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Separation of intact proteins on porous layer open tubular (PLOT) columns

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ABSTRACT

Porous layer open tubular (PLOT) polystyrene divinylbenzene columns have been used for separating intact proteins with gradient elution. The 10 μm I.D. × 3 m columns were easily coupled to standard liquid chromatography–mass spectrometry (LC–MS) instrumentation with commercially available fittings. Standard proteins separated on PLOT columns appeared as narrow and symmetrical peaks with good resolution. Average peak width increased linearly with gradient time (t_G) from 0.14 to 0.33 min (t_G 20 and 120 min, respectively) using a 3 m column. With shorter columns, peak widths were larger and increased more steeply with gradient time. Theoretical peak capacity (n_c) increased with column length (tested up to 3 m). The n_c increased with t_G until a plateau was reached. The highest peak capacity achieved (n_c = 185) was obtained with a 3 m column, where a plateau was reached with t_G 90 min. The within- and between column retention time repeatabilities were below 0.6% and below 2.5% (relative standard deviation, RSD), respectively. The carry-over following injection of 0.5 ng per protein was less than 1.1%. The retention time dependence on column temperature was investigated in the range 20–50 °C. Proteins in a skimmed milk sample were separated using the method.

1. Introduction

The "bottom-up" procedure [1] in LC-based proteomics is often preferred compared to the "top-down" [2] procedure, since peptides in general are easier to separate, ionize and fragment than proteins. If the separation and detection systems were more appropriate for proteins, on the other hand, the top-down approach would undoubtedly be a larger benefit to proteomics. Protein separations are at present mostly performed with one or two-dimensional (2D) gel electrophoresis (GE) [3]. Although 2D GE is a highly resolving technique, it requires significant amounts of manual handling, is prone to smearing, and is often a challenging technique for separating membrane proteins [4]. Liquid chromatographic separation of intact proteins with conventional packed columns is problematic, often associated with adsorption, broad peaks and carry-over [5].

An alternative to packed columns may be PLOT columns, which has been shown to provide a higher column efficiency compared to conventional packed columns [6]. Open tubular columns for LC use were first investigated by Tsuda et al. in 1978, using an I.D. of 60 μm [7]. Jorgenson and Guthrie [8] published the first report on open tubular columns with an inner diameter close to what is theoretically required for efficiency similar to packed columns (~15 μm). Although PLOT columns have been shown to produce over one million theoretical plates [6], the use of narrow PLOT columns has been problematic due to difficulties with dead volumes, high pressures, and detector couplings. Some of these problems are less present when using PLOT columns in capillary electrochromatography (CEC), and Eeltink et al. reported column efficiencies up to 400,000 plates/m for alkylbenzenes with otherwise commercially available CEC instrumentation [9]. PLOT-CEC with UV detection has also been used for separating peptides and proteins, but no efficiency was reported [10].

PLOT columns for LC have gained renewed interest after the coupling to nanospray-MS proved to be simple and efficient [11]. Recently, 10 μm I.D. PLOT polystyrene divinylbenzene (PS-DVB) columns have been designed and used for high resolution, ultra-trace LC–MS separations of peptides [12,13].

In the present study, we have examined the potential of PS-DVB PLOT columns for separating intact proteins. Carry-over, resolution and the repeatability of retention time, peak width and shape on the column(s) were examined. The effect of column length and gradient time on peak width was also investigated, as was the temperature as a separation parameter. The PLOT column LC–MS system was also applied for separation of proteins in skimmed milk.

2. Materials and methods

2.1. Materials and chemicals

All common solvents and additives were of analytical or HPLC grade. Divinylbenzene (DVB) 80% mixture of isomers, styrene (99%),

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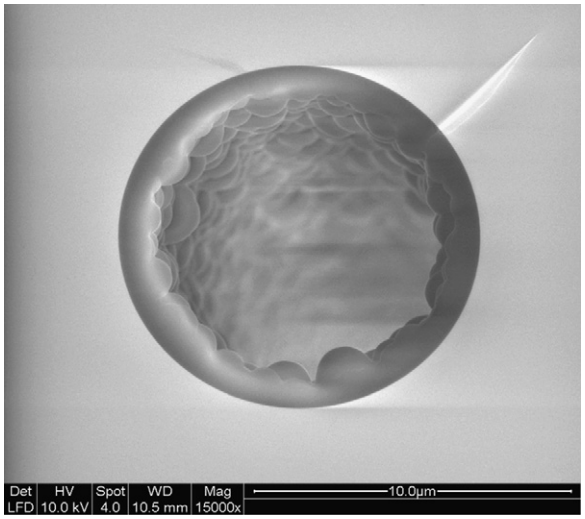

Fig. 1. SEM image of PLOT column used for separation of intact proteins.

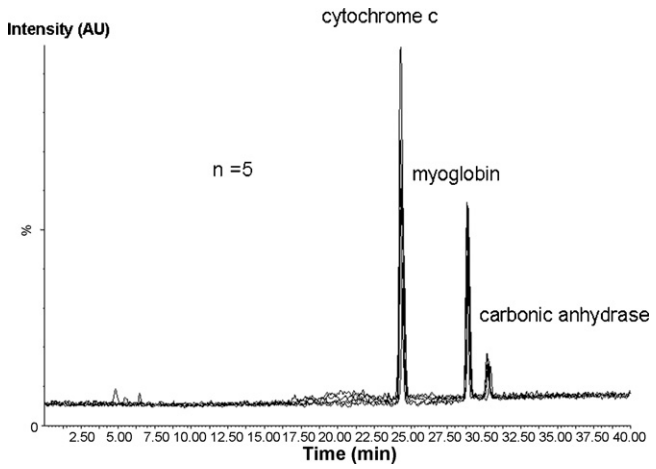

Fig. 2. Overlay of 5 consecutive separations of three standard proteins with PLOT-nanospray-MS. The solvent gradient was 90% A (0.1% FA, 0.05% TFA (*v*/*v*) in water) to 90% B (0.1% FA, 0.05% TFA, 10% water (*v*/*v*) in ACN) in 40 min. The concentration of each protein was 33 μg/mL. The column was 3 m long.

inhibitor 2,2-diphenyl-1-picrylhydrazyl (DPPH) and azobisisobutyronitrile (AIBN) were purchased from Sigma Aldrich (Milwaukee, WI). Polyimide coated fused silica tubing (360 μm O.D., 10 μm I.D.) was purchased from Polymicro Technologies (Phoenix, AZ).

Carbonic anhydrase, myoglobin, cytochrome C, β-lactoglobulin A, and β-lactoglobulin B were purchased from Sigma Aldrich.

2.2. Preparation of PLOT columns

The columns were prepared as described by Yue et al. [12], with only a few insignificant practical deviations. The column lengths were typically 3.0 m, and the inner diameter was 10 μm. A scanning electron microscope (SEM) image of one of the prepared columns is shown in Fig. 1.

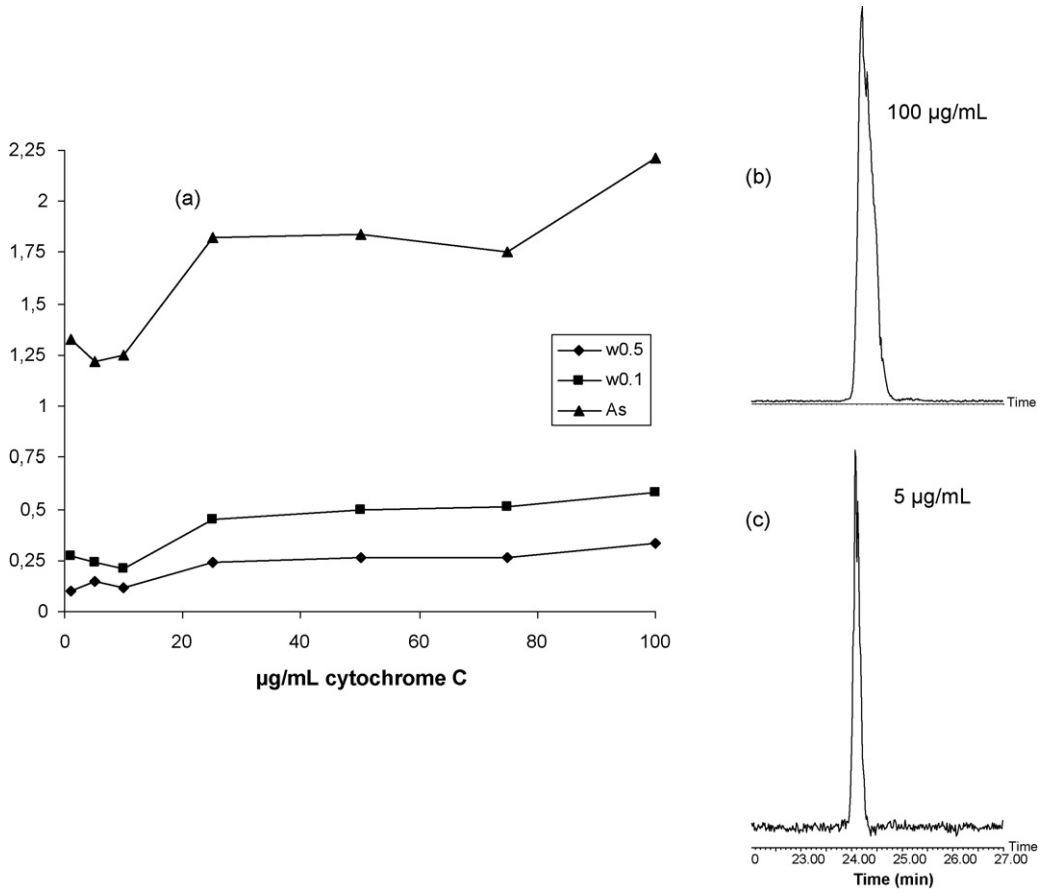

Fig. 3. (a) Plot of $w_{0.1}$, $w_{0.5}$ (min) and tailing factor A_s (10%) versus injected amount of cytochrome *c* on a 10 μm I.D. PLOT column. Conditions were as in Fig. 2. (b) Chromatogram of 100 μg/mL solution (intensity = 8.3×10^3 Arbitrary Units (AU). (c) Chromatogram of 5 μg/mL solution (intensity = 1.2×10^3 AU). The chromatograms were normalized for easier comparison.