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Notes

Purification of tryptic peptides for mass spectrometry using polyvinylidene fluoride membrane

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A simple procedure for the purification of tryptic peptides, prior to mass spectrometric analysis, using polyvinylidene fluoride membrane (PVDF) is described. The sensitivity of mass spectrometric analysis is such that minor impurities in tryptic peptide digests suppress the signal obtained. However, we obtained useful signal, from a sample that did not yield any spectra earlier, by purifying the sample using PVDF membrane. For this, the tryptic peptide digest was first spotted on the membrane which was then air-dried and washed. Further, the membrane was extracted with trifluoroacetic acid (TFA) and acetonitrile and subjected to mass spectrometric analysis. This procedure enabled us to identify a cross-reactive D1 antigen on the neutrophil surface that bound antibodies that targeted 60 kD Ro autoantigen in systemic lupus erythematosus, an autoimmune disorder.

Mass spectrometry (MS) has become a fundamental analytical technique for the routine analysis of proteins, peptides, nucleic acids and polysaccharides[1-6]. Proteins analyzed on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) have been excised and subjected to in-gel tryptic digestion and mass spectrometric analysis[7-15]. The sensitivity of MS is such that even subpicomolar levels can be detected, making this procedure vulnerable to the slightest contamination. We have experienced difficulties in obtaining good signals using this procedure owing to sample contamination. Earlier reports have shown severe signal suppression due to residual acrylamide monomer[16], trifluoroacetic acid[17] (TFA) and non-ionic detergents[18]. An automated on-line ionic detergent pre-column system has been described for the removal of SDS contained in trypsin-digested protein samples[19]. However, no simple method has been reported, so far, for the removal of other contaminants. Here we report a quick and simple method, using polyvinylidene membrane, to purify tryptic peptides prior to mass spectrometric analysis when little or no signal can be obtained.

We observed that anti-60 kD Ro[20,21] autoantibodies from patients with an autoimmune disorder, systemic lupus erythematosus, bound to intact normal neutrophils in an *in vitro* fluorescence activated cell sorter assay[22]. In an effort to identify the antigen on the intact neutrophil membrane surface responsible for binding this antibody, we purified human neutrophil membranes by nitrogen cavitation[23]. The purified neutrophil membranes were solubilized with a detergent in the presence of protease inhibitors and further, passed over an anti-60 kD Ro affinity chromatography column[22]. The eluted protein sample was subjected to SDS-PAGE[24] and the electrophoresed proteins were transferred to nitrocellulose non-electrophoretically[25]. The proteins remaining in the gel after the non-electrophoretic transfer were stained with Coomassie brilliant blue. Coomassie stained candidate bands were excised and subjected to in-gel digestion with trypsin[22]. Following tryptic digestion, 150 µl of 60% acetonitrile and 0.1% TFA were added to the gel slices and the mixture shaken for 15 min at room temperature. The supernatant was saved and the procedure was repeated twice. The combined supernatants were dried using a Speed-Vac Concentrator (Savant Instruments Inc, Farmingdale, New York, USA) and the resulting pellets were suspended in 20 µl of 70% acetonitrile containing 5% TFA. A 0.5 µl aliquot was applied to a grid and to it, 0.5 µl of ferrulic acid was added. The sample was air-dried and then subjected to mass spectrometric analysis.

The spectra was obtained using Matrix Assisted Laser Desorption/Ionization Time of Flight (MALDI-TOF) mass spectrometer, Voyager Elite Biospectrometry

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Research Station (Serial No. 130), equipped with a delayed extraction option (PerSeptive Biosystems, Framingham, MA, USA) operated at the accelerating voltage, 20 kV; grid voltage, 75%; guide wire voltage, 0.1%; and pulse delay time, 250 ns. A pulsed nitrogen laser emitting at 337 nm was used as a desorption/ionization source. Mass spectrometry was performed in a reflector with positive ion detection. The ion signal was recorded using a 500 MHz transient digitizer. The data were analyzed using GRAMS/386 (Galactic Industries Corp., Salem, NH, USA).

It was frustrating, however, when no signals were obtained and since we were desperate to obtain useful data from limited sample amounts, it was decided to purify this sample further. Nitrocellulose and polyvinylidene fluoride (PVDF) membranes were chosen for the purification procedure. Nitrocellulose has been shown to bind to proteins[26] and peptides[27]. PVDF membranes have also been reported to have very good protein[28] and peptide binding properties[18,29,31].

A piece of nitrocellulose and PVDF membranes were cut and placed on top of Whatman # 3 filter papers cut slightly larger than the size of the membranes. A 5 µl aliquot of the 70% acetonitrile/ 5% TFA sample (obtained after extraction of trypsin-digested gel slices) was spotted on each of the membrane surfaces and the membranes were air-dried for 30 min at room temperature. The dried spot, in each case, was cut and placed in a microcentrifuge tube containing 1 ml of NANO pure water and shaken gently for 15 min. The water was decanted and this procedure repeated once more. Then 1 ml of NANO pure water was added to the tube, vortexed for one min full speed on a Super-Mixer (Curtin Matheson Scientific, USA) and water was decanted. This was repeated once more. Then the PVDF and the nitrocellulose membrane spots were extracted separately, with acetonitrile and TFA, as described earlier for the gel slices. The combined supernatants, in each case, were dried using the Speed-Vac and the pellets were dissolved in 20 µl of 70% acetonitrile. A 0.5 µl of the sample was then subjected to mass spectrometric analysis as before.

Fig. 1 shows part of the spectra for the tryptic peptides obtained after purification with the PVDF membrane. Useful signal was not obtained from this sample prior to purification with the PVDF membrane. The sample obtained after purification using the nitrocellulose did not produce any spectra. In fact, nitrocellulose was not compatible with the organic solvents used in the extraction procedure.

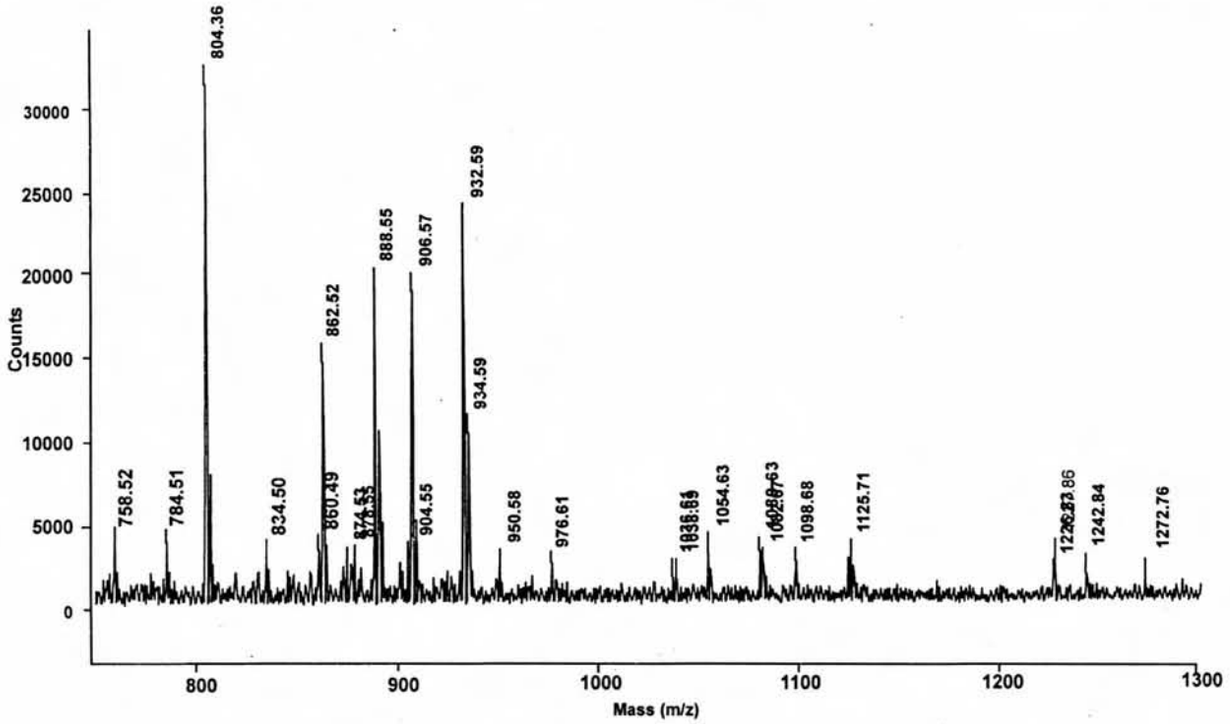

Fig. 1—Mass spectrometric spectrum of peptides obtained following PVDF membrane purification of tryptic peptides extracted after in-gel enzymic (trypsin) digestion. [All the signals with masses indicated correspond to tryptic peptides (only the mass region of *m/z* 750 to 1300 is shown)]

Using the tryptic peptide fingerprint, we sought the identity of the candidate protein through a peptide search using the database compiled by the European Molecular Biology Laboratory (EMBL) protein and peptide group. The search identified a human thyroid autoantigen[22,30].

This report describes a simple procedure for the purification of tryptic peptides obtained after enzymic in-gel digestion of an affinity purified neutrophil membrane protein. Our membrane purification suggests that impurities in the extracted peptide mixture possibly quenched the signal when the sample was analyzed prior to PVDF membrane purification. Acrylamide monomer has been shown to cause severe signal suppression[16] while TFA[17] and non-ionic detergents[18] have also been implicated in signal suppression. The tryptic peptides spotted on the PVDF membrane bound to the membrane while the impurities apparently did not bind. Upon washing, the impurities were washed away leaving behind the pure peptide pool which was then later extracted. PVDF has been reported to possess good peptide and protein binding property[18,29,31]. This method of using PVDF membranes for purifying tryptic peptides is simple, easy and inexpensive and does not involve the use of sophisticated equipment.

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