Automation of micro-preparation and enzymatic cleavage of gel electrophoretically separated proteins

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Abstract To achieve high throughput, protein microcharacterization sample preparation must be automated. We describe a cartesian robot capable of processing 32 protein samples in parallel. The system is based on specially designed flow-through reactors for contamination-free reagent delivery and removal. Washing of excised gel pieces, reduction and alkylation, proteolytic cleavage and peptide extraction are performed in these reactors. Compatibility of the system with HPLC peptide separation and Edman degradation as well as with laser desorption mass spectrometry of the unseparated mixture is demonstrated. This is the first report describing automated preparation and processing of multiple protein samples.

Key words: Automation; In-gel proteolysis; Internal protein sequence analysis; Micro-characterization; Mass spectrometry; Protein identification

1. Introduction

A large and rapidly growing amount of nucleotide sequence information from genomic sequencing projects is now available [1,2]. Likewise, the powerful resolution of two-dimensional gel electrophoresis in combination with 2D gel databases have in principle made thousands of proteins available to biochemical study [3,4]. Techniques to rapidly identify protein spots in sequence databases would link these two 'infrastructure' projects, potentially providing a shortcut to the functional elucidation of many genes and their protein products.

Automation of many of the techniques central to molecular biology has taken place over the past three decades [5], but in protein chemistry the emphasis has been on the automation of characterization techniques such as the Edman degradation, amino acid analysis and mass spectrometry [6,7]. So far no significant automation has been applied to the area of sample preparation.

Enzymatic cleavage of the proteins is performed in order to obtain peptides from which sequence and mass information can be derived, even if the N-terminus of the protein is blocked. The analysis of peptide mixtures by HPLC and Edman degradation is highly automated and mass spectrometric analysis of unseparated peptide mixtures has become very sensitive and rapid. In contrast, the washing of the protein spots, subsequent proteolysis and extraction of the peptides generated, are labor intensive processes that have become rate limiting steps. They require considerable time and manual skill. In order to overcome this bottleneck, the process must be automated.

Here we describe the design and illustrate the application of an automated system for the simultaneous preparation of peptides from up to 32 proteins isolated by gel electrophoresis. Apart from uses in large scale protein identification projects, the system can be applied to the batch control of recombinant proteins, as well as in the sample preparation work typically performed in a protein core facility.

2. Materials and methods

2.1. Materials

All chemicals and reagents were from Sigma, Serva or Lab-Scan, and of the highest grade available. Sequencing grade trypsin was from Boehringer Mannheim. Proteins were quantified using amino acid analysis.

2.2. Gel electrophoresis and sample loading

One- and two-dimensional gel electrophoresis were performed using the Mini Protean II system (Bio-Rad). Gels were stained with Coomassie Blue R250, destained, and protein containing gel pieces were cut into small pieces. These were loaded into a 1.5 ml reservoir (Mueder and Wochele, Berlin) fitted with a 0.45 μm PVDF filter unit (Millipore) and a sealing cap (DEC-cap) on top (ABIMED) (Fig. 1A).

2.3. Temperature controlled reactor block

The reactor block was designed and constructed at the EMBL Workshop and can accommodate 32 reactors. The temperature was controlled by a 4-channel Jumo DICON 1000 process controller (Juckheim, Fulda) connected to 90 W heating wires (Horst, Bensheim). The reactor block was mounted on rails and moved by the robot arm to either the washing or sample collection zone.

2.4. Sample preparation robot

A Gilson 222XL robot with 402 dilutor was used during the experiments (ABIMED). The delivery needle had a conical tip to fit in the DEC caps. This allowed either the delivery of liquid or its ejection from the reactor by 2.6 bar nitrogen gas pressure depending on the insertion depth of the needle (Fig. 1B,C).

2.5. In-gel enzymatic cleavage

Gel washing, reduction/alkylation steps, subsequent proteolysis and extraction of the peptides were implemented on the robotic system but were otherwise as described previously ([8,9], Wilm, M., Shevchenko, A., Houthaeve, T. and Mann, M., unpublished).

2.6. Reversed-phase HPLC and Edman sequencing

Extracted peptides were separated on a 1.6 mm × 250 mm Vydac C18 218TP column with gradient elution at a flow rate of 120 μl/min on a Hewlett Packard 1090 HPLC system equipped with a Gilson 231 XL autosampler (ABIMED). Peptide fractions were sequenced on an ABI 477A sequencer (Applied Biosystems Division of Perkin Elmer).

2.7. MALDI-MS

Matrix assisted laser desorption ionization (MALDI) was performed on a Bruker REFLEX time-of-flight mass spectrometer (Bruker Analytik, Bremen, Germany) equipped with the SCOUT ion source. Sam-

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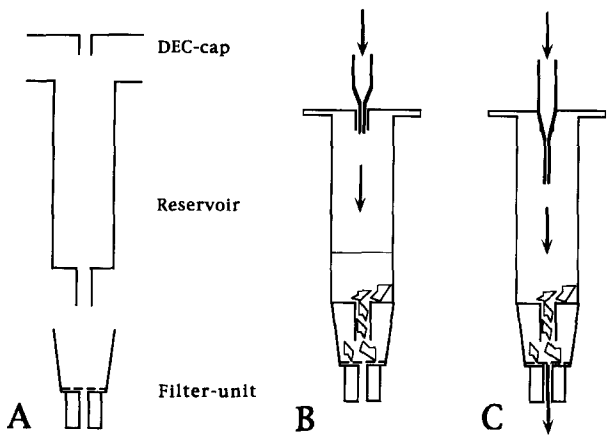


Fig. 1. Detail of a flow through reactor. (A) Parts of the reactor: cap to seal the reservoir with a central hole for the delivery needle ('DEC-cap'), 1.5 ml reservoir and filter-unit. (B) Liquid delivery: the needle tip is inserted such that it does not seal the DEC-cap and liquid is added to the gel pieces. (C) Liquid removal: the needle is inserted such that it seals the DEC-cap and nitrogen gas pressure applied through the needle pushes liquid out of the reactor. Note that the needle tip never comes into contact with the sample.
←

ples were prepared for MALDI by the fast evaporation method [10,11]. Data acquisition parameters, the transfer and subsequent averaging of spectra as well as further data processing were carried out using the computer program LaserOne developed by P. Mortensen and M. Mann in house.

2.8. Protein identification

Peptide mass maps were searched using PeptideSearch 2.7 [12,13] in EMBL-SWISSPROT and in a combined database (nrdb) maintained by the group of C. Sander, EMBL.

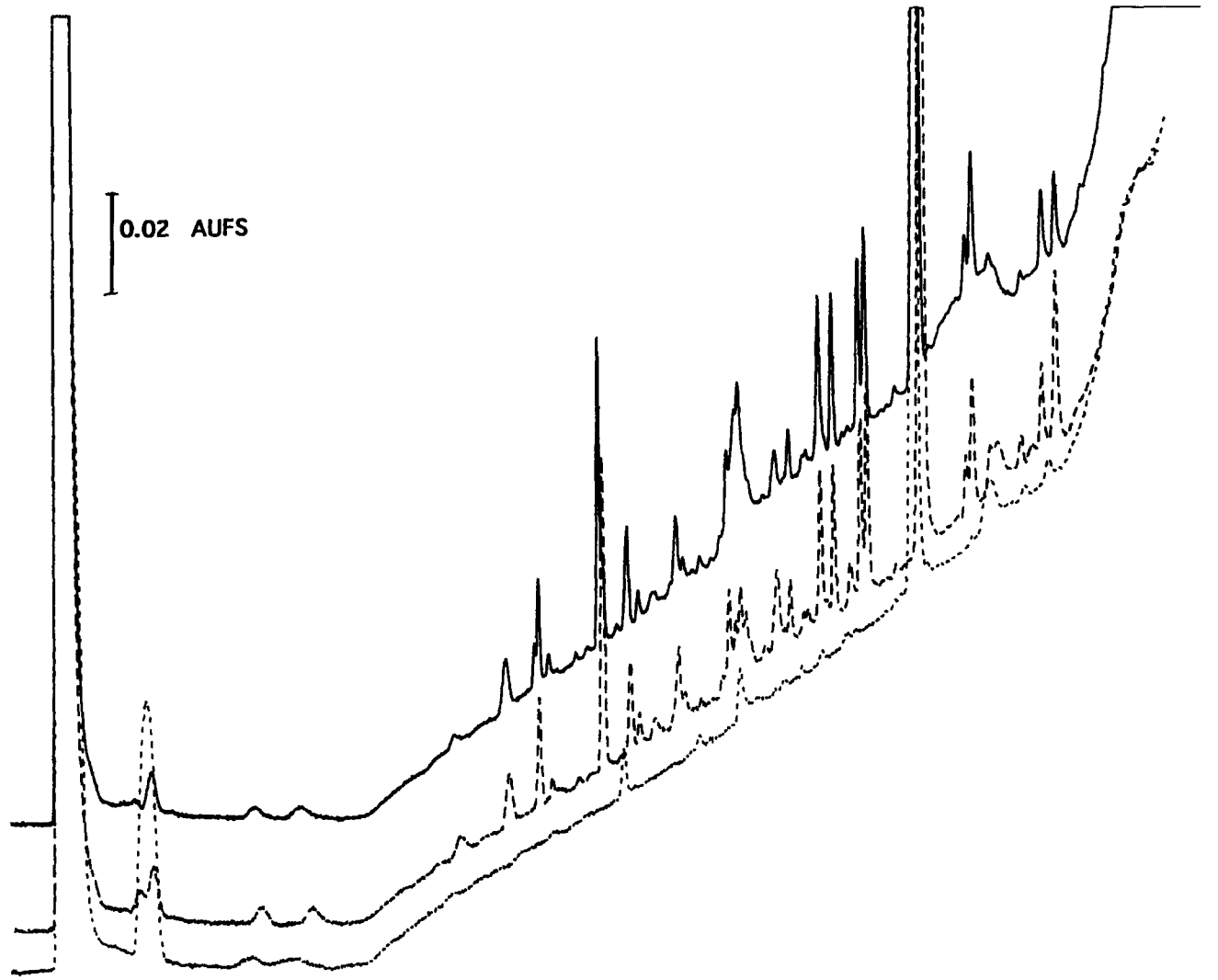


Fig. 2. Reproducibility of automated protein processing: comparison of two UV traces of RP-HPLC separations of in-gel tryptic digests of 50 pmol bovine carbonic anhydrase. The upper chromatogram (——) shows the peptide separation from the first reactor (no. 1), the middle chromatogram (--) shows the one from last reactor (no. 32). The lower chromatogram (....) is the result of processing a blank piece of gel which was included in the run. The chromatogram of the two digests is nearly identical.

3. Results

3.1. Reproducibility

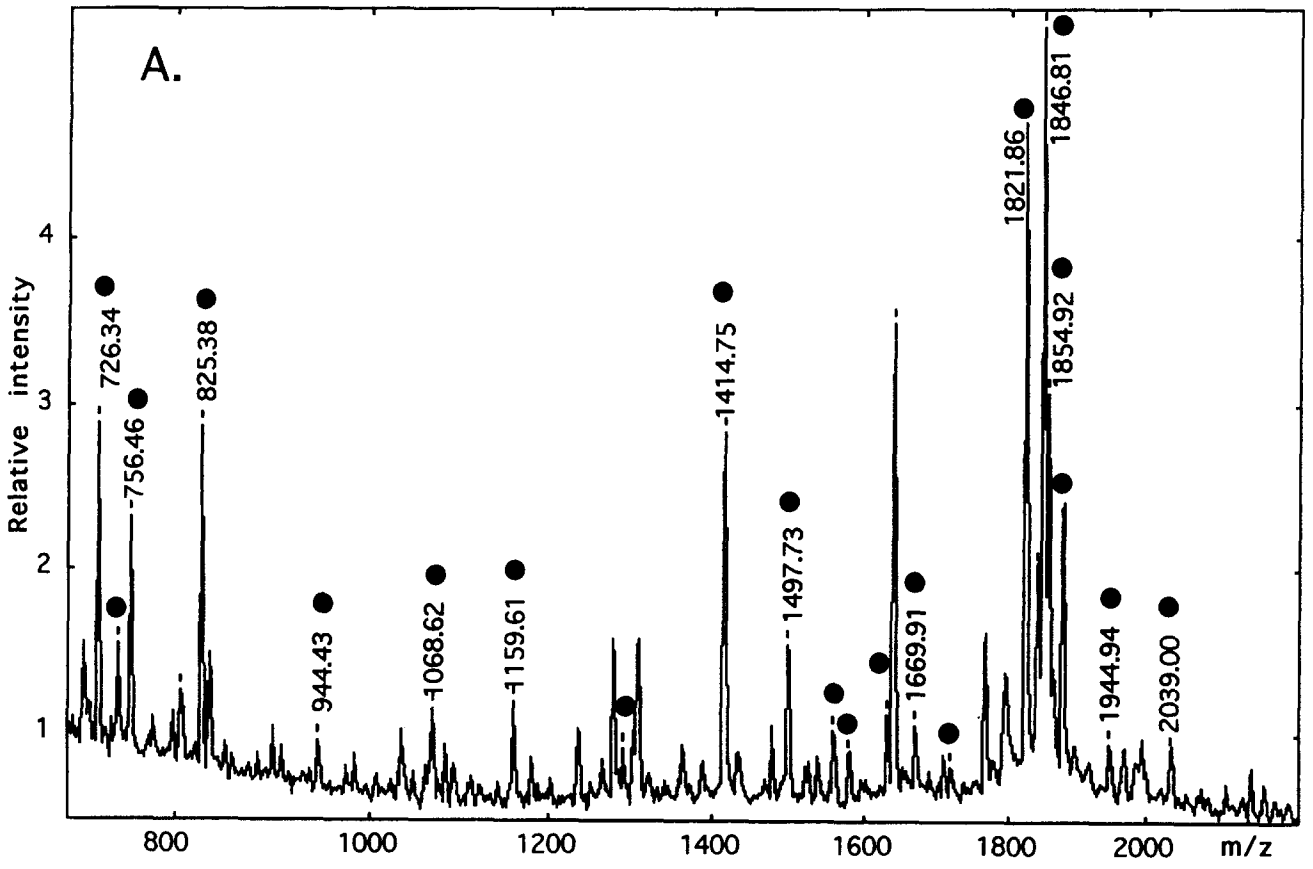
In order to test the reliability and reproducibility of the system, various amounts of several standard proteins were analyzed. Six samples each of bovine serum albumin, carbonic anhydrase, lysozyme, ovalbumin, phosphorylase B were loaded at 50 pmol and 5 pmol. After the automated preparation had been completed (16 h), the resulting 32 peptide mixtures were loaded on an autosampler and HPLC separated. The resulting chromatographic peptide maps of each protein were highly similar. As an example, Fig. 2 shows a comparison of two independent tryptic digests of 50 pmol bovine carbonic anhydrase. Two peptides of one of the runs were Edman sequenced and confirmed to be part of carbonic anhydrase (data not shown).

3.2. Characterization of yeast proteins separated on a 2D-gel

After successful testing on model samples, a series of six samples from a collaborative project on large scale identification of yeast proteins was processed by the robot. MALDI peptide maps of the automatically processed protein samples were similar to the ones prepared manually (data not shown). As an example Fig. 3A shows a MALDI peptide mass maps of one of the spots. Database searching of the spectrum positively identified the sample as Enolase 2 (SW: ENO2_YEAST). The peptide map covered more than 60 percent of the protein sequence (Fig. 3B).

4. Discussion

Automation of the preparation of peptides for the microcharacterization of proteins required several issues to be addressed: reliability, reproducibility sensitivity, reactor design and a solvent delivery method that avoids cross contamination between samples. Cartesian robots similar to the one employed here have been used with success in many areas of laboratory automation so it is not surprising that there are no reliability problems with the system. Automation of protein processing removes the risk of manual errors when performing labor inten-



B.

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  1 AVSKVYARSV YDSRGNPTVE VELTTEKGVF RSIVPSGAST GVHEALEMRD
 51 EDKSKWMGKG VMNAVNNVNN VIAPAFVKAN LDVKDQKAVD DFLLSLDGTA
101 NKSKLGANAI LGVSMAAARA AAAEKNVPLY QHLADLSKSK TSPYVLPVPF
151 LNVLNGGSHA GGALALQEFM IAPTGAKTFA EAMRIGSEVY HNLKSLTKKR
201 YGASAGNVGD EGGVAPNIQT AEEALDLIVD AIKAAGHDGK VKIGLDCASS
251 EFFKDGKYDL DFKNPESDKS KWLTGVELAD MYHSLMKRYP IVSIEDPFAE
301 DDWEAWSHFF KTAGIQIVAD DLTVTNPARI ATAIEKKAAD ALLLKVNQIG
351 TLSESIKAAQ DSFAANWGVM VSHRSGETED TFIADLVVGL RTGQIKTGAP
401 ARSERLAKLN QLLRIEEELG DKAVYAGENF HHGDKL
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Fig. 3. MALDI-MS spectrum of a 2D-gel protein spot from a yeast cell lysate (Shevchenko, A., et al., unpublished). Some 5 pmol protein were present in the spot as estimated by the intensity of Coomassie blue staining. After processing by the robot the extracted peptide mixture was dried down, reconstituted in 20 μl of 0.1% TFA/10% acetonitrile/water and 0.5 μl were applied to the MALDI target. The resulting peptide map (A) was searched in nrdb using PeptideSearch. The search identified the protein as Enolase 2 (SW: ENO2_YEAST). Tryptic peptides matching the expected masses within 0.1 Da are indicated by dots. (B) shows the sequence coverage obtained.

sive and highly repetitive tasks. Reduction of manual sample handling should also lead to lower levels of contamination. The reactor design presented here is simple and all the consumable components are commercially available and disposable.

Data obtained with the system show that individual reactors yield similar results and indicate that the robotic system can produce peptides from starting amounts in the lower picomol range. Further work is in progress to test its adequacy for the 0.1 to 1 pmol sample levels accessible to mass spectrometry. The flow-through design employed here permits the use of a simple reagent delivery and removal technique based on a single needle with a conical tip. Since reagents and peptides are always moved in the same direction there is no need for the needle to come into contact with the samples, thereby avoiding cross contamination.

The system has been tested with practical samples provided by collaborating groups and the robot was found to perform well under conditions which are very close to what is expected in large scale identification projects. Automation of the sample preparation is a key step towards automated protein identification which in turn should allow optimal exploitation of the accumulating information in sequence data libraries. The system presented here provides an important contribution towards this goal.

The automation of multiple protein sample preparation, which is successfully shown here for the first time, also opens the way to increased sample throughput in other fields using protein characterization. We expect it to be useful in core facility work and in the biotechnology industry, for the batch control of proteins.

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References

[1] Boguski, M. (1995) Trends Biochem. Sci. 20, 295–296.
[2] Adams, M. (1995) Nature 377, 3–174.
[3] O'Farrell, P (1975) J. Biol. Chem. 250, 4007–4021.
[4] Celis, J. (1994) Electrophoresis 15, 1349–1579.
[5] Pauwels, R., Azijn, H., Bethune, M., Claeys, C. and Hertogs, K. (1995) Curr. Opinion Biotech. 6, 111–117.
[6] Kellner, R., Lottspeich, F. and Meyer, H. (1994) Microcharacterization of Proteins, VCH, Weinheim.
[7] Crabb, J. (1995) Techniques in Protein Chemistry Vol VI, Academic Press, San Diego.
[8] Kurzchalia, T., Gorvel, J., Dupree, P., Parton, R., Kellner, R., Houthaeve, T., Gruenberg, J. and Simons, K. (1992) J. Biol. Chem. 267, 18419–18423.
[9] Jeno, P., Mini, T., Hintermann, E. and Horst, M. (1995) Anal. Biochem. 224, 451–455.
[10] Vorm, O., Roepstorff, P. and Mann, M. (1994) Anal. Chem. 66, 3281–3287.
[11] Vorm, O. and Mann, M. (1994) J. Am. Soc. Mass Spectrom. 5, 955–958.
[12] Mann, M., Højrup, P. and Roepstorff, P. (1993) Bio Mass Spectrom. 22, 338.
[13] Mann, M. (1994) in: Microcharacterization of Proteins, pp. 223–245 (Kellner, R., Lottspeich, F. and Meyer, H., eds.) VCH, Weinheim.