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APPLICATION NOTE

Digestion Limits of Human Serum Albumin with Trypsin.
Case study of digestion of Human Serum Albumin with StyrosZyme® TPCK-Trypsin Hard Gel Simulated-Monolith™ Enzyme Reactor with the Acquity UPLC *I* class Plus and Final Silica C18 mapping.

Using similar setups as the previous Application Notes for online digestions, we have explored using Human Serum Albumin, to study the limits of automated online digestion of Albumin in comparison with batch digestion.

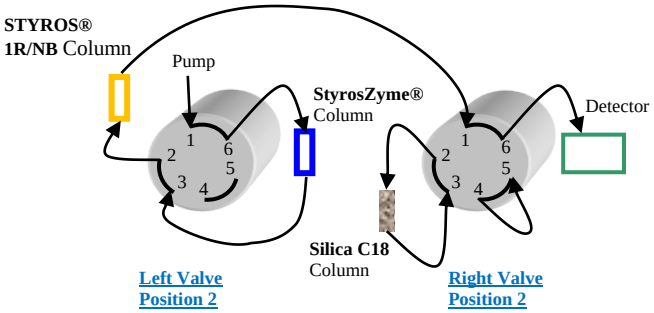
As suggested in the literature

"Extensive digestion of human serum albumin with trypsin at pH 8.8 yields essentially one main fragment which is resistant to further tryptic degradation. The fragment has been characterized by amino acid analysis, N- and C-terminal analyses, cyanogen bromide digestion, electrophoresis, ultracentrifugation and gel filtration, and circular dichroism measurements. The results indicate that the main fragment consists of the amino acids 182 585. Repeated digestion did not degrade the isolated fragment further. The fragment mainly retains the secondary and tertiary structure of intact human serum albumin as well as its capacity to bind bilirubin and diazepam. The localization of the binding sites for these substances is discussed."
Biochimica et Biophysica Acta (BBA) - Protein Structure
Volume 494, Issue 1, 27 September 1977, Pages 61-75

We have shown so far, in Application Notes 142 and 153, that the enzyme column remains stable, and it is not affected during the digestion of the Trypsin itself in solution.
That is now a controlled digestion of the enzyme in solution and not the immobilized enzyme of the enzyme reactor.

In this study, we have used volumetric flow rate of 0.05 ml/min and a normal bore column of 4.6 x 50 mm with a volume of 0.83 ml.
That is a linear flow rate of 18 cm/hr on an empty column to provide the proper conditions for full digestion.

The following shows the starting position of the valves used:



Setup 1

In this first position, the StyrosZyme® enzyme column and the polymeric STYROS® 1R/NB column only, are online and the Silica C18 column is not exposed to any high pH aqueous buffer that is used for the digestion.
It is therefore possible to safely scout the right digestion buffer as well as the optimum conditions for the full digestion prior to the use of the silica column.
Both polymeric columns can tolerate high pH.

The buffer used are as follow:
Buffer A: 0.8 % TFA in H2O: ACN 98:2 (for mapping)
Buffer B: 0.8 % TFA in H2O: ACN 70:30 (for mapping)
Buffer C: 0.1 M CO3HNa, pH = 8.3 (for digestion)

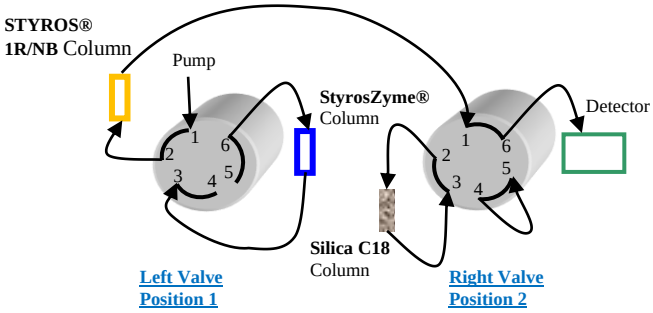
1-Equilibrate the enzyme column with all columns except the silica column, in line, as shown in Setup 1.

Time	% Of buffer C	Flow rate (ml/min)
0	100	0.2
0.01	100	0.2
5	100	0.2

2-With both columns in line as in Setup 1, 0.5 µl of a solution of 10 mg/ml in buffer A is injected, and the resulting digests are dumped on the reversed phase column using the following method:

Time	% Of buffer C	Flow rate (ml/min)
0	100	0
1	100	0.05
8	100	0.05
10	100	0.2

In the second set up, the left valve is switched to position 1 with only the STYROS® 1R reversed phase column online to equilibrate it with the starting gradient solvent.



Setup 2

3-The reversed phase Polymeric column only is now in line as in Setup 2 to wash the leftover salt and prepare it for the reversed phase mapping.

It is also ready for hyphenation with a mass spectrometer.

Time	% of buffer B	% of buffer A	Flow rate (ml/min)
0			0
1	5	95	0.2
10	5	95	0.2

4-The digested peptides are now trapped on the polymeric reversed phase column and can be mapped following a gradient.
The setup is now as Setup 2.

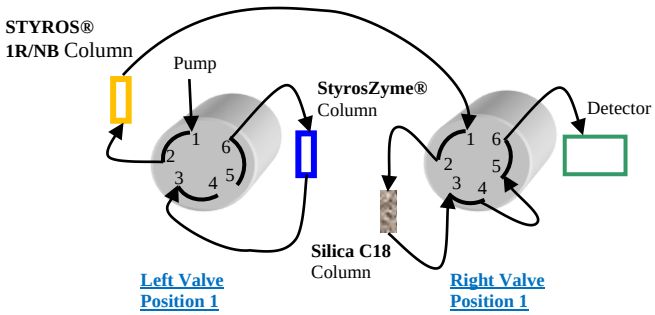
Time	% of buffer B	% of buffer A	Flow rate (ml/min)
0			0
0.1	5	95	0.2
60	60	0	0.2

Once the conditions of the digestion are satisfactory, one can use the second switching valve to bring the Silica C18 column online as shown in Setup 3.

The same gradient as in step 4 can be used for the mapping to run the separation on the C18 Silica column in addition:

4'-The digested peptides trapped on the polymeric reversed phase column can now be mapped on the C18 Silica column in addition.
The setup is now as Setup 3.

Time	% of buffer B	% of buffer A	Flow rate (ml/min)
0			0
0.1	5	95	0.2
60	60	0	0.2



Setup 3

4''-In this step, using the same setup 3, the columns are washed with high organic to make sure they are devoid of any leftover proteinaceous species.

The setup remains Setup 3.

Time	% of buffer B	% of buffer A	Flow rate (ml/min)
0			0
0.01	60	40	0.2
1	100	0	0.2
2	100	0	0.2
4	5	95	0.2
8	5	95	0.2

To re-equilibrate both reversed phase columns that are now in line, the following step 5 is used.

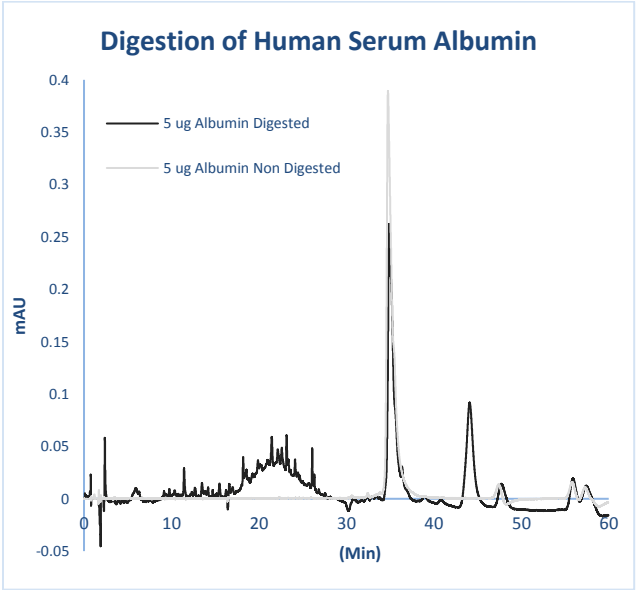
5- Both reversed phase columns are preequilibrated to the initial low organic prior to getting in contact with the digestion buffer.
The setup is now Setup 3.

Time	% of buffer B	% of buffer A	Flow rate ml/min
0			0
0.01	5	95	0.2
10	5	95	0.2

6-In the final step the line and the reversed phase column are flushed clean from any leftover organic going to the enzyme column, still avoiding the Silica column. The setup therefore remains as setup 2.

Time	% of digestion buffer C	Flow rate (ml/min)
0		0
0.01	100	0.2
6	100	0.2

The system is now ready for the next cycle to check the reproducibility of the digestion.
The temperature is set at 37°C for all sequences. The absorbance is monitored by the PDA at 214 nm.



The above overlayed chromatograms compare the partly digested Human Albumin with the non-digested equivalent in accordance with the literature observations.

Hyphenation with a mass spectrometer would shed additional light as to the nature of the generated peptides.

Inc.