Protein Digests: a Comparative Analysis

Introduction

Separating protein digests by reversed-phase HPLC is a standard method for the analysis of proteins in research and in developing a "Well Characterized Biotechnology Pharmaceutical." The selectivity for various closely related peptides depends on the bonded phase and the silica more than the pore size. The claim that one pore size is always better for digests is simplistic. To test this claim, lactoperoxidase (MW = 85,000) was digested by trypsin and analyzed by reversed-phase chromatography on three different C18 columns: Vydac 218TP54, YMC A-303 and Zorbax 300SB-C18. A very shallow gradient was used to maximize the resolution of peaks.

Materials and Methods

Lactoperoxidase was dissolved at 1 mg/mL in 50 mM Tris, pH 8.0, 10 mM dithiothreitol. Trypsin was dissolved at 1 mg/mL in 1 mM HCl. Trypsin is stable in 1 mM HCl but not active. This allows the use of microliter pipettes to add accurate amounts of trypsin to reaction mixtures.

The time course of digestion was studied by analysis on a short 50mm-long Vydac 218TP5405 column (C18, 5µm, 4.6 mm ID x 50 mm L). The comparison of separations was performed on three columns, all 250mm long.

An EZChrom™ Chromatography Data System, Version 6.6 (Scientific Software Inc., San Ramon, CA) was used to collect data and integrate the peaks.

Results

Figure 1 is an overlay of chromatograms from the short 218TP5405 column (4.6 mm ID x 50 mm L) used to follow the kinetics of the digestion. Note the decrease in size of the lactoperoxidase peak (at 24 minutes) and the increasing size of earlier peptide peaks with time. This figure illustrates the utility of a short reversed phase column for rapid peptide chromatography.

Figure 2 is an overlay of the digest analyses on Vydac, Zorbax and YMC C18 reversed-phase columns. The large number of peaks was difficult to analyze subjectively, so comparisons were done objectively by automated analysis using the EZChrom software. The chromatograms were integrated with four different methods, varying the values for the threshold (T), peak width (W) and minimum area (Min). Valley-to-valley (v/v) was used to determine the baseline for all integration methods. The following four value-sets were used with the data system to calculate the number of peaks: T = 100, W = 1, Min = 20,000; T = 200, W = 1, Min = 10,000; T = 200, W = 2, Min = 10,000; and T = 100, W = 1, Min = 10,000. The results of these integrations are summarized in Table 1.

Conclusion

The data in Table 1 shows that smaller pores do not give more resolution of the small peptides generated by trypsin digests. The pore size relates to surface area and carbon load, which correlate directly with retention time but not resolution. The resolution on Vydac 218TP54 is the result of a quality synthetic silica and a unique polymeric bonding chemistry.

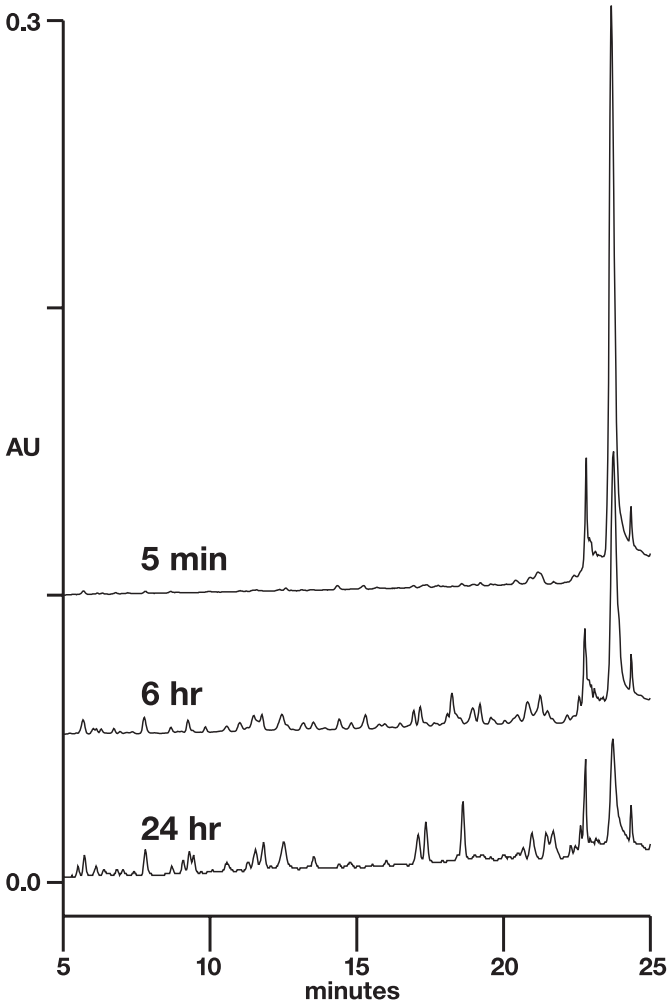

Figure 1. Kinetics of lactoperoxidase digestion by trypsin . An autosampler was used to follow the time course by injecting samples at various times after addition of a 1:50 mass ratio of trypsin to the lactoperoxidase solution. The lactoperoxidase peak (retention time = 24 min) can be seen to decrease in size and the earlier peptide peaks to increase with digestion time. Column: Vydac 218TP5405 (C18, 5µm, 4.6 mm ID x 50 mm L). Conditions: 3 mL/min. 215 nm. Gradient from 0 to 30% ACN over 20 min. then 30 to 90% ACN in 5 min. A uniform concentration of TFA, 0.1% (w/v), was present in the mobile phase throughout the gradient.

The Nest Group, Inc. • 45 Valley Road • Southborough, MA 01772-1306 • USA
Tel: 800-347-6378 or 508-481-6223 • Fax: 508-485-5736 • Email: info@nestgrp.com

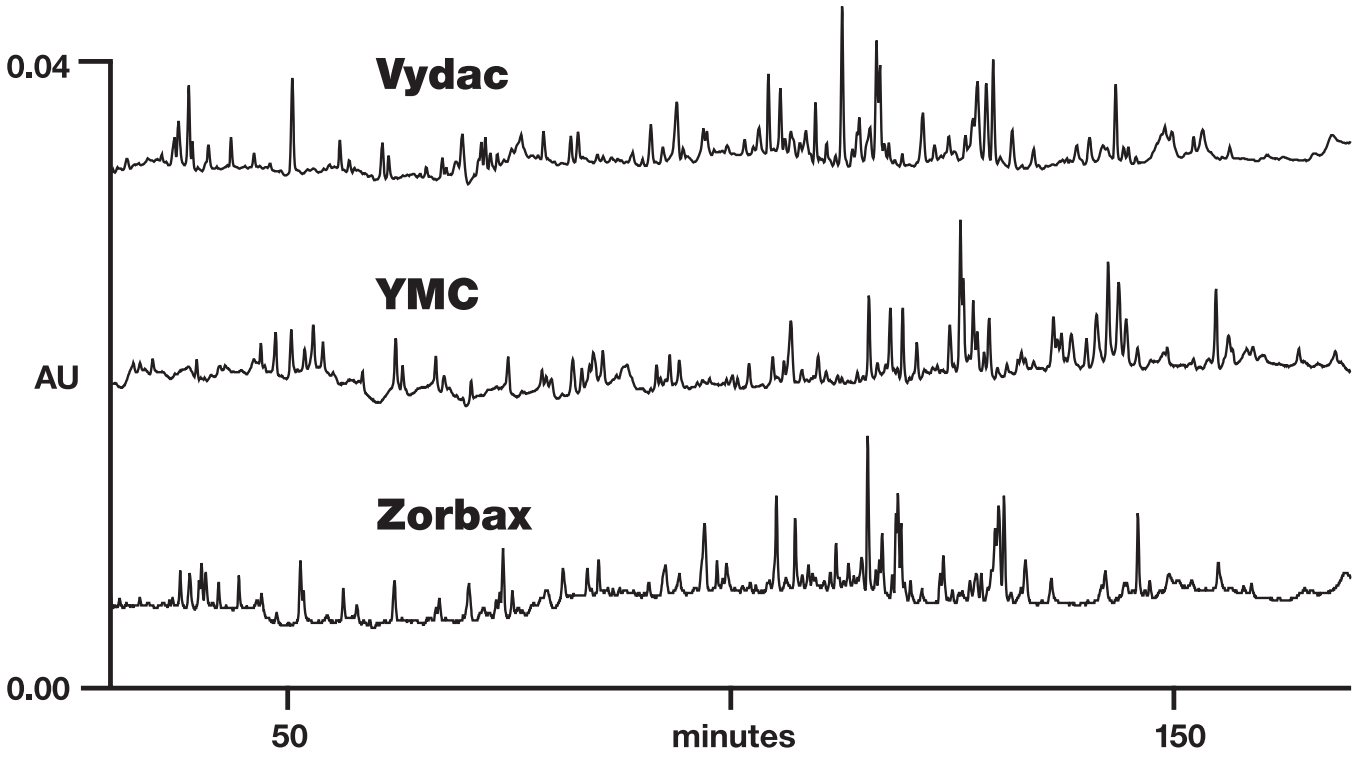

Figure 2. Comparison of three different C18 columns for separation of trypsin digest of lactoperoxidase. The digest was diluted 1:1 with 0.4% TFA in water and 50 µL was injected on each column. Conditions: 1mL/min. 215 nm. Column temperature controlled at 30° C. Gradient from 0 to 50% ACN in 200 minutes. TFA concentration 0.1% (w/v) throughout. Columns: Vydac 218TP54 (S/N E960425-9-1 #20), YMC A-303 (S/N 4255461) and Zorbax 300SB (Part # 880995.902, S/N HH1179) column. All three columns are C18 reversed-phase with 5µm spherical particles and 4.6 mm ID x 250 mm L. The Vydac 218TP has 300Å pores. The YMC A-303 has 120Å pores, and Zorbax 300SB has 300Å pores.

Table 1:

C18 Column	Pore Size	Number of Peaks
Vydac 218TP54	300Å	63, 107, 102, 105; Avg = 94
YMC A303	120Å	61, 98, 100, 98; Avg = 89
Zorbax SB300	300Å	59, 97, 92, 91; Avg = 85

ORDERING INFORMATION:

Vydac Cat. #	Description
218TP54	C18 Reversed-Phase Column. 5µm, 300Å, 4.6mmID x 250mmL
218TP5405	C18 Reversed-Phase Column. 5µm, 300Å, 4.6mmID x 50mmL

Larger columns are also available.

To place an order, contact The Nest Group toll-free at 800-347-6378.

Vydac began producing 300Å synthetic silica from purified organic silicates over 20 years ago. Before this invention, HPLC-quality silica was either mined (diatomaceous earth) or made by acidifying silica sols. These earlier methods do not yield the same consistent high quality silica matrix as Vydac's synthetic silica process. That is why many HPLC companies are now turning to this newer silica technology.

The high resolution for peptides and proteins provided by Vydac reversed-phase columns is due not only to the high purity silica matrix but also to Vydac's "polymeric" bonding chemistry. Vydac 218TP silica is made with a multifunctional silane that bonds the C18 chains three ways: 1) C18 bonded to one silanol; 2) C18 bonded to two silanols; and 3) C18 bonded to another C18 at the silane moiety. This type of bonding chemistry results in a unique hydrophobic surface with some polymer-like characteristics. This polymer-like surface affects selectivity and increases column stability. It is the combination of high quality synthetic silica and multifunctional bonding that gives higher resolution.