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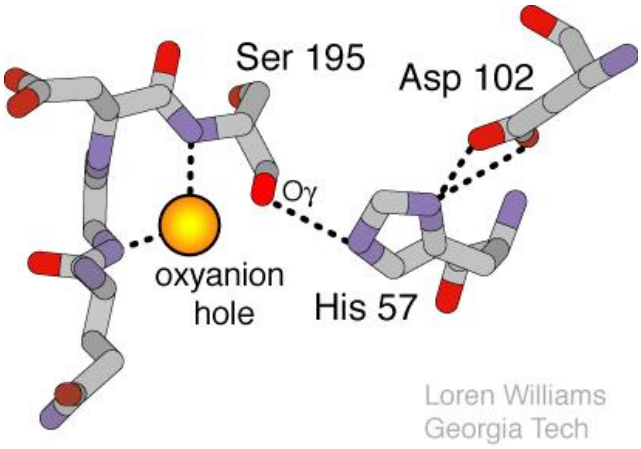
APPLICATION NOTE

Enzyme Digestion in Batch Compared to Digestion Online.

Case Study: StyrosZyme® TPCK-Trypsin, Immobilized Enzyme on Polymeric Hard Gel Simulated-Monolith™ Enzyme Reactor used with the Acquity UPLC *I* class Plus and Final Silica C18 mapping.

Enzyme digestion is usually performed in batch during a tedious and lengthy process using free enzymes.
To circumvent the subsequent cleaning and removal of the leftover, specifically for biopharmaceuticals, one requires to resort to immobilized enzyme to prevent their inclusion at any stage of the process.
This would avoid all ensuing operations in purifying the resulting digests.
This, at present, is a totally unsuccessful operation as witnessed with the recent Covid19 vaccines and the subzero temperature requirements.
The stability and non-leaching of the stationary phase to be used as a support for enzyme immobilization is the initial criteria to be considered.
No stationary phase on the market can satisfy such basic criteria.
The fact that no immobilized enzyme is being used by biomanufacturers is the obvious proof of such assertion.
Then understanding that enzyme digestion also involves high pHs, and only polymerics can tolerate it, that adds an additional barrier to eligibility.
Moving further, we notice that the slow pace of diffusion needs a dramatic update to the fast rhythm of convection offered by monoliths.
However, the shortcomings of monoliths are a major impediment that only Simulated-Monoliths™ can circumvent.
The only logical answer to such scientific and technical challenges is therefore StyrosZyme® series offered by OraChrom Inc. with no sales pitch detected anywhere in the literature.

The use of enzyme is common in biomanufacturing.
The process of digestion in batch, is a slow process in which the so-called “Catalytic Triad” of enzyme among other of chymotrypsin, trypsin, and elastase is required for the enzyme digestion to takes place.
It lowers the bonding energy of the lysing sites to be ruptured and for the digestion process to occur.



In the present application note the enzyme reactor used is a short narrow bore column of 3 cm length and 2.1 mm of diameter. It is run at a volumetric flow rate of 0.2 ml/min that corresponds to over 300 cm/hr of linear flow rate calculated for an empty column.
The actual linear flow rate of the packed column is far higher considering the void volume of the packed column.
Suffice it to say that such a short contact between the immobilized enzyme and the substrate is sufficient to digest the full amount injected
It is not realistic to consider the digestion to resort to any catalytic triad to proceed.
There is, however, room to consider a shear factor to be involved in assisting the breaking of bonds at specific lysing sites that are clearly detected and are reproducible regardless of the amounts of samples injected as substates. The superimposed chromatograms provided in this application note clearly demonstrates this point.

The bigger challenges are no longer scientific, rather purely financial as well as administrative:
How to convince BARDA to approach this simple but decisive issue in getting the manufacturers to rethink

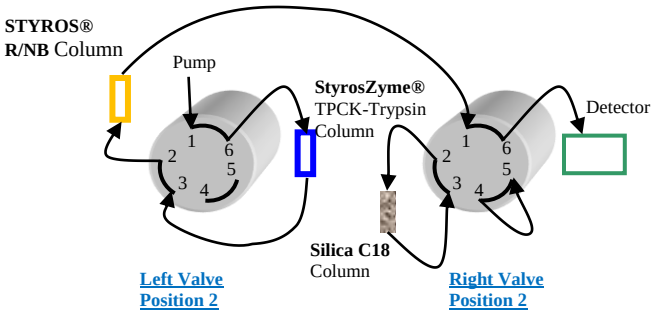
the over 80 % profit earned by using leaching products that cannot be used to immobilize enzyme, or provide purity, the key to circumvent all issues related to contamination.
An issue BARDA is presently tasked with in its Project Bioshield.
The multitudes of application notes provided by OraChrom is to convince BARDA that the answer that the entire world has been hoping for is within our grasp here in the USA.
By successfully using stable polymerics and immobilizing enzymes on them we have demonstrated that indeed it is possible to make every process in which enzymes are used to become a single stage where immobilized enzyme are replacing the batch digestion, no longer requiring costly recombinant enzyme to satisfy the non-animal origin nor require any after and additional purification and re-purification euphemistically called “polishing” to run the purification.

Stable polymeric media is the future of the industry specifically for biopharmaceuticals, where purity is essential.

The enzyme column used here is a narrow bore column of 2.1x30 mm stainless Steel (StyrosZyme® TPCK-Trypsin) to run the digestion.
Two narrow bore reversed phase columns, one polymeric (STYROS® R) with high capacity, high pH tolerance and high performance (2.1x50mm) column as well as a Silica C18 column (Acquity UPLC® BEH C18 1.7 µm 2.1x50mm column) with high performance and similar size.

The buffers used are:
Buffer A: 0.1 % TFA in DI H2O: ACN 98:2 (for peptide mapping)
Buffer B: 0.1 % TFA in ACN: H2O, 70:30 (for peptide mapping)
Buffer C: 0.1 M Tris pH= 8.8 (for digestion).

The UPLC *I* class Plus is fitted with 2 six port valves. The following shows the starting position of the valves used:



Setup 1

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

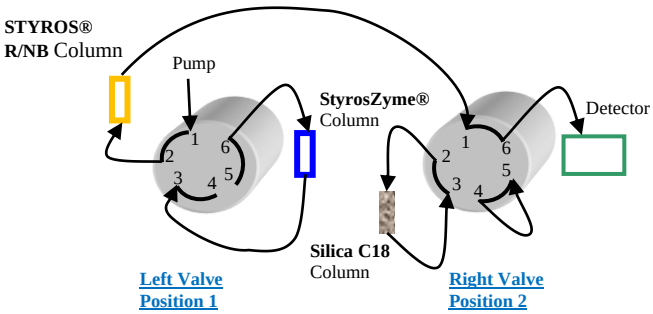
1-Equilibrate the enzyme column with all columns except the silica column, in line, as shown in Setup 1.

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

2-With both columns in line as in Setup 1, 5 and 10 µl of a solution of 10 mg/ml protein in buffer C is injected, and the resulting digests are dumped on the reversed phase polymeric column using the following method:

Time	% Of buffer C	Flow rate (ml/min)
0	100	0.2
7	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 solvent gradient.



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	5	95	0.2
0.01	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.

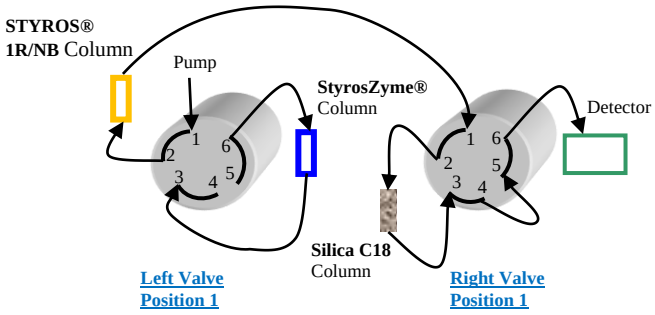
Time	% Of buffer B	% of buffer A	Flow rate (ml/min)
0	5	95	0.2
0.01	5	95	0.2
90	70	30	0.2

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	5	95	0.2
0.01	5	95	0.2
90	70	30	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.

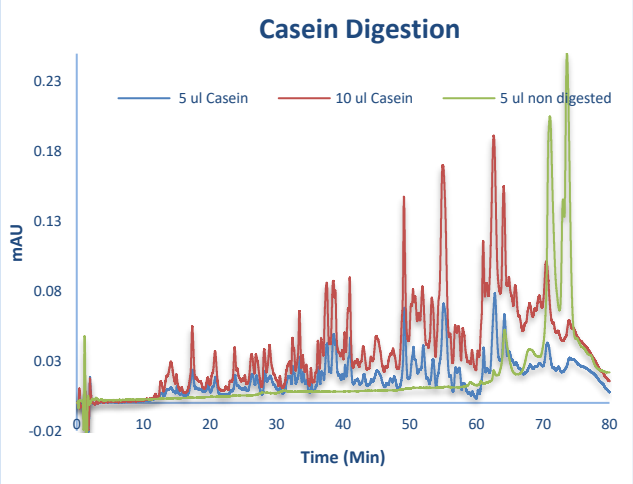
Time	% Of buffer B	% Of buffer A	Flow rate (ml/min)
0	5	95	0.2
0.01	100	0	0.2
4	100	0	0.2
4.1	5	95	0.2
8	5	95	0.2

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	5	95	0.2
0.01	5	95	0.2
5	5	95	0.2

The system is now ready for the next cycle to check the reproducibility of the digestion under the present conditions.
5 and 10 µl of a solution of 10 mg/ml of protein in buffer C is injected as sample to digest.
The temperature is set at 37°C for all sequences and the absorbance is monitored by the PDA at 214 nm.
The digestions are done under similar conditions



The previous Application Note showed the digestion can be carried at different pH's.
The present Application Note clearly shows the reproducibility of the digestion using different loads.
This is a clear assessment of the stability of the enzyme as well as the polymeric Simulated-Monolith™ support on which it is covalently tethered to.
Not only can it be used as a simple way to assess the stability of a candidate media, but it would also be a way to make sure it would not be a source of leaching and contamination avoiding costly additional steps of purifications.
The digestion is complete regardless of the amount of injection compared to the non-digested sample.
The digestion process is by no means random as the ratios of resulting peptides follows similar proportions.