Optimization of enzymatic digestion by LC-MS-MS

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Introduction

MNowadays, biomarkers identification and quantification in the plasma are important. Biological characteristics allowed us to detect and prevent a disease. Results with a good sensitivity and selectivity can be obtained with a mass spectrometry with Single Reaction Monitoring (SRM) mode. However the peptide quantity is function of samples preparation based on the digestion. That is why its efficiency must be increased to have a maximum of signal. Thanks to that, many proteins in a wide dynamic range can be detected.

Materials and method

All the products are LC-MS quality to certify the reliability of the results. The SRM mode is used with a precursor ion is selected in Q1. Then, it is fragmented in collision cell. Finally the ions of interest are chosen in Q3.

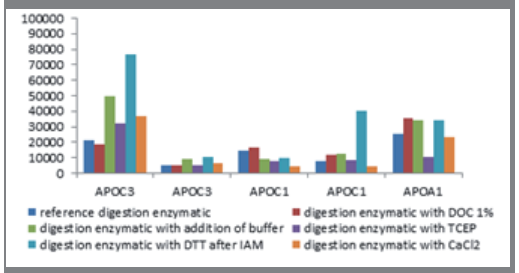
Discussion

A reference digestion enzymatic method with 4 successive stages is used. First of all, the plasmatic proteins were denatured. Thus, they lost their three-dimensional structure after breaking non-peptide bonds by a denaturant agent, the urea. Secondly, the disulfide bonds were reduced with DTT. In the third step, the IAM was added to alkylate proteins, without it, the disulfide bonds could reform. Finally, proteins were digested by the trypsin. This enzyme specifically breaks a kind of peptidic bonds. A shorter amino acid chain, called peptides was obtained.
To optimize all stages, parameters of reference protocol were changed one by one. We could observe their impact on digestion efficiency.
First, a buffer (AMBIC) was added to the plasma. It allowed the pH to be adjusted at 8-9 before starting the different digestion stages. In fact, the primary structure (amino acid chain) arranges in space (tertiary structure) with hydrogen, ionic and disulfide bonds. In the plasma, this structure was stable. The environment change was experiment in order to disorganize the proteins.
Then, the urea denaturant was replaced by the Doc and the DTT reducing agent nature was substituted by the TCEP. The aim was to determine which agent was the most suitable.
DTT was added after alkylation because it allowed the IAM to be eliminated. Indeed, instead of totally consuming, it could alkylated the trypsin too. Its primary structure could be modified and the enzyme lost its specific activity. Thus, the peptidic bonds were not cut.
Finally, the enzymes act in fixing temporarily on the substrates to create a complex which is possible, thanks to a shape compatibility between the two proteins. So, the activity is due to the tertiary structure. The trypsin organization may decrease. Therefore CaCl2 was introduced to maintain the tertiary structure.

To look at the impact of each modified parameters, proteins were identified with mass spectrometry in SRM mode. Two peptides were selected with three transitions for each. After injecting all solutions, the peptides areas (fig1) were compared. To obtain the best enzymatic digestion, the Doc had to be used instead of urea due to the high efficiency.

However DTT and TCEP had the same action. And the increasing of pH allowed the tertiary structure to be disorganized. Then the DTT really eliminated the IAM excess. Finally it was important to use CACl2 to maintain the trypsin. All selected parameters allowed the digestion to be increased.

Figure 1 : Comparison of the effect of different parameters



Conclusion

With this optimization, lower concentration proteins can be detected. It would also be interesting to decrease the analysis time as more patients' plasma could be studied in a shorter time.

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