Characterization of Bacteriophage Derived Anti-staphylococcal Protein(P128) From Production to Purification Using Microfluidic based LC System Coupled to an Advanced QTOF-MS

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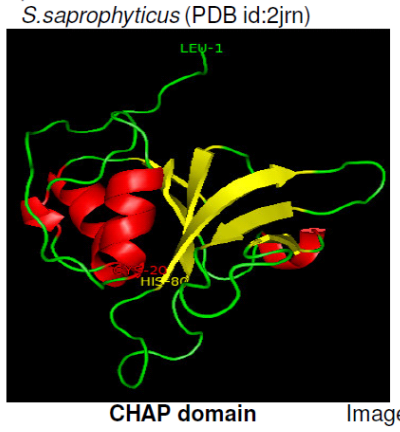
Introduction

P128 is recombinantly expressed in E.coli. P128 has potent bactericidal activity against methycillin resistant Staphylococcus aureus (MRSA) and is currently under early development for use in humans. The Staphylococcal phageK ORF56, a tail associated muralytic enzyme was earlier shown to exhibit anti-Staphylococcal activity. Active portion was found to reside in the C-terminal 16kDa region by deletion analysis of ORF56. To improve the specific activity of ORF56 and to develop it into a potent anti-Staphylococcal molecule for therapy, we combined the muralytic activity of 16kDa ORF56 with a bacterial cell wall binding domain. The chimeric protein termed P128 displayed significantly higher activity than ORF56 and was found to be highly potent against drug resistant Staphylococcus(Figure 1). The P128 was also found to be active in body fluids indicating its potential use as both topical and systemic antibacterial. P128 is a Staphylococcus peptidoglycan hydrolase with an unknown site of action. It is assumed to cleave the peptidoglycan cross bridges at Gly-Gly linkages.

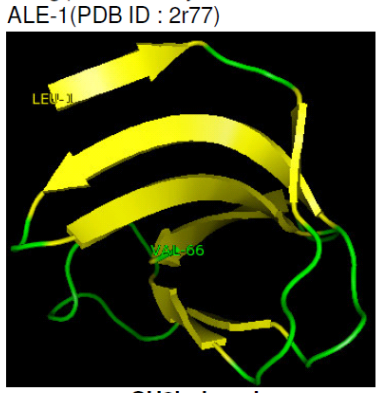
Liquid chromatography/mass spectrometry (LC/MS) technology is a powerful and sensitive technique for characterization and identification of proteins. Chemical modifications and degradation that can potentially occur during manufacturing, formulation, and storage of proteins, necessitate reliable and sensitive methods for the characterization of purity and structural integrity. If suboptimal characteristics of biologics are discovered after the initiation of clinical trials then serious and costly delays can occur in the time to market. Here we present accurate molecular mass measurement, impurity profiling and peptide mapping for P128 which are some of the biophysical characterization for biopharmaceutical selection.

P128 chap domain was modeled using a published NMR structure of CHAP from *S.saprophyticus* (PDB id:2jrn)



CHAP domain

P128-SH3b domain was modeled using published crystal structure of ALE-1(PDB ID : 2r77)



SH3b domain

Image generated using Pymol

Figure 1. Modeled structure of domains of P128

Experimental

Materials and Instrumentation

HPLC-Chip with C-8 and C-18 packing material,6520 QTOF, Chip- Cube and 1200 LC system were from Agilent (Figure2). In-process (Figure 3) and purified protein samples were from GangaGen.

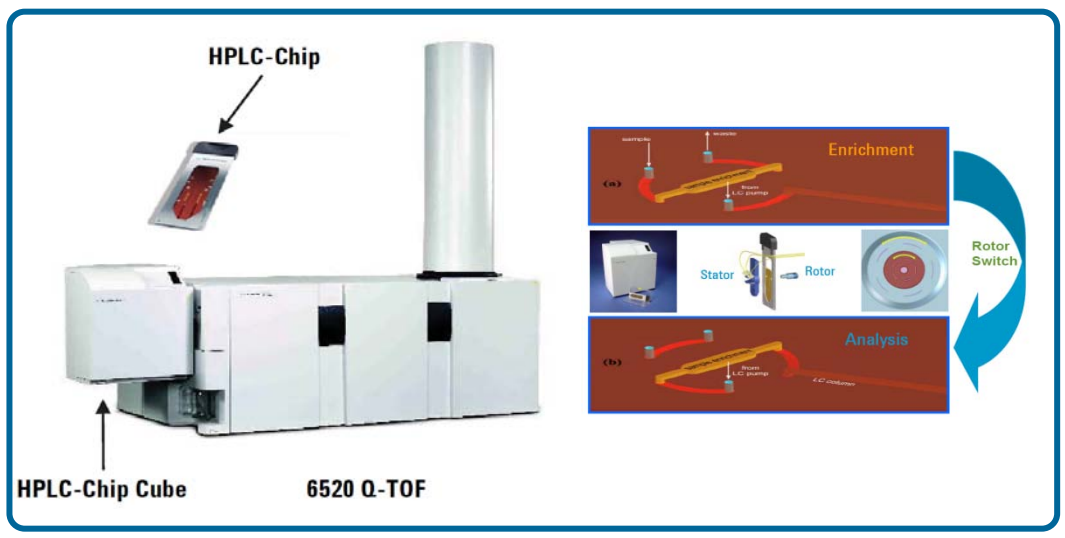


Figure 2. Microfluidic based Chip system coupled to QTOF MS

Data Acquisition: Spectra were recorded in positive ion and in profile mode. Vcap was 1900V and drying gas flow of 5 L/min at 350ºC was used. Data were acquired on extended dynamic (3200 m/z), 2GHz mode. MS only mode was used for in-process samples: range 300-3200m/z. MS/MS scan range was from m/z 50–3,000 at 3 spectra/sec. Precursor selection criteria: active exclusion after 2 spectra for 0.5 min, preferred charges 2, 3, >3, unknown . LC solvents: 0.1% formic acid in water (A); 90% acetonitrile in water with 0.1% formic acid (B).LC gradient :3% B to 95% B in 50 minutes.

Data analysis: Agilent Mass Hunter Qualitative Analysis software with protein deconvolution and large molecular feature extraction (LMFE) was used. Agilent technologies Spectrum Mill was used to assign the MS/MS spectra of the trypsin digested peptides of P128.

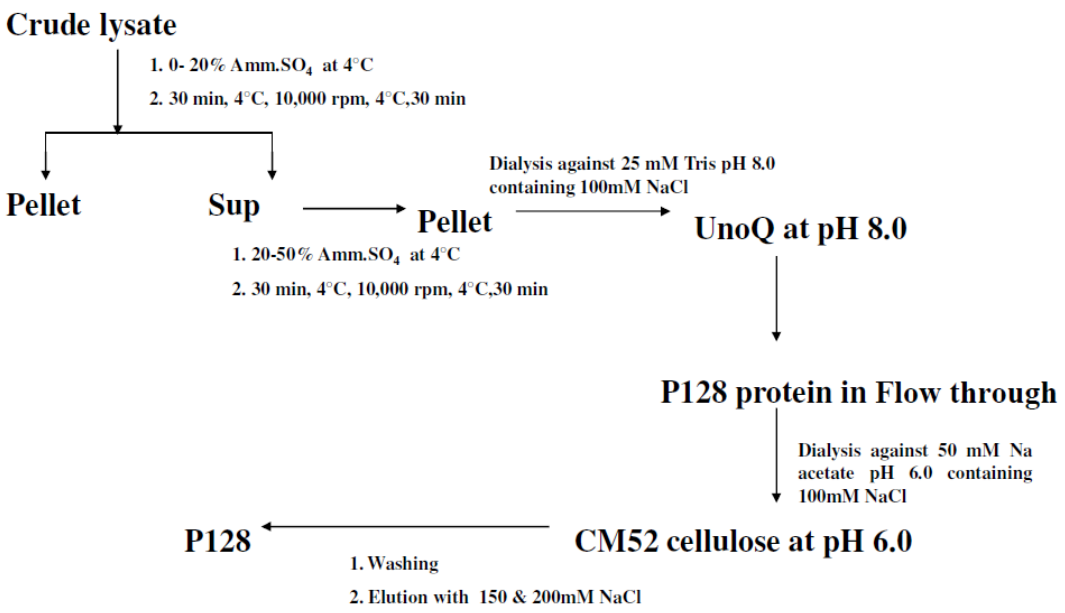


Figure 3. Scheme of purification of P128 from crude cell lysate which was treated with polyethyleneimine(PEI)

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Results and Discussion

In-process sample analysis

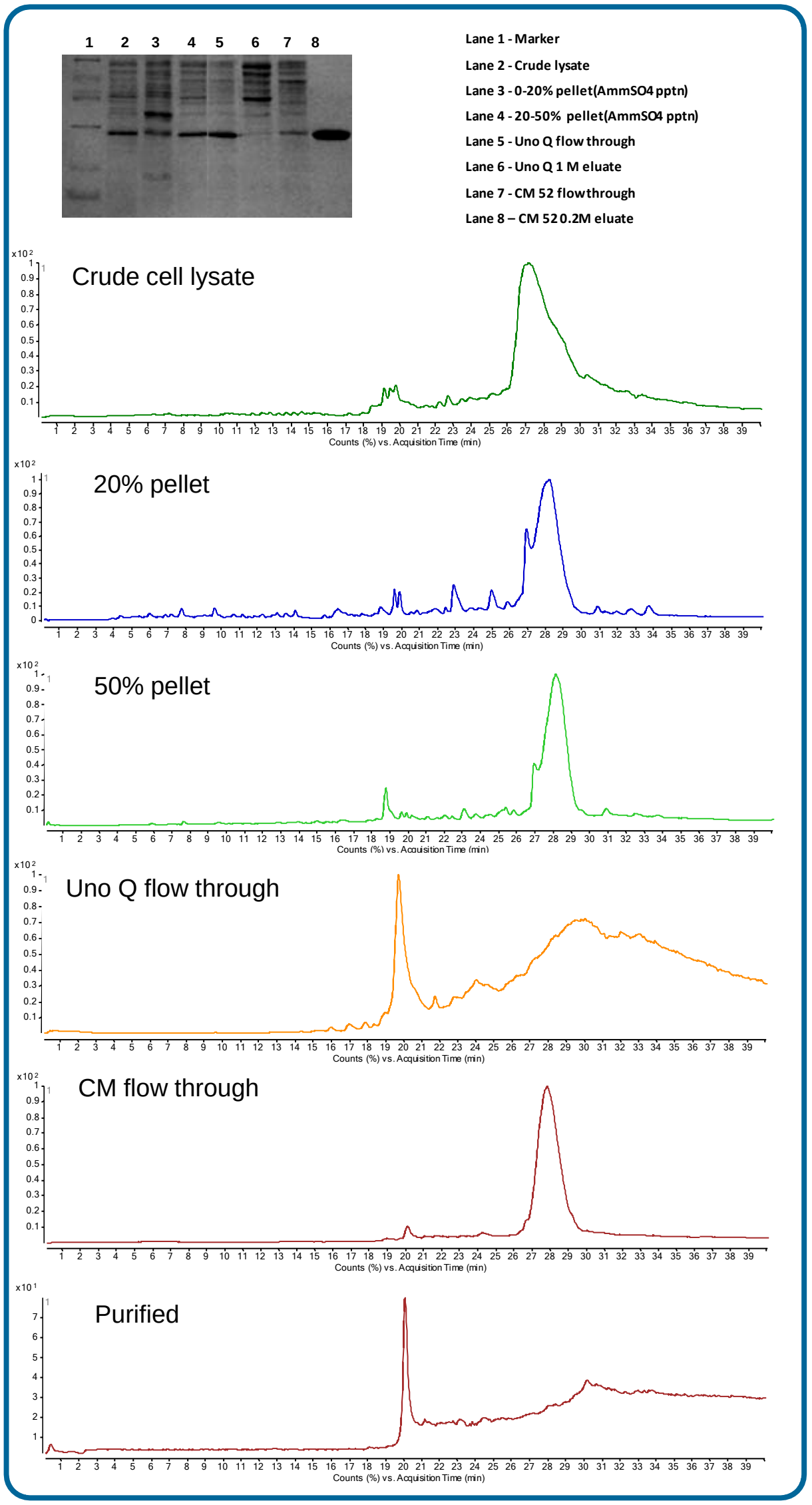

Figure 4. Total ion chromatogram for different in-process samples. Also show at the top SDS-PAGE analysis of these samples.

In-process samples at different stages of P128 purification was analyzed on HPLC Chip coupled to the advanced QTOFMS. The total ion chromatogram of these samples are shown in Figure 4. The broad peak eluting around 27-30 min corresponds to polyethyleneimine(PEI) used to precipitate DNA in the crude cell lysate. The gel analysis does not reveal such details, showing the importance of LCMS analysis. As further steps of purification are continued most of the unwanted proteins as well as other small molecules are removed yielding to enrichment of purified protein. LMFE was used to determine the number of components at each step in the purification. The LMFE algorithm is specifically designed for the analysis of complex mixtures of intact proteins or large oligonucleotides. Data processing with the LMFE algorithm proceeds with exceptional speed and yields substantially more information for complex intact protein mixtures than maximum entropy deconvolution.

The results obtained from using LMFE are tabulated in Table 1. Figure 5 shows the analysis of PEI treated and untreated crude cell lysate.

In-process sample	Number of compounds*
Crude cell lysate	256
20% pellet	199
50% pellet	113
Uno Q flow through	151
CM flow through	60
Purified	6

Table 1. Number of Compounds at different stages of purification from LMFE.* Average of four replicates

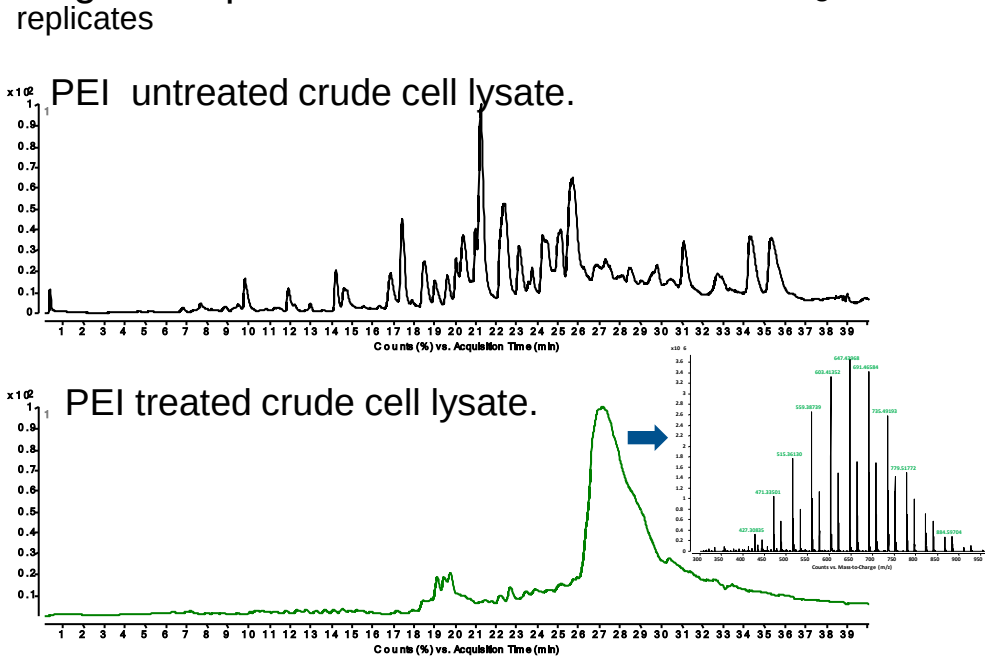

Figure 5. Total ion chromatogram for two differently processed crude cell lysate. The inset shows the spectrum of polyethyleneimine(PEI).

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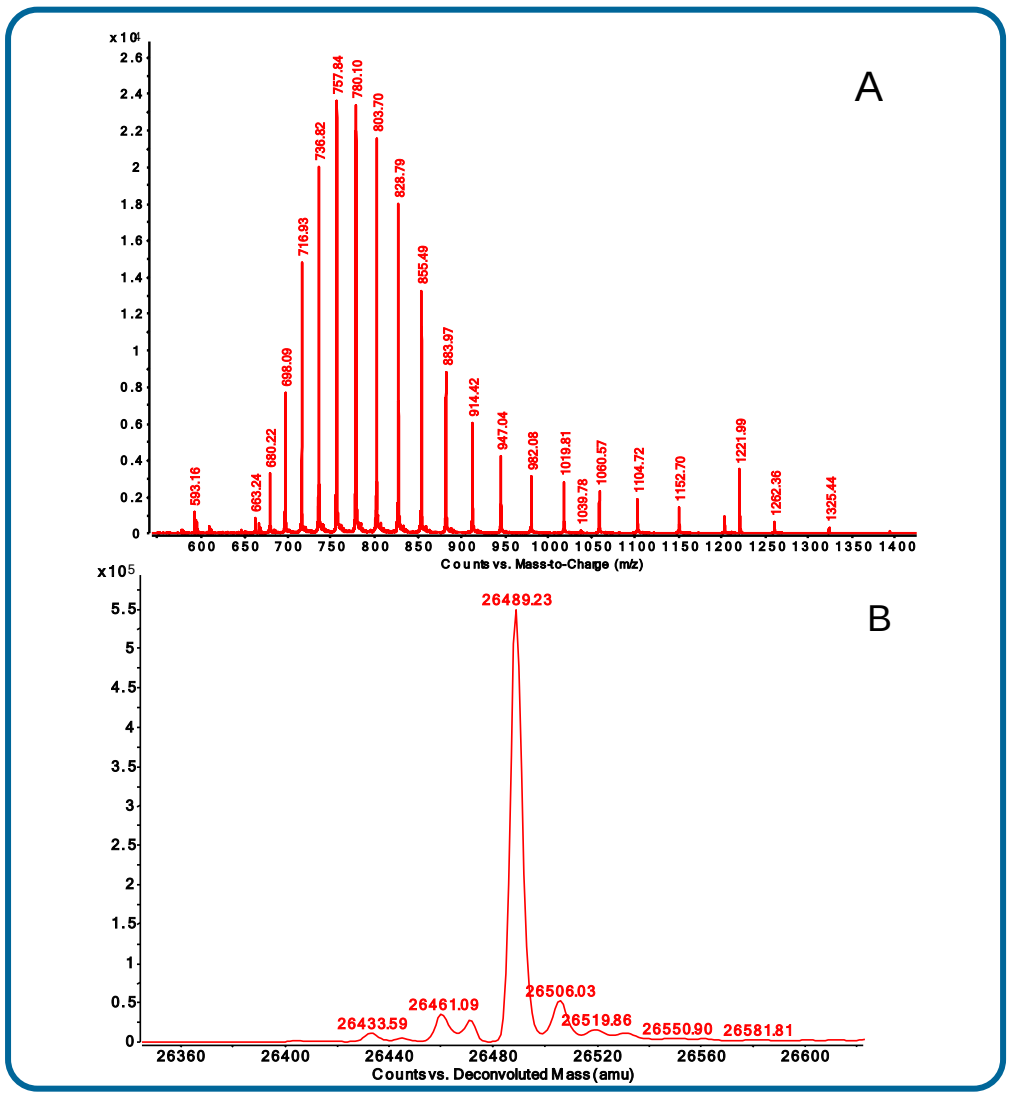
Results and Discussion

P128 Protein Characterization



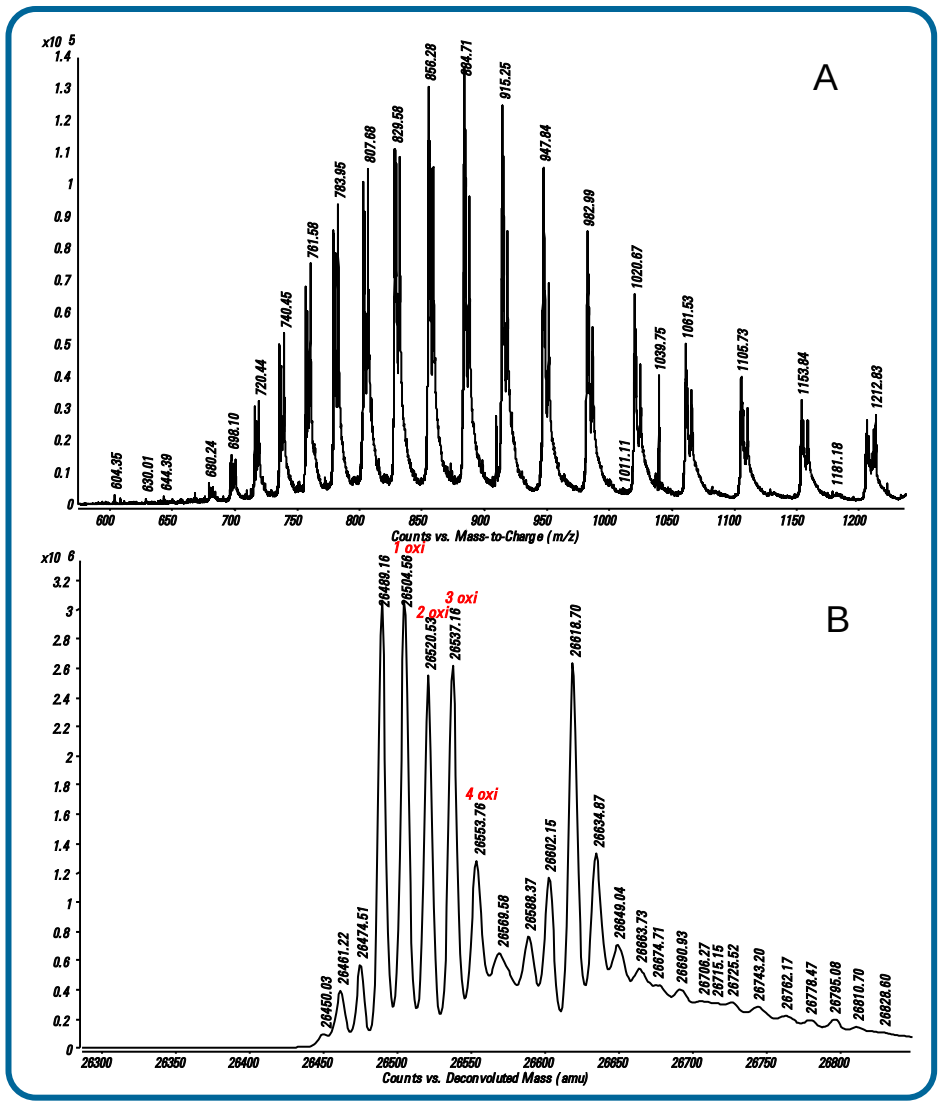


Figure 8. Mass spectrum(A) and deconvoluted spectrum(B) of oxidized P128

Figure 6 shows the measured intact mol wt of P128 to be 26489.23Da while the theoretical mass is 26489.13Da. Therefore the error in mass measurement is around 4ppm. The peptide map generated using trypsin gives 95.85% sequence coverage, the sequence identified from MS only were further validated by MS/MS experiments as shown in Figure 7. The effect of oxidation of P128 is shown in Figure 8 where 4 oxidation species can easily be deduced.

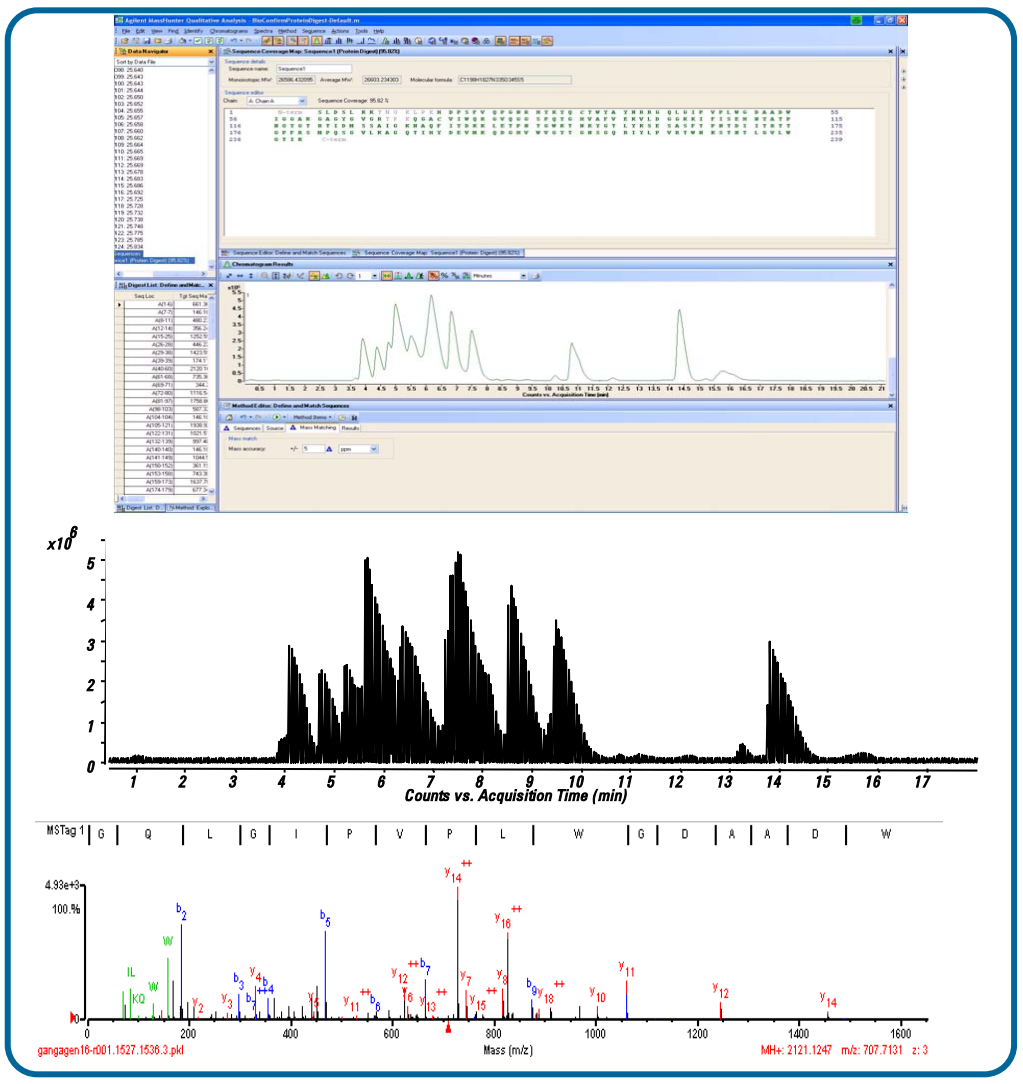


Figure 7. Bioconfirm window showing the peptide map of P128(A). Also shown is the MS/MS analysis of the peptide digest with a representative assigned sequence of peptide from P128(B)

Conclusions

•Micro-fluidic based LC separation coupled with QTOF provides a very convenient and detail way of studying in-process samples as well as for protein characterization. It helps to indentify the additives such as the polyethyleneimine added during purification which are UV inactive as well as not seen on SDS-PAGE.

•Large Molecule Feature Extractor (LMFE) algorithm provides a faster and more information on the complex nature of samples. Hence providing quick analysis of the protein biologics production process saving valuable time in decision making.