DTT stock) and were allowed to cool down for 4 min. Samples were then alkylated using 4 mM IAA (2 µl of 200 mM IAA stock), which was added at room temperature in dark for 1 h. Another 2 µl of DTT was added to scavenge the excess of IAA. 2 µl of 1 mM **EDTA** was added to inhibit the MMP-13. Finally, 2 µl of (0.5 µg/ µl) of trypsin gold was added at 37 ºC for 19 h, then the samples were saved at -20 ºC to be prepared by **SAX** (strong anion exchange), C18 spin column for Ion trap reversed phase chromatography (identification), Q-TOF (qualitative

analysis) and MRM (targeted quantification). The cartilage tissues were washed with AMBIC, vortexed and incubated on a shaker for 1 h at +4 ºC twice. The solution was removed with pipette to the waste. The cartilage tissue was dried in a speedVac for 10 min to remove all AMBIC buffer. 100 µl of 4 M GuHCl, pH 5.8 was added to the cartilage tissues at +4 ºC for 24 h on a shaker. The samples were centrifuged for 30 min at 14000 rpm. The supernatants were transferred to new 2 mL axygen tubes then reduced with 2 mM µl of DTT (1 µl of stock) for 30 min at 56 ºC and alkylated with a 4 mM IAA solution (2 µl of stock, freshly prepared) for 1h in dark then 2 µl of DTT to the excess of IAA as before. 1.8 mL of cold EtOH 96% was added to the samples that were pooled in a rack overnight at -20 ºC (1st precipitation). The samples were centrifuged for 30 min at 14000 rpm at +4 ºC. The supernatants were removed carefully with a small green needle (0.8 mm, Fig. 2) to the waste. 25 µl of sodium acetate (1 M NaAc) was added to the samples then vortexed, another 25 µl of DD H_2O was added then vortexed and again 1.8 mL of EtOH 96% was added for 4 h at -20 ºC (2nd precipitation).

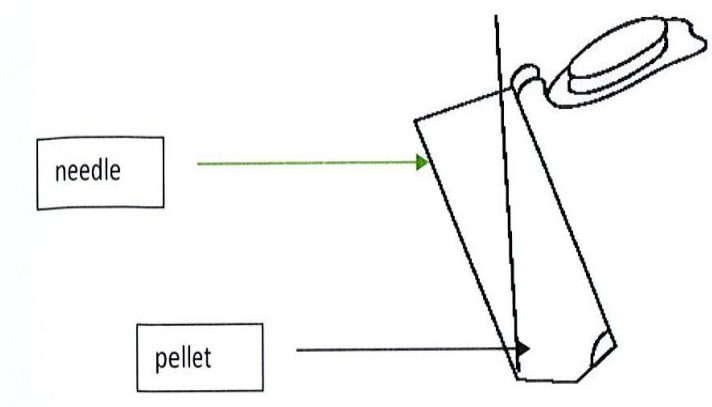

Fig.2. Shows where the pellet should be put in the axygen tube and how it looks like after centrifugation and removal the solution.

The samples were centrifuged again for 30 min at 14000 rpm at +4 ºC. The supernatants were removed with a green needle to the waste leaving a small pellet in the farrest bottom corner edge of the axygen tubes. To keep track of sample volumes, the samples were dried in speedVac for 20 min to be completely dried. The pellet was then dissolved in 150 µl of 25 mM AMBIC, small aliquots 20 µl were saved for SDS-PAGE at -20 ºC and small aliquots were

(reduced & alkylated) as before then digested (trypsinized) with 2 µl of trypsin gold (0.5 µg/ µl) for 19 h at 37 ºC to be prepared by SAX, C18 spin column.

SDS-PAGE

Samples that were saved for SDS-PAGE (as mentioned above) were dried in a speedVac for 10 min. The gel was a 4-16% gradient (separating gel) and stacking gel (4%) was prepared (see Table 1). The samples were dissolved in 20 µl of SDS Sample Buffer**,** SB **(**4 mL of DD H2O, 0.5 M tris-HCl, pH 6.8,10% w/v SDS (1.6 mL), 0.2 ml of 0.05% w/v bromophenyl blue (BPB) and 0.4 mL of 2-mercaptoethanol**),** the sample buffer was diluted 1:4 with MQ water. The samples were heated for 3 min in water bath at ~ 90 ºC. Samples were added to the samples wells and the gel was placed in the gel electrophoresis system with the electrode buffer, pH 8.3 **(**tris base 15 g/L, glycine 72 g/L, SDS 5g/L and dilute the stock 1:5 before use**)**, then connected with a power supply (BIO RAD, model no, power pac HC) current at 75 V, for 3h. The gel was incubated in a FIX (40% methanol and 10% acetic acid) solution for 45 min. The FIX was removed and the gel was washed 3 times with MQ water then in blue silver (staining solution:1 2 % Coomassie G-250, 10% ammonium sulfate, 10% phosphoric acid and 10% methanol) overnight.

Table 1 shows the separating gel & stacking gel ingredients.

Stacking gel (4%)	16% separating gel	4% separating gel
4900 µl of M.Q water	1600 µl of M.Q water	1490 µl of M.Q water
2000 µl of 1.5M tris, pH 6.8	2000 µl of 1.5M tris, pH 8.8	500 µl of 1.5M tris, pH 8.8
1000 µl of acrylamide (30%)	4300 µl of acrylamide (30%)	315 µl of acrylamide (30%)
80 µl of 10% SDS	80 µl of 10% SDS	20 µl of 10% SDS
50 µl of 10% ammonium per sulfate (APS)	50 µl of 10% (APS)	20 µl of 10% (APS)
8 µl of tetramethylethylenediamine (TEMED)	8 µl of TEMED	6 µl of TEMED

MMP-13 activity test on type I collagen

The experiment was carried out at two different concentrations of MMP-13 (2 µg at high concentration for 18 h at 37 ºC, and 0.4 µg at low concentration for 3 h at 37 ºC). Long MMP13 incubation is for ca 18 h and short incubation is for 3h at 37 ºC. Control is 6.6 µl of type I collagen (20 µg). The stock was 3.3 µg/ µl. Two tube of active enzyme were prepared (the MMP13 (18 µl) was activated with 3 µl of APMA and 29 µl of MMP-buffer). The high concentration was a (38.9 µl of &, 6.6 µl of type I collagen and 4.5 µl of MMP-buffer). The low concentration was a (7.8 µl of (the MMP13 (18 µl) was activated with 3 µl of APMA and 29 µl of MMP-buffer), 6.6 µl of type I collagen and 35.6 µl of MMP-buffer). The control was a (6.6 µl of type I collagen and 43.4 µl of MMP-buffer).

Experiment 2

The second part was the comparison between control and MMP13 treated samples using tissue plugs. The same procedure as before but the MMP13 was properly actived (2 µl of APMA, 12 µl of MMP13 (0.143 µg/ µl) and 26 µl of MMP-buffer) in this part by heating for 90 min at 37 ºC. There was one control (5 mg) sample and two MMP13 treated samples, one **(**S1**)** was (4.8 mg, 144 µl of MMP-buffer and 11.66 µl of active MMP13 (0.5 µg/ µl)) and the other MMP13 sample **(**S2**)** was (5.2 mg, 156 µl of MMP-buffer and 11.66 µl of active MMP13 (0.5 µg/ µl)). All reagents volumes (DTT, IAA, EDTA and trypsin) were doubled in this experiment**.**

Experiment 3, tissue plug and powder comparison.

The third part of the project was to see how the powderisation can be utilised and how the differences are between plugs vs powder. The previous cartilage plugs were powderized using **(Retsch Mixer Mill, MM400, Fig. 3).** The cartilage discs were cut with a scalpel to small pieces then put inside the metal sample vial for powderisation (MGP) (a in Fig. 3). The sample vial was covered with liquid nitrogen in a beaker. The MGP was fixed in the MM400

instrument, and the instrument was turned on for 1 min at 25 Hz/sec. The same method was repeated twice more.

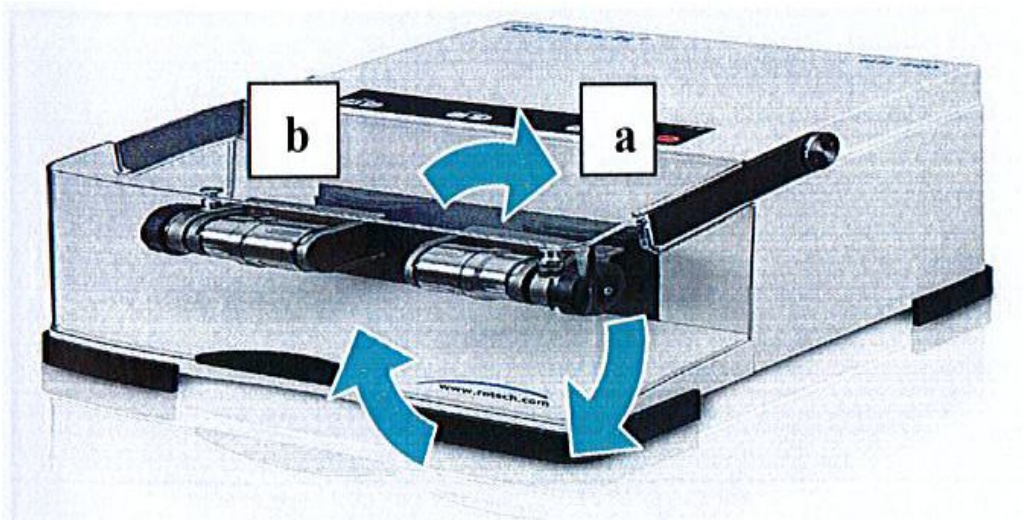

Fig. 3. Shows the instrument used for powderization, the marked metal ground (a) contains the sample and the non marked one (b) is for balance.

The powder was transferred to the tube and saved at -20 ºC. Sample amounts of 1.2 mg of powdered sample were weighed in both control and MMP13 samples. 100 µl of MMP-buffer was added to the control. Then 50 µl of MMP-buffer was added to the other sample followed by 50 µl of active MMP13 (incubated 90 min at 37 ºC and 350 rpm) corresponding to 2 µg of active enzyme **(**15 µl of pro-MMP13 (0.143 µg/ µl), 2.5 µl of 1 mM APMA and 32 µl of MMP-buffer. The samples were incubated in a water bath for at 37 ºC for 19 h. The samples were centrifuged for 20 min at 14000 rpm at +4 ºC. The supernatants of two samples were transferred to new 0.5 mL safelock tubes. The cartilage tissues of two samples were washed with 50 µl of MMP-buffer for 1 h at +4 ºC on a shaker, twice. Then the solutions were added to the supernatants. The cartilage tissues were dried in a speedVac for 10 min. Then 100 µl of 4 M GuHCl with 5 mM NEM was added to the tissues followed by incubation for 24 h on a shaker at +4 ºC. The cartilage tissue samples were centrifuged for 30 min at 14000 rpm at + 4 ºC. The supernatants (ca 80 µl) were transferred to 2 mL Axygen tubes then followed by

ethanol precipitation as before and the final pellets were dissolved in 130 µl of AMBIC, then reduced alkylated and trypsinized (digested) as before to be prepared by SAX, C18 spin column to be ready for Ion trap reversed phase chromatography, Q-TOF and MRM. The 20 µl (which is left from 100 µl) of supernatant were transferred to two new 2 mL Axygen tubes, then followed by ethanol precipitation and the final pellets were saved for SDS-PAGE.

The trypsin (digest) samples were prepared for mass spectrometry by strong anion exchange (SAX) and C18 spin column as the following: 1**)** Conditioning step **(**C-step**)**, a 200 µl of 1 M NaCl was added to the 4 SAX columns, then columns were centrifuged for 11 s at 1020 g, then incubated for 1 h at room temperature. 2**)** Wash step **(**W-step**)** a 200 µl of 0.5M AMBIC at pH 7.8 was added to the columns then centrifuged for 1 min at 1020 g, then the solution was discarded to the waste. The W-step was repeated once more. 3**)** Loading sample step **(**L-step**)**, The Axygen tubes were changed for new Axygen tubes. 83.3 µl of a 0.5M AMBIC solution at pH 7.8 was added to the column +16.7 µl (10%) of GuHCl samples. 80 µl of a 0.5M AMBIC solution at pH 7.8 was added to the column +20 µl (10%) of media samples. The columns were centrifuged for 1 min at 1020 g. 4) Washing step **2nd (W-step)**, 100 µl of AMBIC solution was added to the columns, then centrifuged for 1 min at 1020 g (twice). The final flow through (300 µl) of all samples was saved in the Axygen tubes.

Secondly, C18-spin column: C18 columns were placed in the collection 2 mL Axygen tubes. **1) Column wash**, the columns were washed with 300 µl of 100% MeOH, then centrifuged for 2 min at 1600 rpm, the flow through solution was discarded to the waste. **2) Column equilibration**, 300 µl of **buffer A** (2% acetonitrile, ACN in 0.2% formic acid, FA) was added to the columns, then centrifuged for 2 min at 1600 rpm, the flow through solution was discarded to the waste. The equilibration step was repeated twice more. **3) Sample immobilization**, the column tips were dried on a paper tissue. The samples (300 µl from SAX flow through) were added to the columns then centrifuged for 2 min at 1600 rpm. The flow through liquid was reapplied (twice) to the columns then centrifuged for 2 min at 1600 rpm.

The final flow through was discarded to the waste. **4) Sample wash**, 300 µl of buffer **A** was added to the column, then centrifuged for 2 min

Table. 2. Shows difference steps within SAX and C18 spin column

	SAX	**C18, spin column**
condition	200 µl of 1M NaCl	
wash	200 µl of 0.5M AMBIC pH 7.8	300 µl of 100% MeOH
loading	(83.3 µl of 0.5M AMBIC pH 7.8 was added to the column +16.7 µl of (10%) of GuHCl samples)	
wash	100 µl of 0.5M AMBIC pH 7.8	300 µl of buffer A
Elution		300 µl of buffer B

at 1600 rpm. The sample wash was repeated twice more. The final flow through was discarded to the waste. **5) Sample elution**, the column tips were dried on a paper tissue. The columns were placed in new collection 2 mL Axygen tubes. 100 µl of **buffer B** (50% ACN in 0.2%

F.A) was added to the column, then centrifuged for 2 min at 1600 rpm. Another 100 µl of buffer B was added again to the column then centrifuged as before. The final flow through (200 µl) was saved for evaporation step. **6) Sample evaporation**, 100 µl was transferred to MS vial, then completely dried in speedVac, then the other 100 µl was transferred to the MS vial and completely dried as before. **7) Sample resuspension**, a 20 µl of buffer A was added to the samples. The samples were sonicated for 5 min in water bath. The samples (must be closed) were centrifuged for 1 min speedVac. Finally, the samples were stored in fridge at +4 ºC to be ready for the MS runs.

Experiment 4

The fourth part of the project was a comparison between plug vs powder before and after wash with MMP-buffer. A 2 mg of plug sample was weighed in both control **(a)** and MMP13 **(b)** samples. A 1 mg of powder (before wash) sample was weighed in both control **(c)** and MMP13 **(d)** samples. A 1 mg of powder (3 times wash before incubation (sample digestion) with 100 µl of MMP-buffer for 30 min for each time) sample was weighed in both control **(e)** and MMP13 **(f)** samples. 100 µl of MMP-buffer was added to the control samples **(a,c and e)**. 81 µl of MMP-buffer and 19 µl of actived MMP13, 22 µl of pro-MMP13 (0.143 µg/ µl), 3 µl of 1 mM APMA and 35 µl of MMP-buffer, were incubated for 90 min at 37 ºC at 350 rpm before addition to the MMP13 samples **(b,d and f)**. The samples were incubated in a water bath for 19h at 37 ºC. The samples were centrifuged for 5 min at 14000 rpm at +4 ºC. The supernatants of all samples were transferred to new 0.5 mL safelock tubes. The cartilage tissues were washed with 100 µl of MMP-buffer for 1 h at +4 ºC on a shaker, the solutions were transferred to the supernatants. The supernatants were reduced and alkylated as before.

A small aliquot (20 µl) was saved for SDS-PAGE and another 20 µl was saved for western blot. A 4 µl volume stock (concentration of 2 mM EDTA was added to the supernatants (160 µl) of active MMP13 samples in order to inactivate the MMP13, then 2 µl of trypsin gold (0.5 µg/ µl) was added to all samples (controls and active MMP13 samples) and incubated at 37 ºC for 19 h.

200 µl of 4 M GuHCl, 5 mM NEM was added to the cartilage tissues for 24 h on a shaker at +4 ºC. The cartilage tissue samples were centrifuged for 30 min at 14000 rpm at + 4 ºC. The supernatants (ca 160 µl) were transferred to 2 mL Axygen tubes then followed by ethanol precipitation as before and the final pellets were dissolved in 130 µl of AMBIC, then reduced alkylated and digested with 2 µl of trypsin gold (0.59 µg/ µl) as before samples were prepared for MS by SAX and C18 spin column as before. A small aliquot of 20 µl (which is left from 200 µl) of supernatant samples were transferred to new 2 mL Axygen tubes, then followed by ethanol precipitation and the final pellets were stored at -20 ºC for SDS-PAGE.

Database searching

Mascot Daemon

It is a data base searching engine programme for protein identification. The raw data is processed by different softwares ”DataAnalysis” (Bruker) and ”ProteinLynx” (Waters) into mgf and pkl peak lists files respectively, which are then used in the database searching.

Results and Discussion

Sample preparation

Blocking of cystein group

DDT was used as a reducing agent to reduce the cystein group and unfold the proteins as there are many disulfide bonds in proteins that make the matrix very complexed. IAA was used as an alkylating agent to prevent the formation of disulfide bonds again.

Blocking the active enzyme

EDTA was used to block the activity of MMP13 before adding the trypsin digest as it forms a chelating bond with metal ions (Ca^{+2} & Zn^{+2}) in MMP-13. A high concentration of 0.5 M of AMBIC was used in SAX experiment as it breaks the interaction between the peptides in the sample.

SDS was used to unfold the proteins with negative charge so during the gel electrophoresis the proteins will go to the + electrode.

MMP13 was activated with APMA. Matrix metalloproteinases (MMPs) can be activated with many mechanisms such as treatment with detergents, proteases and organomercurials (APMA in this case). The reason for a good activation with APMA is that the cystein group is used for the activation. This explains why there are many mechanisms for activation caused by a dissociation of the single cystein residue in the inactive peptide (propeptide) from the ion in the zinc active site. APMA is working by directly reacting with the sulfydryl group of cystein resulting a displacement from the active site [ref. 8]. **T**he ethanol precipitation step was used to wash away the high amount of GuHCl as this salt forms a white precipitate upon adding sample buffer (SDS). GuHCl should extract all proteins except collagens in the cartilage tissue due to their strong triple helix structure stabilized with covalent cross-links both for plug or for powder tissues. The high salt of 1 M NaAC after the first ethanol precipitation was used to dissolve the protein samples. The second wash was done to make sure that all GuHCl was washed away.

SDS-PAGE results

Different experiments were performed as is mentioned in the Experimental part. Fig. 5 shows the (SDS-PAGE) results of first experiment (plug) samples, as it is mentioned in the Material Methods part), that the bands in media samples are quite similar between control and MMP13 samples.

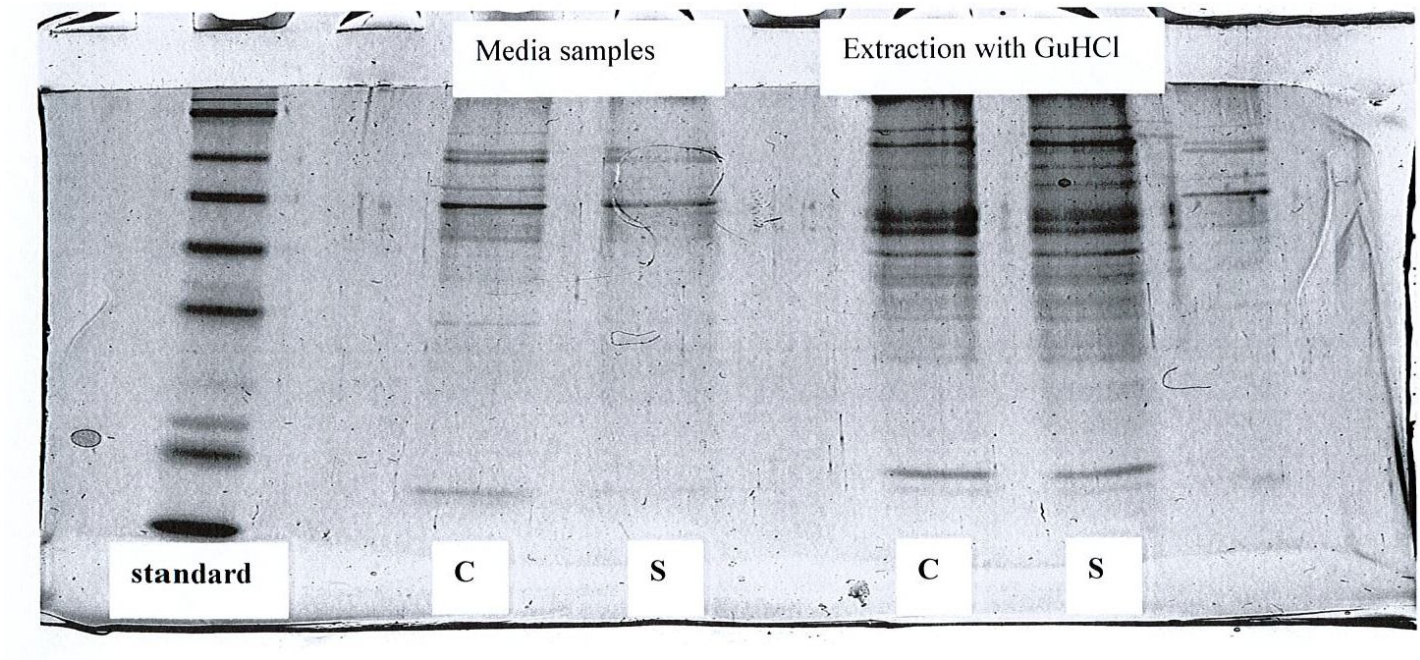

Fig. 5. SDS-PAGE in case of tissue plug show the first experiment. The enzyme (MMP13) was not properly activated.

It is obvious that more proteins in the case of the extraction with GuHCl, but the control and MMP13 samples are quite similar as well and the reason for this, it could be that MMP13 was not efficiently activated.

The MMP13 activation was therefore tested on type I collagen, Fig. 6. to make sure that it was ready for the next experiment to do its job. The MMP13 (18 µl) was activated with 3 µl of APMA and 29 µl of MMP-buffer. The MMP13 was very active according to the mentioned figure.

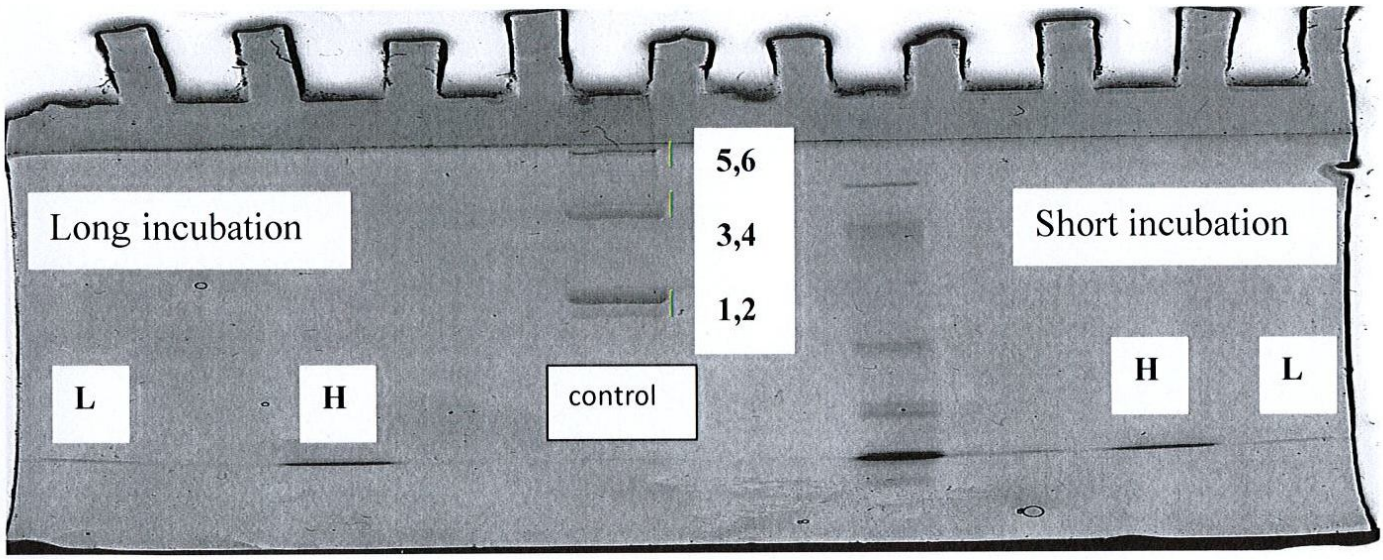

Fig. 6. SDS-PAGE, shows that the MMP13 activity on type I collagen was highly active. Abbreviations, H, is high concentration (2 µg) and L, is low concentration (0.4 µg).

The control sample shows six bands, the first band from the bottom is alpha-2 (α2-chain), the next one is covalently cross-linked band, it could be (α1α1). The next bands (3rd&4th) are dimers **(**(α1α1), (α2α2) or (α1α2)**)**. The last two bands are multimers (more than 2 chains), (Fig. 6, light yellow colors).

Fig. 7 shows that there is a visual difference (in media samples) between the control and the samples with active enzyme MMP13 (S1 & S2) as expected. There is quite a difference between S1and S2, and the reason for this could be that some of sample plugs were full depth and some were half depth, it could be also that the two samples released different amount of proteins.

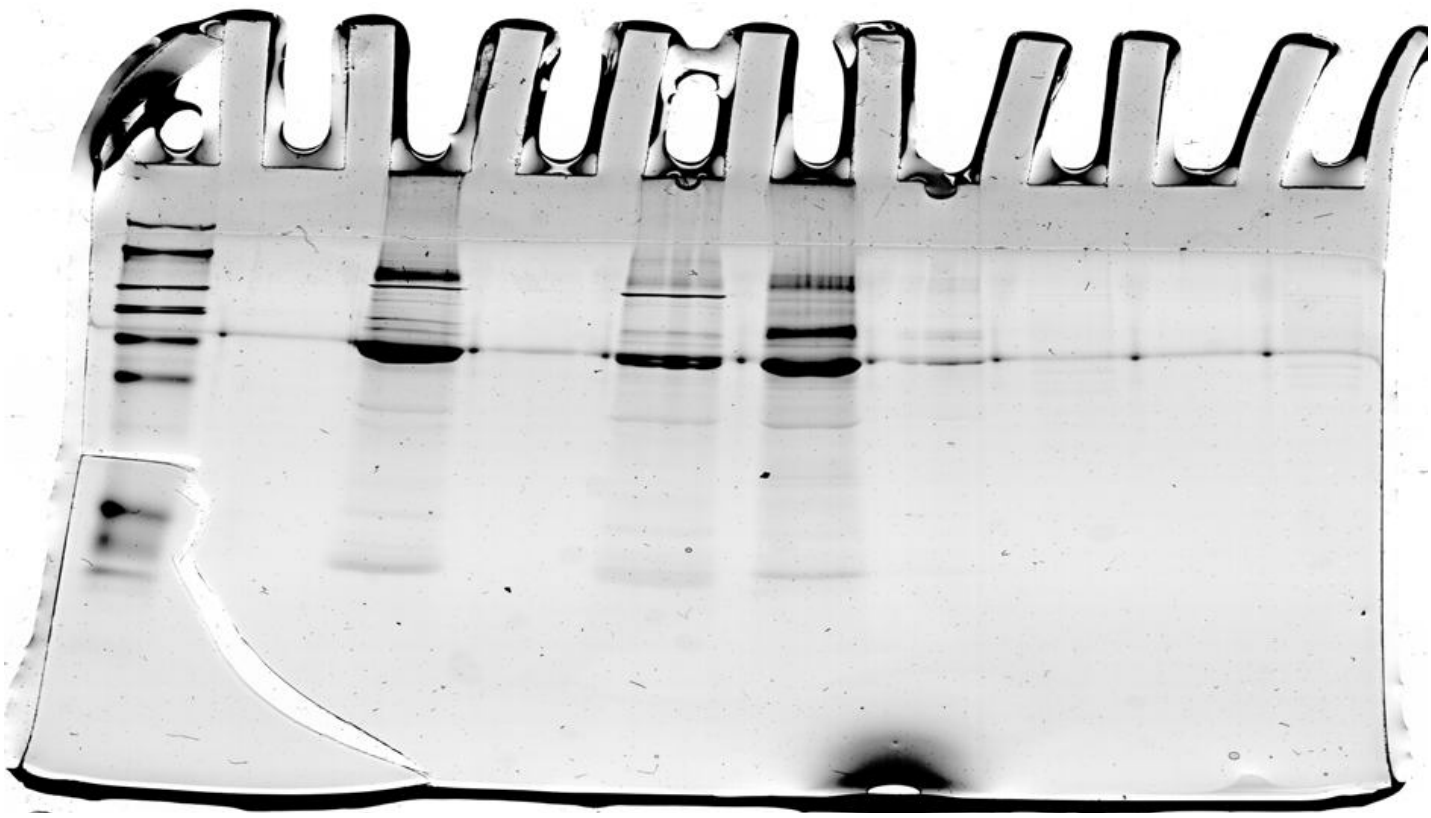

Fig. 7. SDS-PAGE for control, media and GuHCl samples in case of plugs.

There were no visual results after extraction with GuHCl. This might be due to the fact that the precipitated samples were dissolved in AMBIC, which is not a good solvent for the sample. The question now, why has it been obtained a result with GuHCl in the first experiment?, the reason for this is that any portion of the solution when the sample was dissolved in AMBIC entered the pipet before it was run on the gel so it gave good results, or perhaps the pellet was lost during the sample handling steps in one sample run but not the other.

Mass spectrometry results (Plug vs Powder)

(2nd & 3rd experiment)

Ion trap MS/MS reversed phase chromatographic results

1- Powder vs plug tissue (Control media)

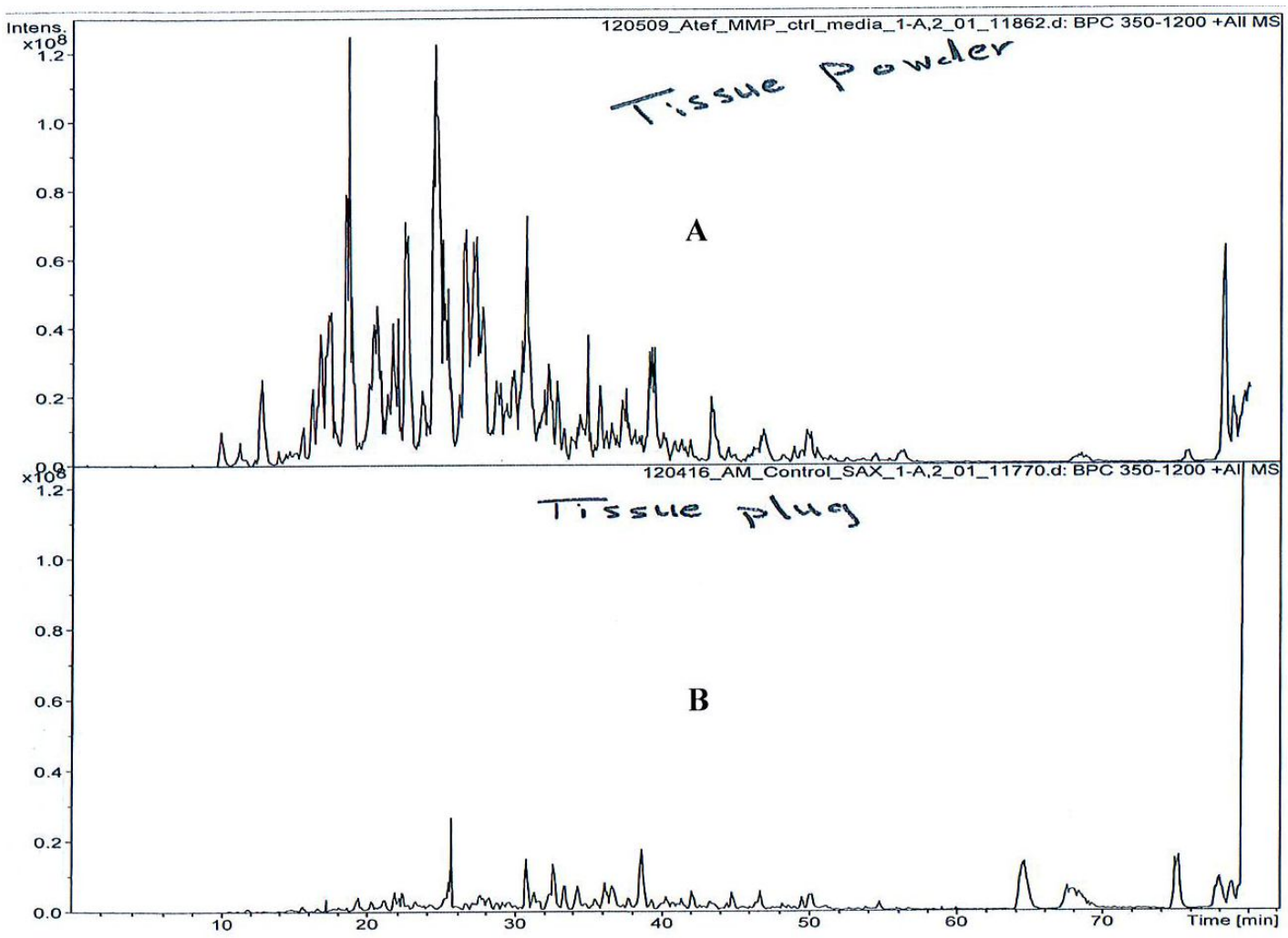

Fig. 8. Show the base peak chromatogram difference for control media samples between plug vs powder.

A big difference is obvious (Fig. 8) between control media samples in case of plug and powder. More proteins were released in case of powder than plug, where ~15 proteins were found in plug tissue while ~25 proteins in powder tissue.

2- Control vs MMP13 (Powder tissue)

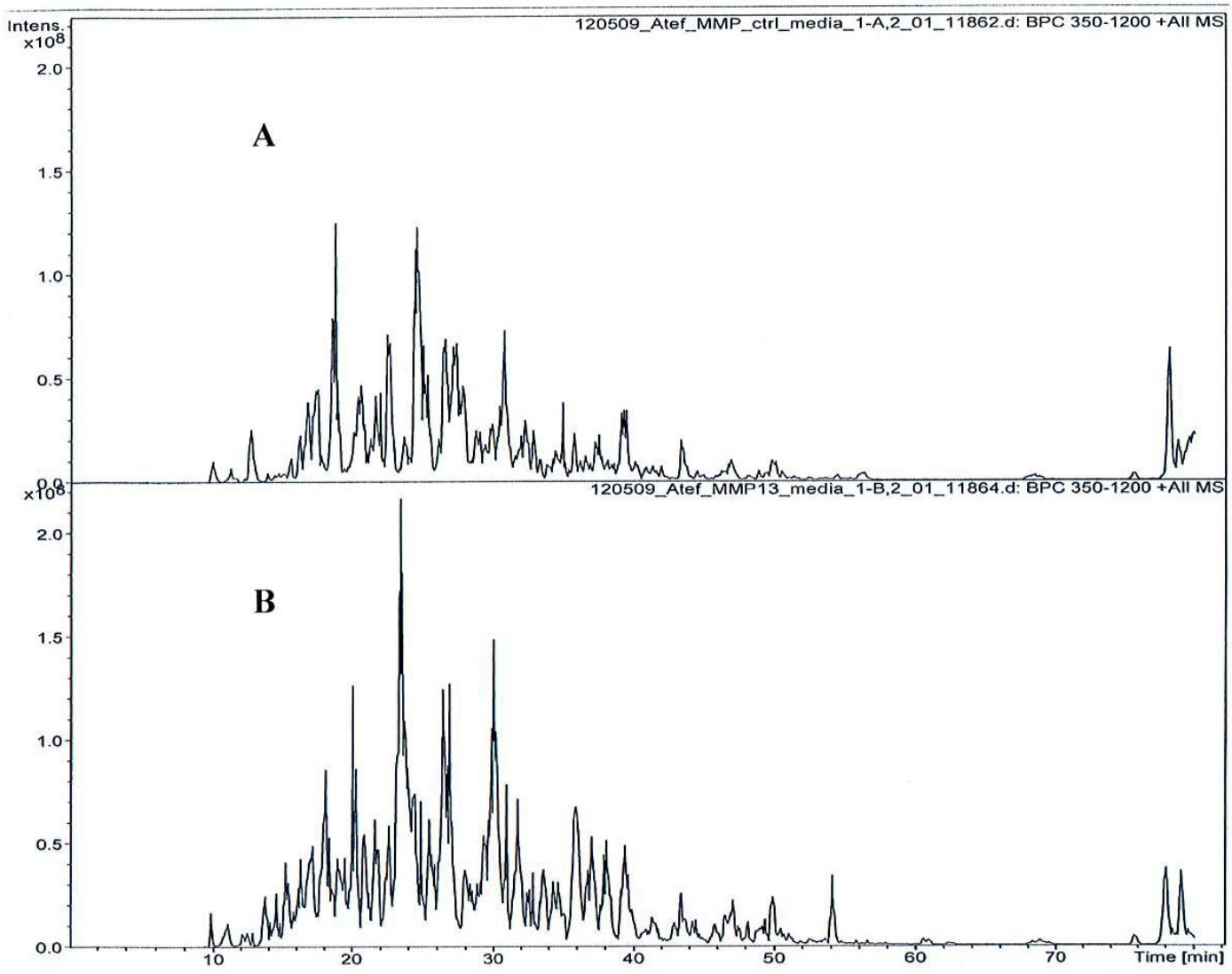

Fig. 9. Shows the difference between control and active MMP13 media samples in case of powder tissue.

Figure 9 shows an obvious difference between the control (A) and active MMP13 (B) media samples, as more proteins are found in B than in A as is expected as it seems that the enzyme was active and did its job.

In the figure below (Fig. 10), more proteins were found in control with GuHCl (A) than control sample media before extraction (B), as more proteins were extracted with GuHCl as is expected and also shown in the previous experiment.

3- Control vs MMP13 (media), Control vs MMP13 (GuHCl), (Plug)

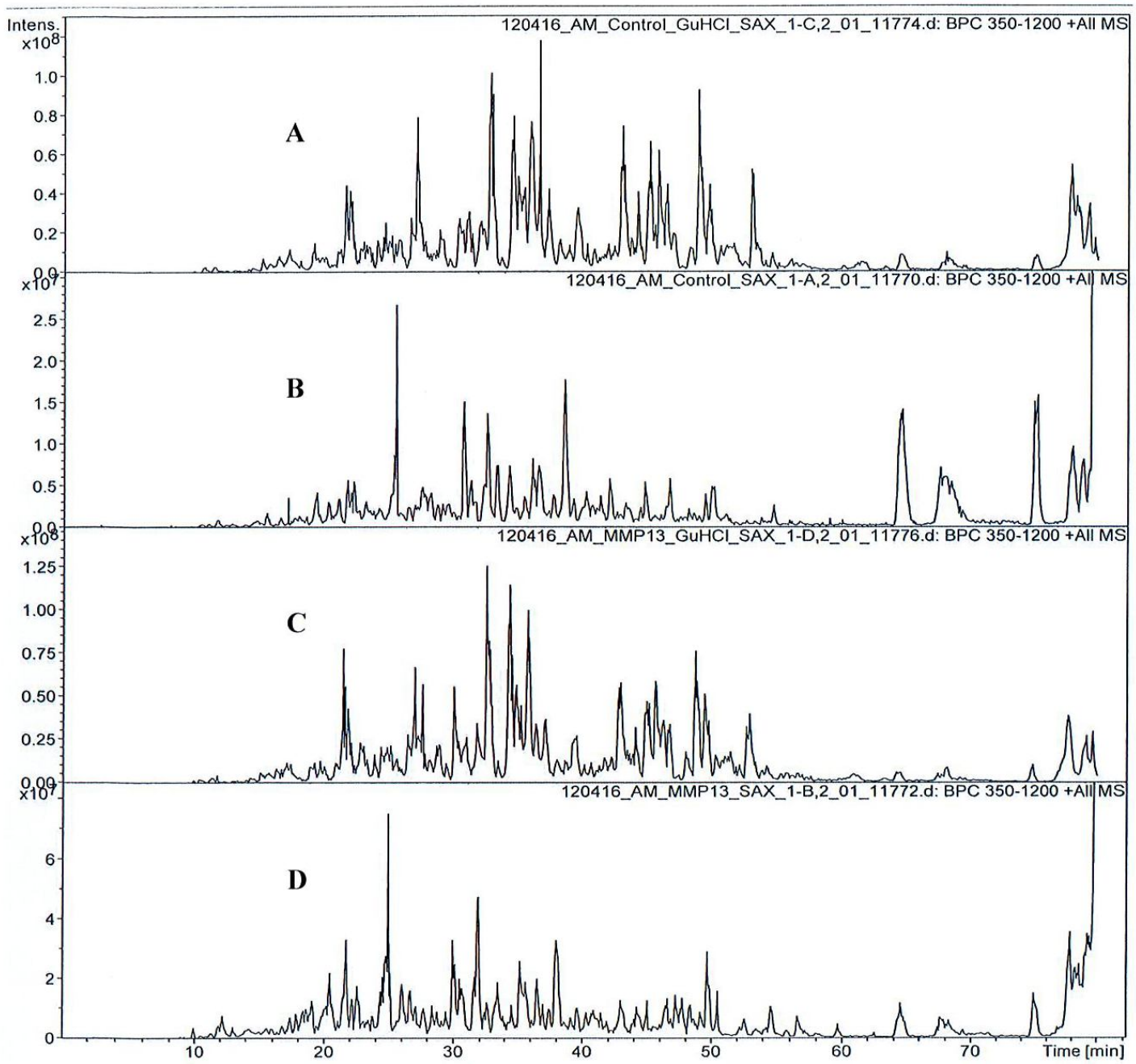

Fig. 10. Shows the base peak chromatogram for control and MMP13 samples before and after extraction with GuHCl.

In the slides MMP13 with GuHCl (C) & MMP13 media sample (D), it is seen that we have more proteins in C than in D also when there is active MMP13.

Results

In the (current) third experiment (powder tissue), there were 20 proteins in MMP13 sample media and 15 proteins in control sample media in the case of powder tissue with semi trypsin as expected with **semi trypsin**. The cleavage consists of one side in C-terminal in R or K amino acids and the other cleavage is in a different amino acid. There were 20 proteins in MMP13 sample after extraction with GuHCl and 16 proteins in control sample after extraction with GuHCl in the case of powder tissue with semi trypsin. **S**ome proteins found in the MMP13 media sample that were not found in control media sample with non-tryptic cleavages, includes PRELP, HPLN1, PGS2 and CO6A1. PGS1 was found in both control and MMP13 samples but with different cleavage sites.

Results of the last experiment

SDS-PAGE

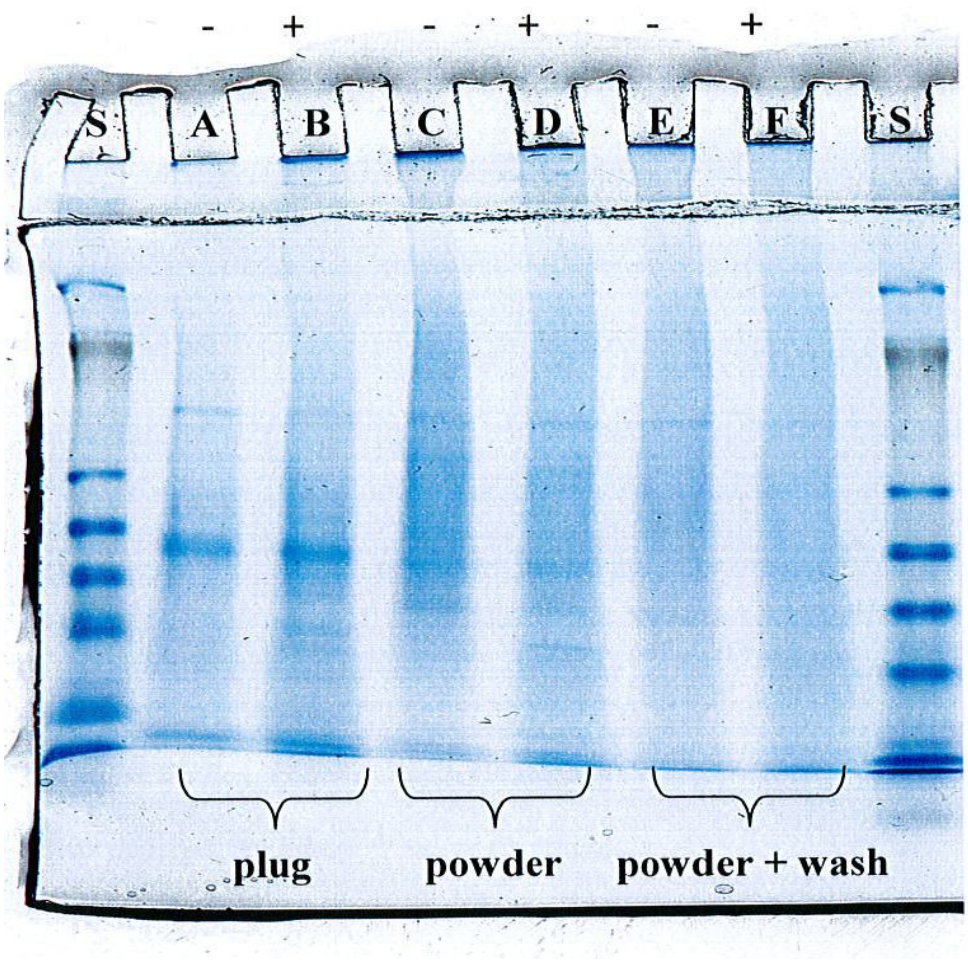

Fig. 11. Shows the SDS-PAGE for control and MMP13 media samples in plug (A&B), powder (C&D) and powder after wash (E&F), (+) represents the sample with MMP-13 and (–) represents the sample without MMP-13.

In Figure 11, there is a difference between the control and MMP13 media samples, where, in case of the plug tissue sample (2 mg), **A** is the control and **B** is the active enzyme (MMP13) media samples. In case of powder tissue sample C, is the control and **D** is the MMP13 media samples. In case of powder after wash with MMP-buffer, **E** is the control and **F** is the MMP13 media samples, **S** is the standard.

There is a quite obvious difference between control and MMP13 media samples in case of plug and powder before wash as expected, but not a big difference in case of powder after wash.

Mass Spectrometry results.

1- Ion trap reversed phase MS/MS

1.A. (Control samples)

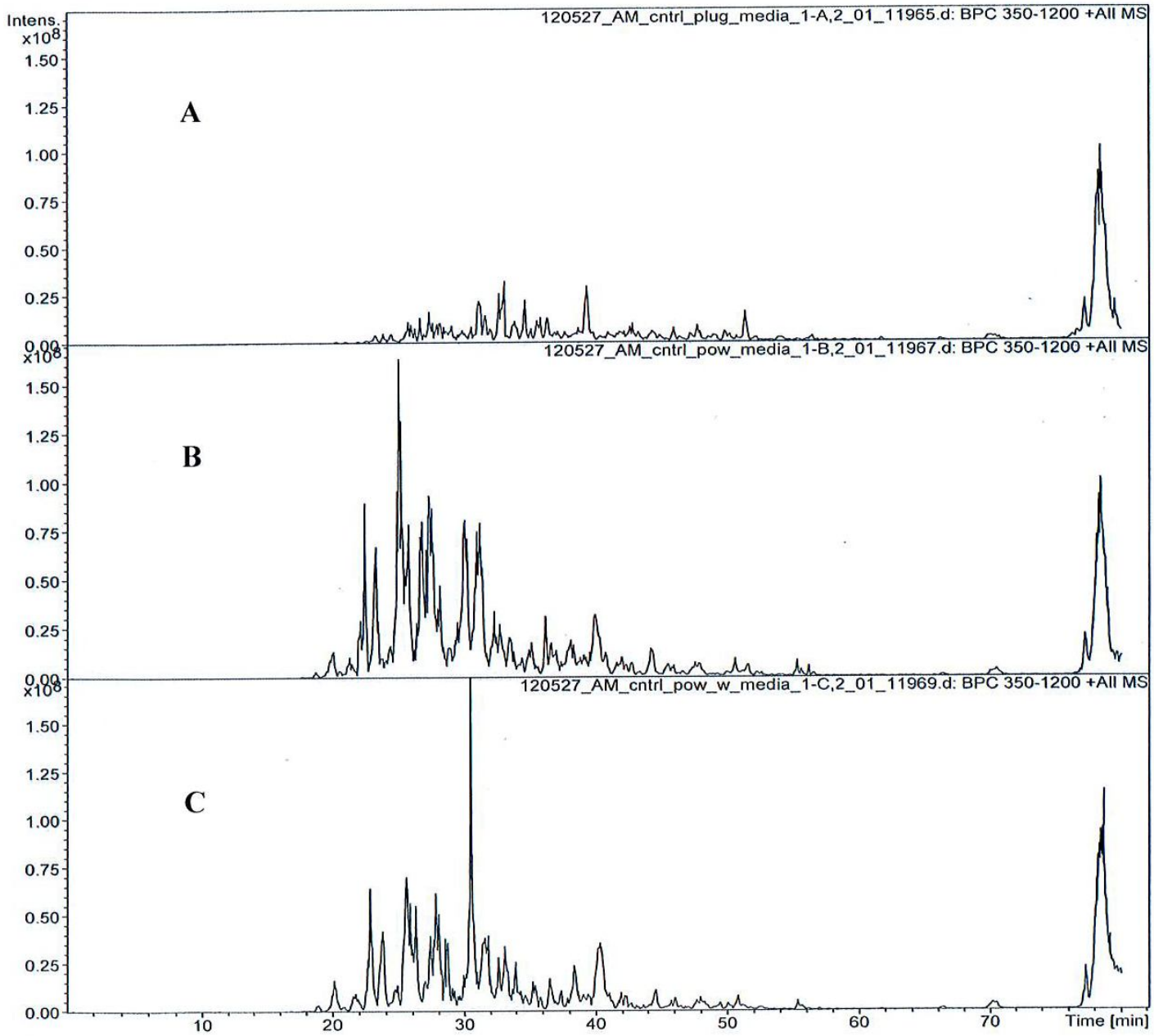

Fig. 12. Ion trap base peak chromatograms (BPC) in case of plug (A), powder (B) and powder after wash (C).

In Figure 12 above, it is very obvious that more proteins were released by powderization (B) as found before and surprisingly also after 3 times wash (C) than plug (A) in case of media

samples (and even without adding the active MMP13). ~22 proteins were found in control, where as ~29 proteins were found in MMP-13 sample.

2.A. Powder tissue before wash (Control vs MMP13)

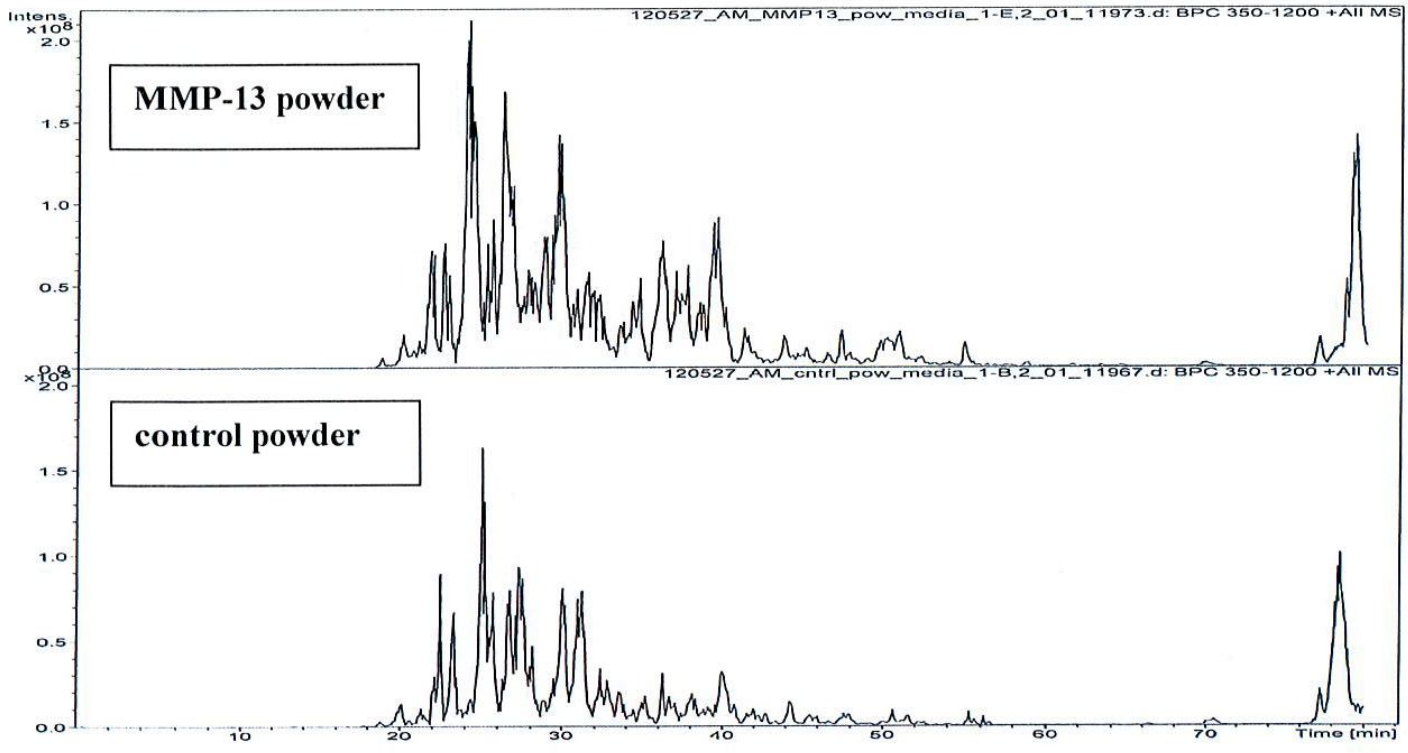

Fig. 13. Control (bottom) and MMP13 (top) media samples in powder tissue without wash.

In Figure13, it is shown that there are more proteins with higher signals in MMP13 than in control media samples in case of powder tissue before wash. This confirms that MMP13 has done its job well.

2- Q-TOF data

More proteins were identified by Q-TOF (see Appendix 1) due to better mass accuracy (on-line calibration), a wider MS/MS mass range and higher resolution than Ion trap but similar sensitivity. With both instruments a data-dependent protocol is utilized where a cycle of one MS scan (see Fig. 14b) is followed by 4 MS/MS experiments (see Fig. 14c) before the next MS scan is made. This is exemplified in Figure 14 for a fibromodulin peptide.

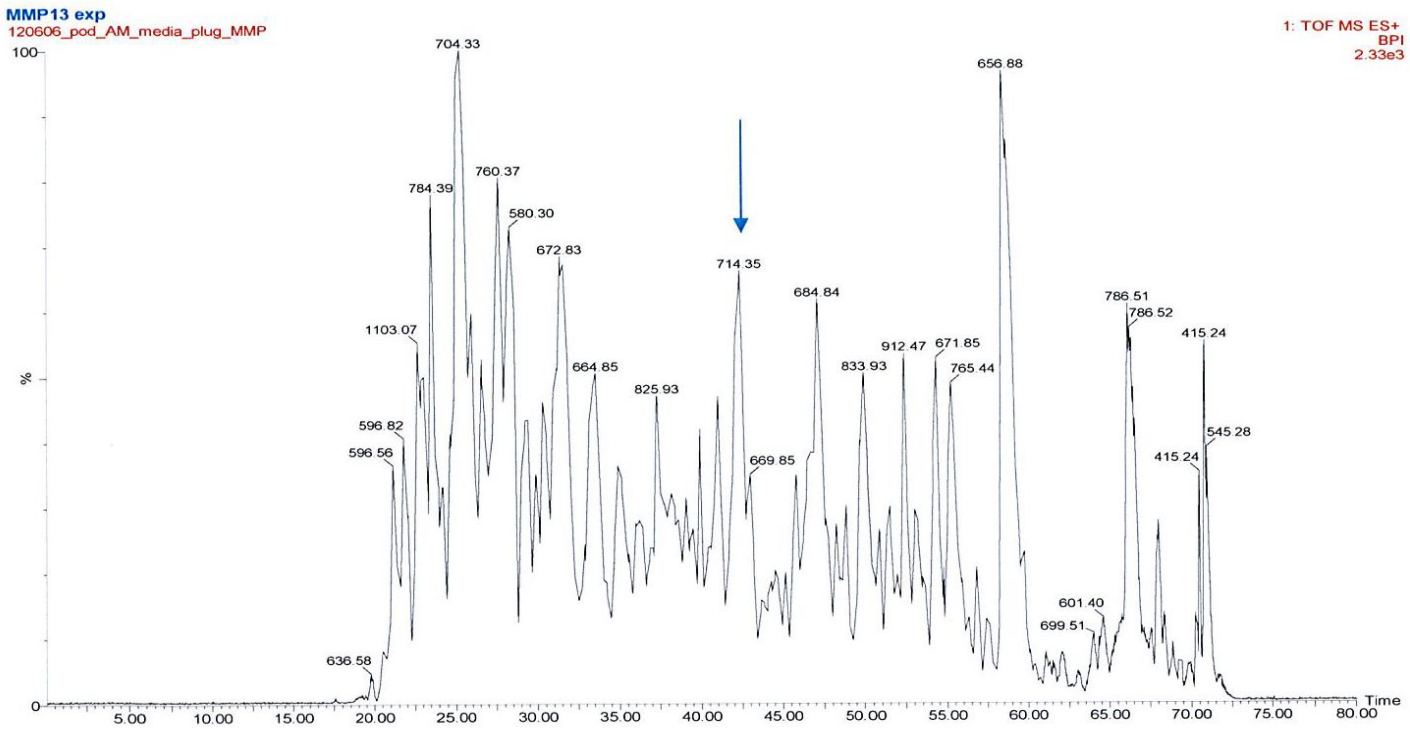

Fig. 14a. Q-TOF Base Peak Chromatogram (BPC) of the MMP13 media plug sample (fibromodulin) in case of BPI. The arrow points at an ion of 714.4 (m/z) which is the base peak (highest peak) at t_R ~42 min. The corresponding MS scan is shown in Figure 14b.

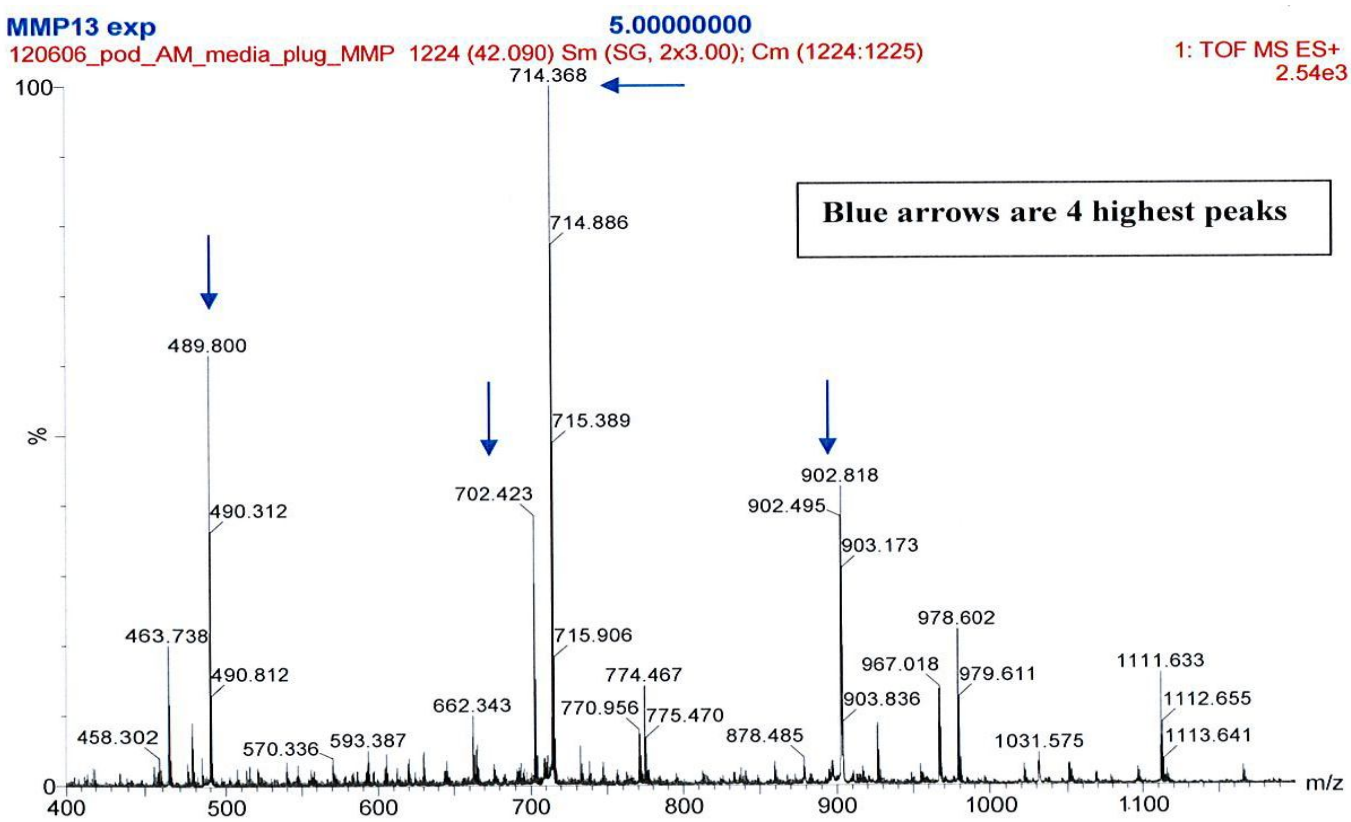

Fig. 14b. Ms spectrum at 42 min retention time that has molecular weight (mass) of 489.8, and the four highest peaks 489.8 (+2 charge), 714.3 (+2), 702.4 (+2) and 902.8(+3) which are doubly or triple charged (as singly charged represents shorter peptides which is not very unique and often results in poor MS/MS spectra). y ion represents the positively charged fragment ion counting from (contain) the C-terminal and b ion represents positively charged fragment ion counting from (contain) the N-terminal.

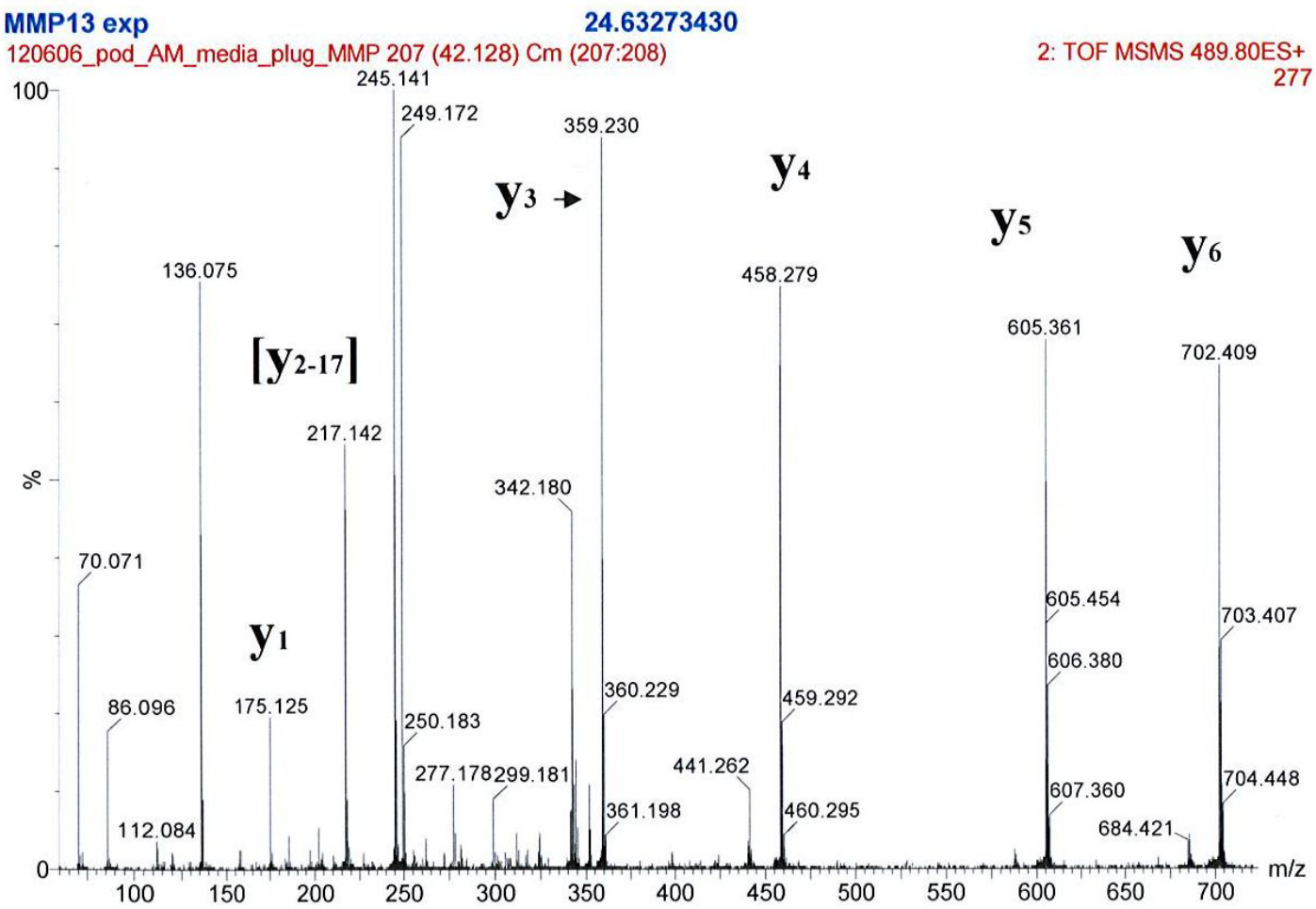

Fig. 14c. Shows the first MS/MS from total of four MS/MS of 489.8 (identified as fibromodulin peptide "YLPFVPSR" in a database search) in control media plug from mascot daemon research with trypsin, where is the m/z (702.4) represent y_6 (PFVPSR) and The m/z (605.4) represent y_5 (FVPSR), The m/z (458.3) represent y_4 (VPSR), the m/z (359.2) represent y_3 (PSR), the m/z (217.1) represent y_{2-17} (SR), and the m/z (175.1) represent y_1 (R).

3- MRM results (quantitative analysis)

Protein name: CO6A3_HUMAN Collagen alpha-3(VI)

Peptide sequence: K.LSDAGITPLFLTR.Q [2532, 2544]

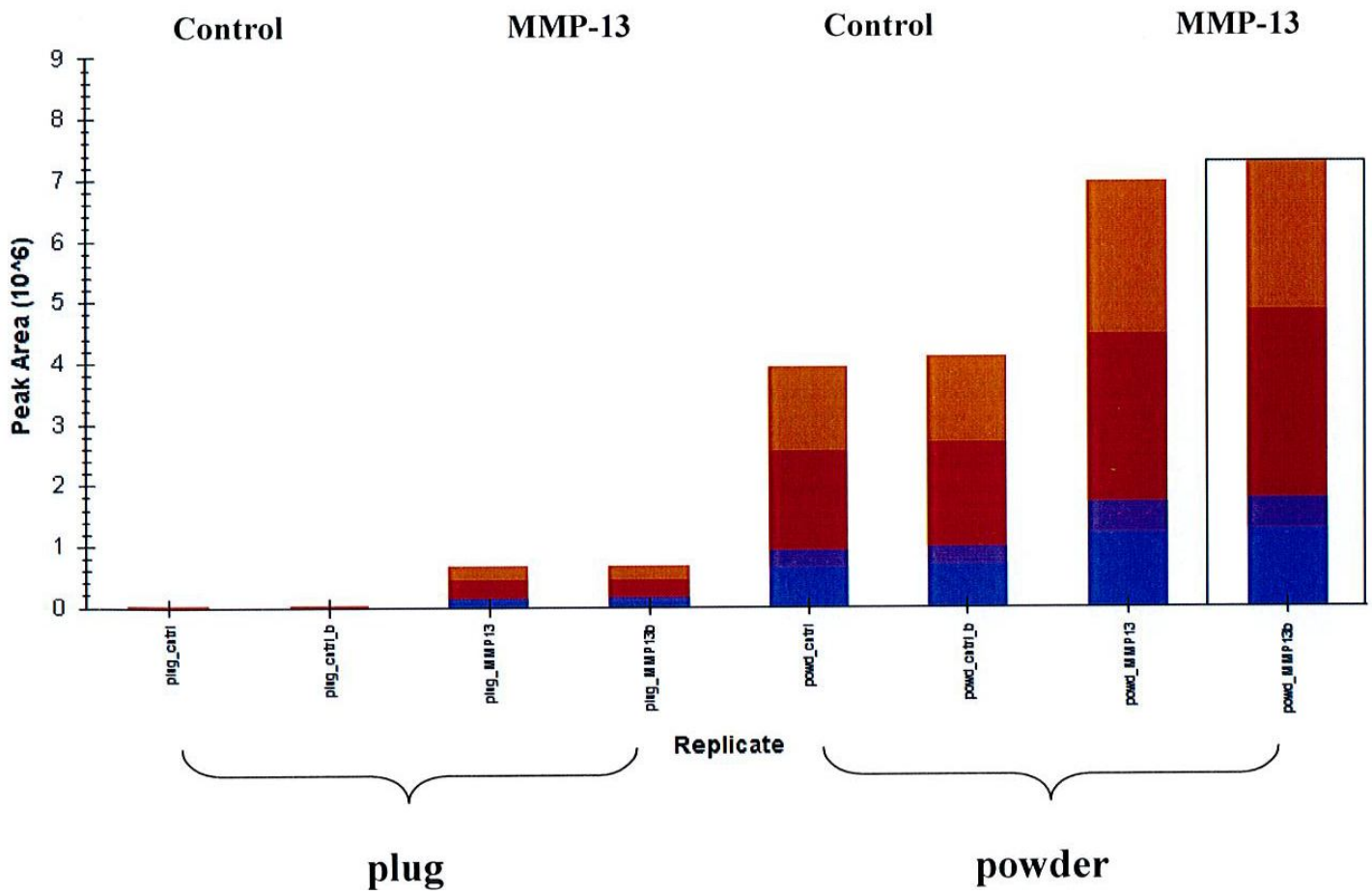

Fig. 15 shows that more proteins are released with MMP13 than control in both plug and powder tissue.

The selected peptide contains four transitions (y9, y8, y7 and y6). Each peak area is a summation of four transitions, where y7 represents the highest rank brown color, y6 represents the second rank dark (brown color), y9 represents the third rank (violet color) and y8 represents the highest rank (blue color). There is an obvious effect of MMP-13 in both plug and powder tissue (more proteins are released with MMP13**)**.

Conclusion

MMP13 is well activated with APMA. MMP13 is a good enzyme for some proteins in the cartilage tissue but not as good for others. **T**he effect of MMP13 was not so marked, which it could be due to the accessibility of substrate being low as MMP-13 is not able to penetrate the tissue for either plug or powder. The presence of highly charged aggrecan and dense extracellular matrix are possible reasons to hinder MMP13.

More proteins were released in the case of powder tissue than in plug tissue as expected as there is more contact surface area of the powder. Q-TOF data analysis gave a better mass resolution than Ion trap mass instrumentation. More proteins were identified by Q-TOF (~39 proteins) than by Ion trap (~22 proteins), even regarding that 2.5% of the sample was injected in Ion trap and 3.4% was injected in the case of Q-TOF.

MMP13 showed a good activity for cleavage of type I collagen both for long (18 h) and short incubation (3 h) times at 37 ºC.

More proteins were released in the case of powder both in the case of control and MMP13 media samples. The ratio of MMP13 to the tissue was 1:100 (w/w) in case of powder (last experiment), which probably would be sufficient for an in solution digest but it could be a limiting factor in case of tissue samples.

Acknowledgement

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I would like to thank the group leader Professor **Dick Heinegård.**

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Appendix (1)

Q-TOF analysis (mascot research)

A) Powder tissue (control media) with trypsin

Cartilage powder control release	
Accesion number	**Protein name**
P02458|CO2A1	Collagen alpha-1(II) chain
P49747|COMP	Cartilage oligomeric matrix protein
P02461|CO3A1	Collagen alpha-1(III) chain
P16112|PGCA	Aggrecan core protein
P02768|ALBU	Serum albumin
P02452|CO1A1	Collagen alpha-1(I) chain
P21810|PGS1	Biglycan
P08123|CO1A2	Collagen alpha-2(I) chain
P51888|PRELP	Prolargin
Q8IUL8-22|CILP2	Cartilage intermediate layer protein 2-2
O15335|CHAD	Chondroadherin
P02751-1|FINC	Fibronectin
P07585|PGS2	Decorin
O75339-12|CILP1	Cartilage intermediate layer protein 1-2
P12111|CO6A3	Collagen alpha-3(VI) chain
P10915|HPLN1	Hyaluronan and proteoglycan link protein 1
P14555|PA2GA	Phospholipase A2, membrane associated
Q06828|FMOD	Fibromodulin
P61626|LYSC	Lysozyme C
P12109|CO6A1	Collagen alpha-1(VI) chain
P51884|LUM	Lumican
P20774|MIME	Mimecan
Q14055|CO9A2	Collagen alpha-2(IX) chain
P12110|CO6A2	Collagen alpha-2(VI) chain
P10909|CLUS	Clusterin
P20849|CO9A1	Collagen alpha-1(IX) chain
P68871|HBB	Hemoglobin subunit beta
P13942|COBA2	Collagen alpha-2(XI) chain
Q9NVH2|INT7	Integrator complex subunit 7
P69905|HBA	Hemoglobin subunit alpha
Q969H0|FBXW7	F-box/WD repeat-containing protein 7
P12107|COBA1	Collagen alpha-1(XI) chain

356 peptides were identified and matched to 32 proteins

B) Powder tissue (MMP13 media) with trypsin

Cartilage powder MMP-13 release	
Accesion number	**Protein name**
P02458|CO2A1	Collagen alpha-1(II) chain
P49747|COMP	Cartilage oligomeric matrix protein
P02461|CO3A1	Collagen alpha-1(III) chain
P21810|PGS1	Biglycan
P02452|CO1A1	Collagen alpha-1(I) chain
Q8IUL8-22|CILP2	Cartilage intermediate layer protein 2-2
P16112|PGCA	Aggrecan core protein
P51888|PRELP	Prolargin
P12111|CO6A3	Collagen alpha-3(VI) chain
P02768|ALBU	Serum albumin
P02751-1|FINC	Fibronectin
P08123|CO1A2	Collagen alpha-2(I) chain
O15335|CHAD	Chondroadherin
P12109|CO6A1	Collagen alpha-1(VI) chain
P45452|MMP13	**Collagenase 3**
P10915|HPLN1	Hyaluronan and proteoglycan link protein 1
Q06828|FMOD	Fibromodulin
O75339-12|CILP1	Cartilage intermediate layer protein 1-2
P20774|MIME	Mimecan
P07585|PGS2	Decorin
P07996|TSP1	Thrombospondin-1
P68871|HBB	Hemoglobin subunit beta
Q14055|CO9A2	Collagen alpha-2(IX) chain
P51884|LUM	Lumican
P14555|PA2GA	Phospholipase A2, membrane associated
P20849|CO9A1	Collagen alpha-1(IX) chain
P10909|CLUS	Clusterin
O60687|SRPX2	**Sushi repeat-containing protein SRPX2**
P12110|CO6A2	Collagen alpha-2(VI) chain
P61626|LYSC	Lysozyme C
P13942|COBA2	Collagen alpha-2(XI) chain
P07355|ANXA2	**Annexin A2**
Q9H1Z8|AUGN	**Augurin**
O75339-11|CILP1	Cartilage intermediate layer protein 1-1
P01033|TIMP1	**Metalloproteinase inhibitor 1**
P04264|K2C1	**Keratin, type II cytoskeletal 1**
P06733|ENOA	**Alpha-enolase**
P04083|ANXA1	**Annexin A1**
P12107|COBA1	Collagen alpha-1(XI) chain
P69905|HBA	Hemoglobin subunit alpha

305 peptides were identified matched to 40 proteins

Bold lines are the proteins found in MMP-13 and not found in control.