

Poroszyme™ Immobilized Pepsin Cartridge

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WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from thermofisher.com/support.

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Your Poroszyme™ cartridge is unique!

Thermo Scientific™ Poroszyme™ Immobilized Pepsin cartridges perform online peptic digests in a flow-through environment.

Benefits of the flow-through environment include:

- The cartridge can be connected directly to an HPLC or LC/mass spectrometry system for analysis of the digest.
- The degree of digestion can be optimized by changing flow rate or temperature.
- Sample handling is reduced because of the direct connection to the analysis device.

Poroszyme™ Immobilized Pepsin cartridges contain the enzyme attached to POROS™ media (a hydrophilic polystyrene support). Additional advantages provided by using POROS™ media as the support include:

- Autodigestion of the enzyme is minimized, because the enzyme is immobilized instead of free in solution.
- Fast flow rates allow quick, efficient washings.
- The cartridge can be used many times for digestion.

Product description

Poroszyme™ Immobilized Pepsin cartridges are packed with POROS™ media consisting of pepsin covalently bound to POROS™ 20 µm beads. The solid support consists of cross-linked poly(styrene-divinylbenzene) flow-through particles with a patented bimodal pore size distribution for rapid mass transport of substrate to the enzyme immobilized within the pores of the POROS™ media particles.

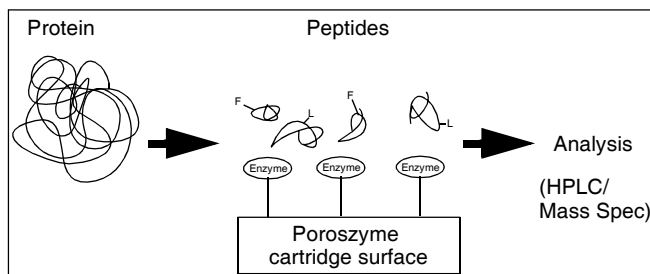


Fig. 1 Poroszyme™ pepsin digestion

Poroszyme™ Pepsin cartridges are designed for protein characterization work. Pepsin [EC 3.4.23.1] cleaves proteins or peptides at the carboxy side of aromatic and hydrophobic amino acids, particularly phenylalanine and leucine. Poroszyme™ Pepsin cartridges allow cleavage of immunoglobulins into (Fab')₂ and Fc fragments.

Materials provided

Poroszyme™ Immobilized Pepsin cartridge packages include the following:

- Packed cartridge (2.1 mmD/30 mL) with sealing end caps
- Product *Operating Instructions*

Materials recommended but not provided

- INTEGRAL™ Micro-Analytical Workstation, BioCad™ workstation or BioCad™ Sprint™ System. If these systems are not available, use an HPLC system with 2-solvent capability, UV detector and injection valve. A system capable of tandem column work greatly facilitates automation.
- Digestion buffer (100 mM sodium acetate, pH 4.5)
- NaOH or acetic acid to adjust the pH of the digestion buffer
- Column heater
- Reversed-phase column, if peptide separation is desired
- Aqueous reversed-phase buffer to equilibrate the reversed-phase column
- Concentrated organic solvent to elute and wash the reversed-phase column

Overview of digestion process

To enhance digestion, protein can be reduced or alkylated.

Digestion on a Poroszyme™ Pepsin cartridge involves:

- **Equilibration**—The Poroszyme™ Pepsin cartridge and the reversed-phase column are equilibrated with the appropriate buffers.
- **Injection**—The protein is injected onto the Poroszyme™ Pepsin cartridge.
- **Digestion**—The protein is digested by cleavage at aromatic and hydrophobic amino acids (phenylalanine, leucine). Flow rate and temperature can be adjusted to control digestion. Effluent from the Poroszyme™ Pepsin cartridge flows onto the reversed-phase column.

After digestion, peptides can be analyzed using reversed-phase HPLC, mass spectrometry, or other analytical method.

General considerations

Temperature and flow rate

Different proteins digest at different rates. Temperature and flow rate are two variables you can modify to control the degree of sample digestion and achieve optimal digestion conditions. Different temperatures and flow rates may generate different size fragments and allow monitoring of intermediate species.

- Temperature—Digestion can be carried out between 25°C and 60°C. However, the recommended temperature range for reproducible digestion is 37 to 60°C. Increase the temperature to increase digestion. Decrease the temperature to decrease digestion.
- Flow rate—Decrease the flow rate to increase digestion (increases the amount of time the protein is in contact with the enzyme). This usually results in enhanced digestions, but longer digestion times. Increase the flow rate to decrease digestion.

You can also stop the flow when the protein solution is inside the cartridge to increase digestion.

The INTEGRAL™ and BioCad™ Workstations include flow rate templates that can help you easily determine the optimum flow rate for your digestion process.

Recovery of hydrophobic peptides

Peptide recovery is enhanced if 5 percent methanol or acetonitrile is added to the digestion buffer. The 5 percent organic reduces binding of hydrophobic peptides to the Poroszyme™ Pepsin cartridge without compromising peptide binding on the reversed-phase column.

Note: If you are not using a reversed-phase column to capture peptide fragments, you can increase acetonitrile or methanol concentration to more than 5 percent. However, higher organic concentration may compromise cartridge lifetime.

Protein treatment to enhance digestion

Proteolytic digestion of most proteins is facilitated by denaturation and removal of disulfide bridges before digestion. “Reductive Alkylation” is a general term that describes a sequence of steps that:

- Denatures the protein
- Reduces disulfide bonds
- Alkylates the free -SH groups to prevent their reassociation.

Reductive Alkylation yields a protein that digests more rapidly, and generates peptide fragments that are free of disulfide bridges.

Note: A reductively alkylated protein may be less soluble in aqueous buffers than it was initially. You may need to add reagents such as urea, or guanidine to keep the protein soluble for digestion in the Poroszyme™ cartridge.

Your Poroszyme™ Pepsin cartridge digests proteins that have been reduced and alkylated with 4-vinyl pyridine, iodoacetamide, or iodoacetic acid. After alkylation, scavenge excess alkylating agent with a molar excess of soluble thiol, such as cysteine or thiomalic acid. This prevents alkylation of primary amines (such as lysine residues).

Typical steps involved in reductive alkylation include:

- Incubation of protein with a 40-fold molar excess of dithiothreitol (DTT) in 3 M urea for 15 minutes at 50°C
- Addition of excess alkylating agent
- Incubation for 15 minutes
- Addition of excess thiol

You can inject the entire mixture of protein and reagents in urea directly onto your Poroszyme™ Pepsin cartridge for digestion.

Each protein can be tested to determine conditions sufficient to completely reduce and alkylate the disulfides without reacting with primary amines.

Automating the process

In traditional methods, reductive alkylation has been a manual procedure performed offline before enzymatic digestion. Digestion has then been carried out in a second procedure, and reversed-phase separation and analysis of peptide fragments in a third procedure.

The INTEGRAL™ Microanalytical Workstation allows you to automate and combine these procedures. Consult your Thermo Fisher Scientific representative for details.

Solvent compatibility

Your Poroszyme™ Pepsin cartridge is compatible with many of the common solvents used to keep proteins in solution including 3 M urea or 2M guanidine hydrochloride.

Note: When using organics in combination with elevated temperatures (>40°C), you will see a gradual loss of enzymatic activity and a shorter cartridge lifetime.

For maximum cartridge lifetime, use the following solvents and conditions when possible:

- Use methanol instead of acetonitrile
- Use urea instead of guanidine hydrochloride
- Keep temperatures below 37°C when using organics

Preparing digestion buffer

1. Dissolve 8.2 g of anhydrous sodium acetate in 800 ml of deionized water.
2. Adjust the solution to pH 4.5 with NaOH or acetic acid as required.
3. Bring the volume up to 1 liter in a graduated cylinder. Filter before use.

Digesting the protein



CAUTION! To preserve the activity of immobilized pepsin cartridges, do not expose the cartridge to solutions with pH greater than 4.5, or to denaturants, detergents, or organic solvents.

Initial cartridge equilibration

The first time you use the Poroszyme™ Pepsin cartridge, wash it with 20 column volumes of digestion buffer to equilibrate and pretreat the cartridge.

Digesting without a reversed-phase column

1. Set the column heater temperature to 37°C (or the optimum temperature for your protein) and allow temperature to equilibrate.
2. Wash and activate the Poroszyme™ Pepsin cartridge with 10 column volumes of digestion buffer at 3 to 5 ml/min.
3. Inject the protein.
4. Starting with a flow rate of 50 µl/min, wash the protein through the cartridge with at least 3 column volumes of the digestion buffer. If your system does not run at low flow rates, you can:
 - Use the lowest flow rate at which your system can run
 - Stop flow with the protein in the cartridge
5. Collect the material from the Poroszyme™ Pepsin cartridge in a polypropylene tube for subsequent analysis.
6. Wash the Poroszyme™ Pepsin cartridge with 10 column volumes of digestion buffer at 3 to 5 ml/min.
7. Repeat step 2 through step 6 for subsequent samples.

Digesting with a reversed-phase column

1. Set the column heater temperature to 37°C (or the optimum temperature for your protein) and allow temperature to equilibrate.
2. Take the reversed-phase column out of line.
3. Wash and activate the Poroszyme™ Pepsin cartridge with 10 column volumes of digestion buffer at 3 to 5 ml/min. Take the cartridge out of line.
4. Place the reversed-phase column inline. Equilibrate with aqueous buffer.
5. Place the Poroszyme™ Pepsin cartridge and the reversed-phase column inline. Inject the protein.
6. Starting with a flow rate of 50 µl/min, wash the protein through the cartridge with at least 3 column volumes of the digestion buffer. If your system does not run at low flow rates, you can:
 - Use the lowest flow rate at which your system can run
 - Stop flow with the protein in the cartridge
7. Take the Poroszyme™ Pepsin cartridge out of line.
8. Elute the peptides from the reversed-phase column with appropriate gradient conditions at 1 ml/min.
9. Wash the reversed-phase column with a high concentration of organic solvent. Take the reversed-phase column out of line.
10. Place the Poroszyme™ Pepsin cartridge inline and wash with 10 column volumes of digestion buffer at 3 to 5 ml/min.
11. Repeat step 2 through step 10 for subsequent samples.

Optimizing Digestion

Analyze the digest to determine whether the desired degree of digestion has occurred. Every protein is different, and you may need to adjust temperature and flow rate to optimize digestion:

Option	Description
To increase digestion	<ul style="list-style-type: none">• Increase temperature• Decrease flow rate or stop flow• Reductively alkylate the protein
To decrease digestion	<ul style="list-style-type: none">• Decrease temperature• Increase flow rate

Testing your cartridge for enzyme activity

You can quantify the amount of enzyme activity remaining on your Poroszyme™ Pepsin cartridge using a synthetic peptide. The peptide, H-phe-gly-his-p-nitro-phe-phe-ala-phe-OMe (BACHEM catalog number H-4540), yields two peptides of four amino acids each when hydrolyzed by Pepsin. The peptides (product) and undigested starting peptide (substrate) can be separated on a reversed-phase column.

To test the amount of enzyme activity:

1. Dissolve the H-phe-gly-his-p-nitro-phe-phe-ala-phe-OMe in a small volume of 30 percent acetic acid.
2. Dilute it to a working solution of 1 mg/ml in digestion buffer.
3. Connect your Poroszyme™ Pepsin cartridge and reversed-phase column to your HPLC system.
4. Configure digestion buffer as the mobile phase and set the UV detector to 214 nm and 280 nm.
5. Start flow of the peptide substrate at 0.2 ml/min flowing through the Pepsin cartridge and the reversed-phase column.
6. Take the Pepsin cartridge offline and elute the peptide from the reversed-phase column in a gradient of 5 percent to 70 percent organic solvent.

The smaller and less hydrophobic peptide (product) fragments elute before the undigested starting peptide (substrate).

It is good practice to check the enzyme activity of your cartridge when it is new, and periodically check activity over time. Compare subsequent peak areas to the original peak area to determine the relative amount of enzyme activity remaining on the cartridge.

Storing the cartridge

Short-term storage

Store the cartridge at 4°C in digestion buffer when not in use.

Store the cartridge with the endcaps in place, carefully sealed to prevent drying.

Long-term storage

Store the cartridge in 0.02 percent sodium azide as a preservative.



CAUTION! Sodium azide is toxic. Follow precautions and decontamination procedures recommended by the National Institute for Occupational Safety and Health.

Accessories, spare parts, and ordering information

These accessories are available for your Poroszyme™ columns:

Table 1 Poroszyme™ cartridge accessories

Description	Quantity	Part Number
Poroszyme™ Immobilized Pepsin Cartridge, 2.1 mmD/30 mmL	1	2-3131-00
Poroszyme™ Immobilized Pepsin Bulk Media	0.1 ml	2-3132-00
POROS™ R2 Column for Reversed-Phase Chromatography 2.1 mmD/100 mmL PEEK	1	1-1112-16
Frits, PEEK, 2.1mmD	Package of 5	1-9124-05
EZ™ Grip Fittings (SS)	Package of 5	5-1011-05
Fitting Adaptor Kit ^[1]	1	1-9532-00

^[1] The Fitting Adaptor Kit lets you connect POROS™ columns to M-6 (FPLC™) and 1/4-28 low pressure fitting systems. Includes two 10-32 fittings, two low pressure ferrules, two M-6 nuts, two 1/4-28 nuts and 1/16-inch OD PEEK tubing. Kit is included with all F- and P-Series columns.

Support

For service and technical support, go to thermofisher.com/poros or call toll-free in US: 1.800.831.6844.

For the latest service and support information at all locations, or to obtain Certificates of Analysis or Safety Data Sheets (SDSs; also known as MSDSs), go to thermofisher.com/support, or contact your local Thermo Fisher Scientific representative.

Limited product warranty

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Revision	Date	Description
B	27 March 2017	Baseline for this revision history.

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