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Short communication

Nanogram scale separations of proteins using capillary high-performance liquid chromatography with fully-automated on-line microfraction collection followed by matrix-assisted laser desorption ionisation time-of-flight mass spectrometry, protein sequencing and Western blot analysis

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Abstract

Capillary HPLC was applied for highly sensitive protein separations on a nanogram scale. A crude extract of acid soluble proteins from maize kernels was used as a model extract and separated on a 300-μm I.D. reversed-phase capillary column. Protein fractions of 1–4 μl volume were fully automatically collected with a new robot microfraction collection system. Fraction collection was performed onto matrix assisted laser desorption ionisation time-of-flight targets for mass spectrometric analysis, onto sequencing membranes for automated Edman degradation and onto nitrocellulose membranes for Western blot analysis. © 1998 Elsevier Science B.V.

Keywords: Matrix-assisted laser desorption mass spectrometry; Proteins

1. Introduction

Micropurification of proteins on a nanogram scale for further structural analysis, e.g. by Edman degradation, is mostly performed via gel electrophoresis followed by electrotransfer of the proteins onto membranes. Edman degradation performed from electroblotted protein samples is a standard technique for about 10 years [1–3]. However mass spectrometric analysis, like matrix assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS), from electroblotted samples is not a routine technique yet, because an infrared laser is often required [4,5]. So far, commercially available MALDI instruments are only provided with UV lasers.

As an alternative to gel electrophoresis, chromatographic methods can be used for micropurification of proteins. Protein purification using high-performance liquid chromatography (HPLC) is usually performed on columns with I.D. of 1.0–4.6 mm. If small sample volumes or limited amounts of sample, such as nanogram quantities, are available increased detection sensitivity is required. This is typically achieved by the usage of smaller I.D. columns. Capillary columns of 300 μm I.D. are often used for highly sensitive peptide mapping nowadays. These columns can also easily be applied for highly sensitive protein separations as shown in this paper. Since the flow-rate is typically about 4 μl/min for these columns,

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protein fractions are usually in the same volume range. The collection of such low volume fractions into eppendorf tubes is not appropriate, because any pipetting step required to transfer the sample onto a membrane or onto a MS target will result in a significant loss of sample. In this paper we show the fully automated on-line fraction collection of 1–4 μl fractions using a new robot microfraction collection system which has full flexibility in the *x*, *y* and *z*-axes. Low pmol protein fractions representing less than 500 ng protein were collected fully automatically onto MALDI-TOF-MS targets, sequencing membranes and nitrocellulose membranes and analysed by MALDI-TOF-MS, Edman degradation and Western blot analysis, respectively.

2. Materials and methods

2.1. Buffers and chemicals

All buffers and chemicals used were purchased from Sigma (St. Louis, MO, USA), if not otherwise stated.

2.2. Preparation of the maize kernel protein extract

A crude extract of acid soluble proteins from immature maize kernels was prepared essentially as described previously [6]. Frozen kernels were homogenised in liquid nitrogen and the frozen homogenate was taken up in 10 volumes of a buffer containing 20 m*M* Tris–HCl (pH 7.0), 10 m*M* 2-mercaptoethanol, 1 m*M* EDTA, 0.5 m*M* phenylmethylsulphonylfluoride (PMSF) and 2% (w/v) trichloroacetic acid. The suspension was centrifuged and the supernatant proteins were precipitated with 25% (w/v) trichloroacetic acid, washed twice with acetone, vacuum dried, and resuspended in a buffer containing 10 m*M* sodium phosphate (pH 7.0), 1 m*M* EDTA, 1 m*M* dithiothreithol (DTT), 0.5 m*M* PMSF. Total protein concentration of the crude extract was determined using a commercial protein assay according to the manufacturer's protocol (Bio-Rad, CA, USA). A 1-μl amount of protein extract corresponded to about 750 ng total protein.

2.3. Capillary HPLC separation

For capillary LC the following HP 1100 HPLC configuration (Hewlett-Packard, Waldbronn, Germany) was used: HP 1100 Series binary pump, HP 1100 vacuum degasser, HP 1100 Series diode-array detector, and an HP 1100 Series autosampler. For system control an HP CHEMSTATION was used. For conversion of the HP 1100 Series HPLC system to a capillary chromatography system a microflow processor (Accurate, LC Packings, Netherlands) was used as described in a previous technical note [7]. Separations were carried out on 250×0.3 mm Vydac C_{18} capillary columns (LC Packings, Netherlands) at a flow-rate of about 4 μl/min applying a gradient from 5 to 60% B within 60 min to the column. The gradient applied for collection onto MALDI targets was from 5 to 60% B in 45 min at a flow-rate of 2 μl/min. Solvent A was water with 0.05% trifluoroacetic acid (TFA) and solvent B was acetonitrile with 0.045% TFA. Injection was 500 nl protein extract for MALDI-TOF-MS and Western blot analyses and 2 μl protein extract for protein sequence analysis.

2.4. Automated microfraction collection

Fully automated on-line microfraction collection was performed using the Probot microfraction collection system (BAI, Bensheim, Germany or LC Packings, USA) which allows fraction collection from HPLC and from capillary electrophoretic separations. The fraction size which can be collected with the robot ranges from nanoliter to microliter volumes. The system consists of a fixed capillary holder which holds the capillary coming from the detector and of a movable table which holds the collection devices such as MALDI targets or sequencing or Western blot membranes. This table can be moved in the *x*, *y* and *z*-axes. The entire system is controlled by windows based software via the HPLC CHEMSTATION. For quantitative fraction collection the table was programmed so that the collection capillary carefully touched the surface of the MALDI targets or of the membranes. A more detailed description of the usage

of the microfraction collection robot is reported elsewhere [8].

2.5. MALDI-TOF-MS analysis

Sinapinic acid used as matrix in our studies was pre-crystallized on top of the MALDI targets. Four MALDI targets with ten positions each were placed into a laboratory-made target holder in the microfraction collector. Fractions of 30 s corresponding to 1 μl were automatically collected and subsequently vacuum crystallized using the Hewlett-Packard G2024A sample prep station. All MALDI measurements were carried out on the Hewlett-Packard G2030A MALDI-TOF-MS system equipped with a nitrogen laser at 337 nm (Hewlett-Packard, Palo Alto, CA, USA). External mass calibration was performed with the HP protein calibration standard G2053A (Hewlett-Packard). Mass accuracy using this method typically is about 0.1%.

2.6. Protein sequencing

Fractions of 1 min corresponding to 4 μl were collected onto a dry Selex-20 sequencing membrane (Schleicher and Schuell, Germany). After collection fractions of interest were excised with a scalpel and applied to the Hewlett-Packard GlOO5A protein sequencing system.

2.7. Western blot analysis

Fractions of 1 min corresponding to 4 μl were collected onto a nitrocellulose membrane and reacted with an anti-HMGa antiserum as described previously [9]. Antibody-binding was detected using an alkaline phosphatase-conjugated secondary antibody.

3. Results

For structural analysis of proteins performed by MS or Edman degradation, proteins do not have to be purified in the native form as is true in most cases of Western blot analysis. Hence proteins cannot only be purified in the nanogram scale or low pmol level by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), but also by reversed-phase capillary HPLC. To make this technique useful online microfraction collection of low microliter to nanoliter volumes onto any collection devices is essential. Here we describe the fully-automated

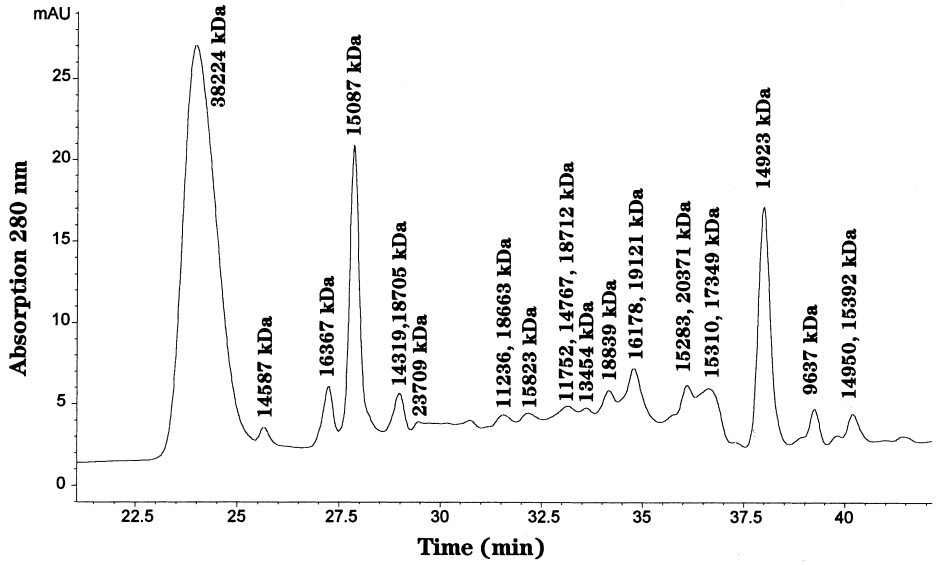
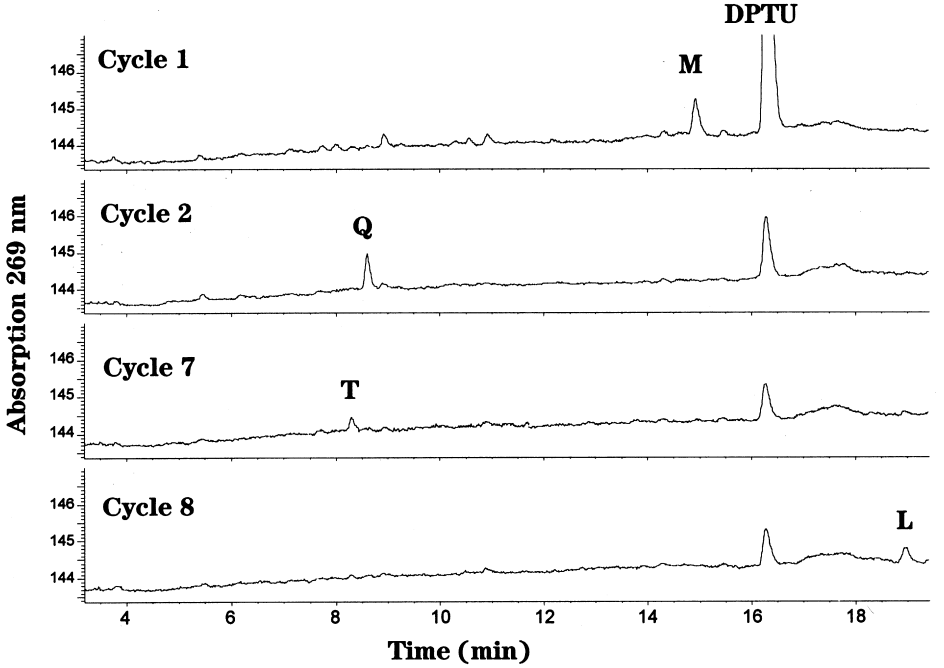


Fig. 1. Reversed-phase HPLC separation of a maize kernel extract with automated microfraction collection onto MALDI-TOF-MS targets. Determined molecular masses are indicated on top of the peaks (kDa=kilo dalton).



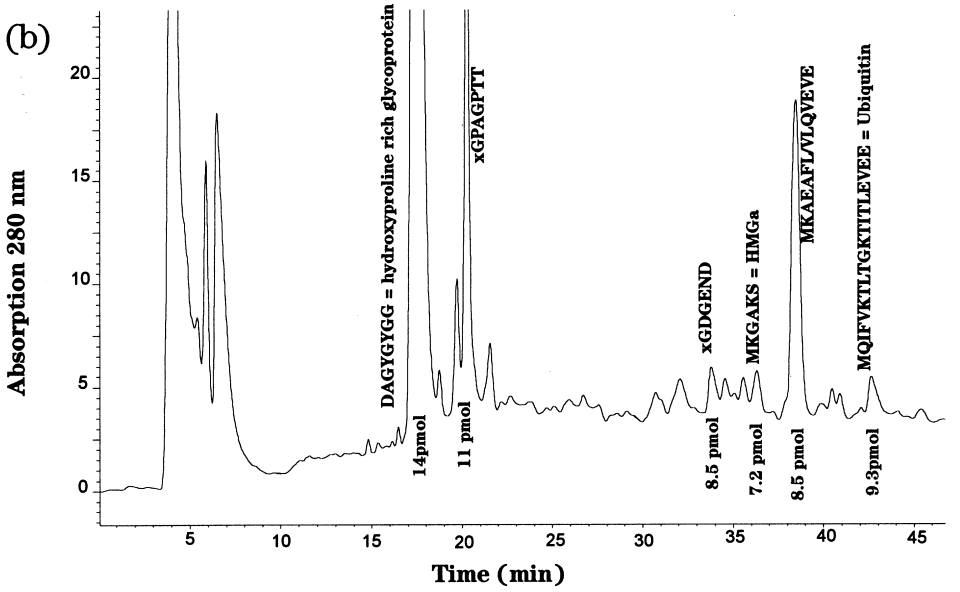


Fig. 2. (a) Edman degradation of protein fraction eluting at 42 min. Degradation cycles 1, 2, 7 and 8 are representatively shown. (b) Reversed-phase HPLC separation of a maize kernel extract with automated microfraction collection onto a sequencing membrane. Determined protein sequences are indicated at corresponding peaks (x=not identified). Determined protein amounts are indicated below the peaks.

microfraction collection of proteins separated by capillary HPLC followed by MS, protein sequence and Western blot analyses.

Firstly, proteins separated from a fairly crude extract can be initially characterized by mass determination of the collected fractions. Since reversed-phase HPLC used TFA as modifier and since it is well-known that TFA suppresses significantly the electrospray ionization (ESI) MS signal intensity [10], MALDI-TOF-MS is the method of choice for mass analysis. Hence, HPLC fractions of 1 μl were fully-automatically collected onto MALDI-TOF-MS targets using the Probot microfraction collection system. As shown in Fig. 1 due to the high sensitivity of MALDI-TOF-MS individual masses could be assigned to every single peak in the chromatogram, even if the signal is lower than 1 mAU at 280 nm. All molecular masses determined were in the range between 9000 and 40 000. Next, fractions were collected onto a chemically inert membrane for protein sequence analysis. Since the size of the fractions was only 4 μl and the HPLC solvent quickly evaporated proteins could be collected as concentrated narrow spots. Fractions of interest were excised with a scalpel and applied to the sequencer. As shown in Fig. 2a, clear sequences were obtained as representatively shown for the protein fraction which eluted at 42 min (see Fig. 2b). All six protein fractions which were randomly selected gave useful aminoterminal sequence information as shown in Fig. 2b. The fraction eluting at 17 min was identified as hydroxyproline rich glycoprotein (sequence: DAGYGYGG). This result is in good agreement with the MALDI-TOF-MS data, because the theoretical molecular mass of the DNA deduced protein sequence is 34 000 whilst the residual 3800 is contributed by the post-translationally added sugar moiety. Interestingly, sequence information could be also obtained from peaks of only 2 mAU at 280 nm as indicated for the fractions eluting at 33.5, 36 and 42 min in Fig. 2b. The sequence of the fraction eluting at 36 min was determined as MKGAKS which corresponds to the HMGa protein [11]. The sequence at 42 min turned out to be ubiquitin. The protein amount analysed ranged between 7 and 14 pmol assuming an initial yield of 50% of the Edman degradation. This corresponds to 80–400 ng of collected protein.

For Western blot analysis the protein fractions were collected onto a nitrocellulose membrane and

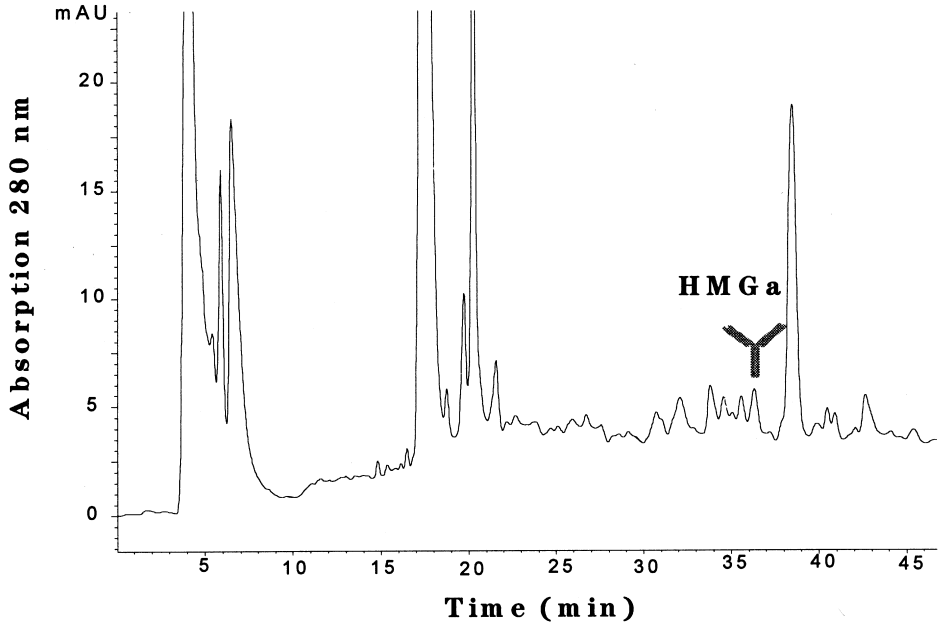


Fig. 3. Reversed-phase HPLC separation of a maize kernel extract with automated microfraction collection onto a nitrocellulose membrane followed by Western blot analysis using a polyclonal anti-HMGa antibody.

processed with a polyclonal anti-HMGa antibody to identify selectively the DNA-binding protein HMGa. Only the fraction eluting between 36 and 37 min was stained by the anti-HMGa antiserum [9] as shown in Fig. 3, which was in full agreement with the sequencing data obtained for the same peak when collected onto the sequencing membrane.

4. Conclusions

In this report we have shown that using capillary HPLC, micropurification of proteins on a nanogram scale can be easily performed. For further structural analysis of individual protein fractions they can be fully-automatically collected onto MALDI targets or onto sequencing or nitrocellulose membranes using a new flexible robot microfraction collection.

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References

[1] J. Vanderkerckhove, G. Bauw, M. Puype, J. Van Damme, M. Van Montagu, Eur. J. Biochem. 152 (1985) 9.
[2] R. Aebersold, D.B. Teplow, L.E. Hood, S.B. Kent, J. Biol. Chem. 261 (1986) 4229.
[3] C. Eckerskorn, W. Mewes, H.W. Goretzki, F. Lottspeich, Eur. J. Biochem. 176 (1988) 509.
[4] C. Eckerskorn, K. Strupat, M. Karas, F. Hillenkamp, F. Lottspeich, Electrophoresis 13 (1992) 664.
[5] M. Schreiner, K. Strupat, F. Lottspeich, C. Eckerskorn, Electrophoresis 17 (1996) 954.
[6] K.D. Grasser, R. Grimm, C. Ritt, J. Biol. Chem. 271 (1996) 32900.
[7] R. Grimm, Application Note 12-5965-1351E, Hewlett-Packard, Waldbronn, 1996.
[8] R. Grimm, Application Note, 12-5965-7745E, Hewlett-Packard, Waldbronn, 1997.
[9] K.D. Grasser, A. Wurz, G. Feix, Planta 185 (1991) 350.
[10] A. Apffel, S. Fischer, G. Goldberg, P.C. Goodley, F. Kuhlmann, J. Chromatogr. A 712 (1995) 177–190.
[11] K.D. Grasser, G. Feix, Nucleic Acids Res. 19 (1991) 2573.