Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 998 (2003) 83–91
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

# Miniaturized on-line proteolysis–capillary liquid chromatography–mass spectrometry for peptide mapping of lactate dehydrogenase

Jenny Samskog[a], Dan Bylund[a], Sven P. Jacobsson[b], Karin E. Markides[a,*]
[a]*Department of Analytical Chemistry, Uppsala University, P.O. Box 599, SE-751 24 Uppsala, Sweden*
[b]*AstraZeneca R&D, S-15185 Södertälje, Sweden*

Received 23 January 2003; received in revised form 15 April 2003; accepted 15 April 2003

**Abstract**

In this study, methodology was developed for on-line and miniaturized enzymatic digestion with liquid chromatographic (LC) separation and mass spectrometric (MS) detection. A packed capillary LC–MS system was combined with on-line trypsin cleavage of a model protein, lactate dehydrogenase, to provide an efficient system for peptide mapping. The protein was injected onto an enzymatic capillary reactor and the resulting peptides were efficiently trapped on a capillary trapping column. Different trapping columns were evaluated to achieve a high binding capacity for the peptides generated in the enzyme reactor. The peptides were further eluted from the pre-column and separated on an analytical capillary column by a buffer more suitable for the following an electrospray ionisation (ESI) MS process. An important aspect of the on-line approach was the desalting of peptides performed in the trapping column to avoid detrimental signal suppression in the ESI process. The developed on-line system was finally compared to a classical digestion in solution, with reference to peptide sequence coverage and sensitivity. It was shown that the on-line system gave more than 100% higher peptide sequence coverage than traditional digestion methods.


*Keywords:* Peptide mapping; Proteolysis; Lactate dehydrogenase; Enzymes

## 1. Introduction

The search for proteins as potential targets for drugs and the increasing amount of therapeutic biopharmaceuticals are two reasons for the escalating development of analytical tools for fast and reliable analysis of proteins. Digestion of isolated proteins followed by separation and mass spectrometric detection of the resulting peptides is a powerful analytical strategy for the characterization of proteins. For quality control purposes, peptide mapping is employed as an identity test to probe for small changes in protein primary structure and to monitor manufacturing processes [1]. As the method is based on comparative analysis of complex patterns, the procedure should be highly reproducible. In addition, the procedure needs to ensure complete solubility of the biomolecules through the entire analysis. For protein identification purposes, the technique also needs to have high peptide sequence coverage. This is especially important for identification of small changes in the biomolecular structure, like post-

*Corresponding author. Tel.: +46-18-471-3691; fax: +46-18-471-3692.
*E-mail address:* karin.markides@kemi.uu.se (K.E. Markides).

0021-9673/03/$ – see front matter 
doi:10.1016/S0021-9673(03)00647-2

translational modifications in endogenic proteins or by-products in synthesised proteins.

Traditionally, enzyme digestion of proteins made in solution or in gels is a fairly time-consuming process, typically around 4 h. In view of the strong interest in high throughput analysis these long sample preparation times should preferably be avoided. One popular approach has been to increase the efficiency of the proteolysis by immobilizing the enzymes onto a solid support media [2]. This will increase the ratio of enzyme:protein from about 1:200 to a ratio of almost 1:1. This enables an efficient and fast proteolysis in a time-scale of a few minutes [3]. Another advantage of an on-line approach is the possibility to automate the method for high-throughput analysis [4,5]. Further, the immobilized enzymes can be used repeatedly for long periods of time without loss of enzyme activity and the enzymes are not mixed in with the peptides in the resulting solution [6].

To date, the enzyme reactors have mostly been used off-line from the separation system. For high throughput analysis, however, it is important that the separation and detection is coupled on-line to the proteolysis. This also minimizes the risk of losses during manual handling of the sample. In many cases it is then necessary to include a desalting and preconcentration step. Enzymatic digestion coupled to both liquid chromatography (LC) [5–8] and capillary electrophoresis (CE) [7,9,10] separation methods has been described by several research groups. The use of mass spectrometry (MS) as a detection method has found increasing interest, either by digestion coupled directly to MS [11–13] or digestion coupled to MS via an on-line separation step [4,5,14]. In order to make this promising approach compatible with proteins that need high amounts of salts or detergents for desolvation and denaturation, a highly effective desalting step would be needed. An efficient desalting is especially important when coupling the proteolysis to electrospray ionisation (ESI) MS. Finally, it should be considered that the advantages of an on-line approach may only be utilized if the coupled functions can be individually optimised also when compiled together.

An earlier reported method for characterising lactate dehydrogenase (LDH) included several steps: (1) digestion with Lys C for 4 h, (2) desalting the protein by ZipTip adsorption, (3) volume reduction by SpeedVac and reconstitution in an MS compatible buffer, and finally (4) μLC–MS analysis [15].

In this paper, a method for characterizing LDH by a miniaturized on-line trypsin reactor-desalting-separation device is reported. An objective with this study was to develop and test a method where desalting was needed together with the ability to simultaneously retain the analytical performance of each step and to decrease the total analysis time. The mobile phase used for the desolvation and digestion was exchanged to the LC mobile phase after peptide adsorption in a desalting device. The performance of the coupled miniaturized system was evaluated in terms of repeatability and peptide sequence coverage.

## 2. Experimental

### 2.1. Sample preparation

A peptide standard (P2693, Sigma, St. Louis, MO, USA) consisting of bradykinin, bradykinin fragment 1–5, substance P, [Arg8]-vasopressin, luteinising hormone releasing hormone, bombesin, leucine enkephalin, methionine enkephalin and oxytocin (25 μg of each) was dissolved in 500 μl Milli-Q water–acetonitrile (ACN) (50:50).

LDH from hog muscle and rabbit muscle were purchased from Boehringer Mannheim (Mannheim, Germany). LDH was reconstituted in 6 *M* guanidine hydrochloride (Gua–HCl; Fluka, Buchs, Switzerland) using microcentrifuge ultrafiltration filters ($M_r$ cut-off 10000, Millipore, Bedford, MA, USA).

The trypsin digestion in solution was accomplished by first pre-incubating 50 μl 2 mg/ml LDH at 37 °C, then adding 50 μl water, 200 μl 15 m*M* Tris(hydroxymethyl)aminomethane (Tris) (Merck, Darmstadt, Germany), pH 8.5, and 2 μl trypsin (sequencing grade, Boehringer) and letting it incubate at 37 °C during 4 h. After that the solution was acidified with 2 μl formic acid and 50 μl of the solution was desalted on a ZipTip device (Millipore), eluted by 80% ACN and evaporated to dryness by a SpeedVac concentrator (Savant Instruments, Hol-

brook, NY, USA). Before injection into the LC–MS system the peptides were re-dissolved in 20 μl mobile phase A.

For the immobilized trypsin digestion, the protein solution was diluted three times with digestion buffer before injecting it to the trypsin cartridge, Porozyme (Applied Biosystems, Framingham, MA, USA). The digestion was performed using a flow-rate of 1 μl/min at room temperature, 25 °C. The digestion buffer consisted either of 10 m*M* calcium chloride (analytical-reagent grade, Merck, 50 m*M* ammonium acetate (analytical-reagent grade, Merck) and 5% methanol adjusted to pH 8 by ammonia or 50 m*M* Tris, pH 8 and 10 m*M* calcium chloride and 5% methanol. LC mobile phase A consisted of 20 m*M* formic acid (analytical-reagent grade, Merck) and 5% methanol (LiChrosolv, Merck) and buffer B consisted of 20 m*M* formic acid and 95% methanol.

### 2.2. Preparation of enzyme reactor column and trapping column

Porozyme immobilized trypsin bulk media (Applied Biosystems) was slurry packed in the laboratory with a polyether ether ketone (PEEK) tube of 5 cm×500 μm I.D. The enzymatic efficiency of the reactors were tested from time to time by injecting bombesin onto the reactor and studying the fragments (*m*/*z* 395 and 1224) by LC–MS and ensuring that no uncleaved bombesin (*m*/*z* 1620) was seen in the MS system. To achieve a qualitative measurement of each reactor, another method was also used. This was performed by injecting a reagent, *N*α-benzoyl-L-Arg-ethyl ester (BAEE) onto the enzyme reactor, separating the product onto a $C_{18}$ column and measuring the area of the product in UV ($A_{249}$).

The trapping columns were slurry packed in fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) of 1 cm×200 μm I.D. The packing materials were 5 μm Kromasil $C_{18}$ particles (Eka Nobel, Bohus, Sweden), 5 μm Grom-Sil $C_{18}$ particles (Grom, Herrenberg-Kayh, Germany), Poros (Applied Biosystems) and μRPC (3 μm $C_2/C_{18}$ particles, Amersham Biosciences, UK). The Vydac trapping column (1 cm×300 μm I.D., guard prototype) was a kind gift from Vydac (GraceVydac, Hesperia, CA, USA).

### 2.3. On-line system

The system set-up is depicted in Fig. 1. Gradient liquid chromatography was performed by a Rheos 2000 pump (Flux Instruments, Basel, Switzerland), an electrical injector (Valco Instruments, Houston, TX, USA) and a PepMap $C_{18}$ column (150×0.3 mm, 3 μm) from LC Packings (Amsterdam, The Netherlands). The linear gradient started at 7% B and increased to 93% B during 40 min and the flow-rate from the pump was split down to 2 μl/min before the injector. Approximately 1.5 μg (2.5 μl injection loop) LDH was injected into the enzyme reactor by use of an LC pump (PU-980, Jasco, Tokyo, Japan) at a flow-rate of 1 μl/min. The trapping column was conditioned with LC mobile phase A (10% methanol) and switched on-line to the enzyme reactor for loading the resulting peptides at a flow-rate of 1 μl/min for 15 min. One enzyme reactor volume was diverted to waste before coupling it on-line to the trapping column. The loading buffer was the digestion buffer, i.e ammonium acetate, pH 8 and 5% MeOH. After that the trapping column was switched on-line to the separation column, the peptides were released from the trapping column in 10% MeOH (93% A), pH 3, and separated.

A PE Sciex API 100 quadrupole mass spectrometer (PE-Sciex, Concord, Canada) with an ionspray interface was used for detection. The quadrupole was scanned between *m*/*z* 250–1500 with a scan speed of 2.5 s/scan in the positive ion mode.

Data pre-processing and principal component analysis (PCA) was performed in Matlab (MathWorks, Natick, MA, USA) according to a previously reported method for classification of LDH [15].

## 3. Results and discussion

The on-line system for proteolytic protein analysis consists of some defined functions with individual optimisation criteria: sample preparation, proteolysis, trapping and desalting of the digestion products, peptide separation and ESI-MS detection. Commonly used salts for denaturation and solubilisation purposes are urea or Gua–HCl. In this study, 2 *M* Gua–HCl was used for this purpose. Proteolysis of proteins demands a certain pH depending on the type

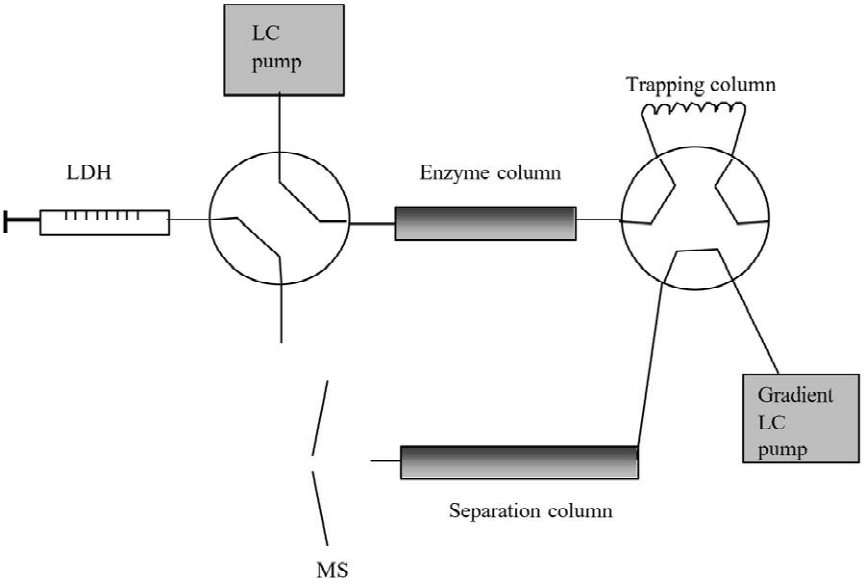


Fig. 1. Set-up of the on-line system. A 2.5-μl volume of protein solution is injected onto the trypsin enzyme column at a flow-rate of 1 μl/min in a digestion buffer consisting of 50 m*M* ammonium acetate, 10 m*M* calcium chloride and 5% methanol. The resulting peptides are trapped on the trapping column, which is coupled in-line to the trypsin enzyme column. The peptides are then eluted from the trapping column to the $C_{18}$ PepMap separation column in mobile phase consisting of 20 m*M* formic acid and 10% methanol at a flow-rate of 2 μl/min. The peptides are separated in a linear gradient and detected in the mass spectrometer.

of enzyme used and the pH of the digestion buffer was as high as pH 8 to ensure full trypsin activity. The trapping column arrangement involved the ability to retain peptides in an environment containing a high salt concentration and to elute the peptides with a low pH, i.e., pH 3, buffer in a slightly higher amount of organic modifier than in the digestion buffer. It is important that the amount of organic modifier in the digestion buffer was kept as low as possible to retain the peptides on the trapping column and to avoid obstruction of the material in the enzyme reactor. The amount of methanol in this buffer was therefore kept at 5%. The gradient for the subsequently separation started at 10% methanol for immediate elution of the peptides from the trapping column. The trapping columns were evaluated with reference to their ability to retain peptides of different hydrophobicities. The LC separation was performed at low pH to enable positive ESI detection of the peptides. The overall system performance was evaluated by measuring the repeatability of consecutive injections and the resulting peptide sequence coverage.

### *3.1. Optimization of the on-line system*

As already mentioned, for the described method, the separation buffer was adjusted to a pH of 3 while the digestion uses a basic buffer (pH 8) for optimal trypsin activity. Using a trapping column between the two systems compensated for this unavoidable pH shift. The buffer used for digestion contained non-volatile constituents, which would hamper the ESI process. Even though the recommended Tris buffer for trypsin digestion was replaced with a more volatile and MS-friendly ammonium acetate buffer, calcium chloride salt and adjustments towards a high pH were still needed. The trapping column therefore accomplished a buffer exchange referring to both ionic strength and pH. Most important, the trapping column was used to desalt the digested peptides from the protein sample. The LDH protein is known to need at least 2 *M* Gua–HCl to be properly dissolved and denatured. Injection of such a high load of salt into the analytical system led to some consequences like the need to wash and regenerate the enzyme reactor and columns at regular intervals. This was

done by washing the system extensively overnight in 50% methanol after 2–3 days of usage. Neglecting this washing procedure led to severe ion suppression during the first 15 min in the LC–MS run. A consequence was loss of the hydrophilic peptides that eluted early in the chromatogram, with a decrease in peptide sequence coverage as a result.

The transport of the peptides dissolved in Gua–HCl to the trapping column resulted in a poor performance of the trapping column. This is illustrated in Fig. 2 where a peptide standard were dissolved in either water or 2 *M* Gua–HCl and injected into the on-line system. Note that the scale is the same in both ion chromatograms. The overall sensitivity decreases and bradykinin 1–5 and bombesin are not found in the total ion chromatogram when the peptides are dissolved in Gua–HCl. In the on-line system, the trapping column loop will contain some remaining digestion buffer and Gua–HCl from the loading of the trapping column. Some Gua–HCl will thus be injected to the separation column, and will, in fact, dominate the spectrum from the total ion chromatogram during the first minutes. This problem could partly be overcome by loading the peptides from the proteolysis reactor for more than 10 min. In this way the salts were washed away from the trapping column loop. The loading time onto the trapping column can thus be identified

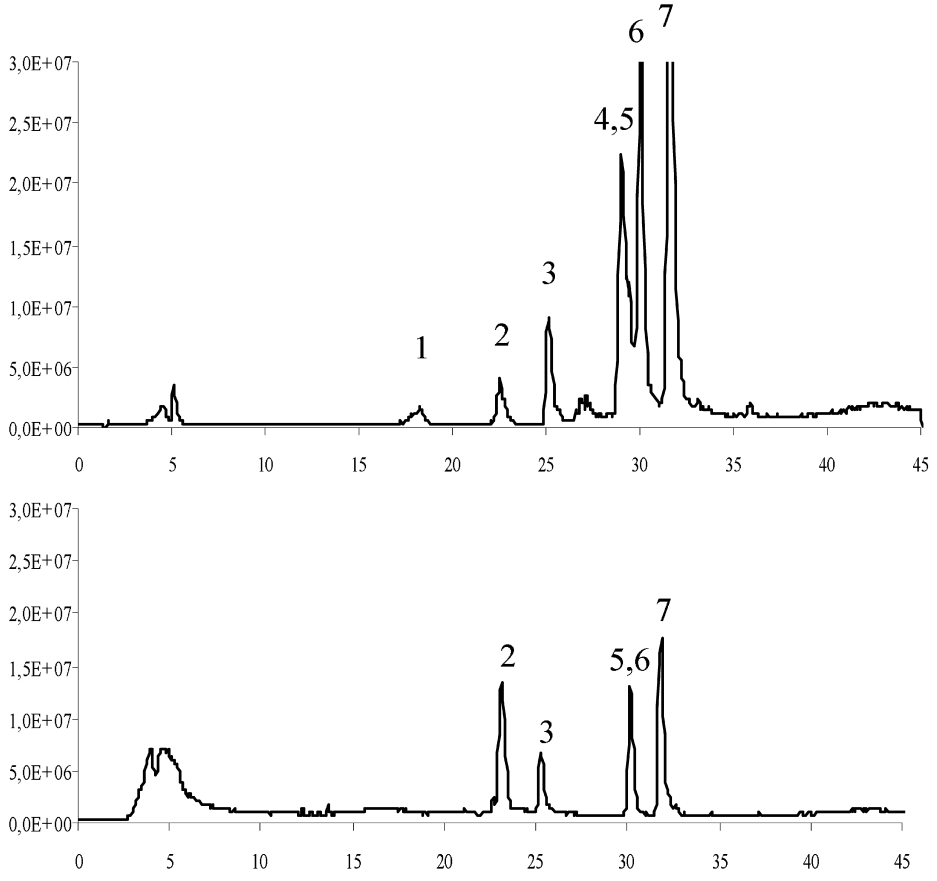


Fig. 2. Ion chromatogram of a nine-peptide standard mixture dissolved in (A) water, (B) 2 *M* Gua–HCl. Conditions: trapping of the peptides was performed on a Kromasil column in Tris buffer, pH: 8, the mobile phase for the LC separation consisted of 10 m*M* formic acid and an increasing amount of methanol starting at 10% methanol. The elution order of the peptides were: (1) bradykinin 1–5, (2) bradykinin, (3) luteinising hormone releasing hormone, (4) bombesin, (5) oxytocin, (6) methionine enkephalin, (7) leucine enkephalin. Time scale in min.

as one of the most critical parameters. Some hydrophilic peptides were lost when the loading time was increased to 20 min or more.

The use of 2 *M* Gua–HCl should not always be necessary for the denaturation of proteins. An alternative would be to use an acid like trifluoroacetic acid (TFA) or an organic modifier. TFA is however not reasonable to use in this case since an acidic environment inside the reactor would result in a very low trypsin activity. With an alternative digestion enzyme, e.g., pepsin, the use of an acidic buffer would be preferred. Further, increasing the amount of organic modifier in the sample zone may result in loss of peptides on the trapping column. Another issue is that the quality of the peptide map, in terms of more reproducible fragments, gets better if the protein is completely solubilised during trypsin digestion [16]. Ionic detergents, like sodium dodecyl sulfate (SDS), may be removed from a peptide sample at a low pH on-line by an anion exchanger [17,18].

The characteristics of the trapping column packing material were, as expected, a critical parameter for the success of the transfer. Different trapping columns were thus tested, like Kromasil, Poros, Grom-Sil, μRPC and Vydac, by breakthrough measurement of peptides with varying hydrophobicity using both UV detection and MS detection. Losses of the hydrophilic peptides during elution of the peptides from a trypsin reactor to a $C_{18}$ separation column [7] had also earlier been reported in the literature. In a first study, the peptides were injected directly onto the trapping column in the digestion buffer at pH 8 with 5% MeOH. Their breakthrough was recorded by UV detection. The buffer was then changed to the LC mobile phase, i.e., pH 3 and 10% MeOH (7% B buffer) and lastly to 80% MeOH (100% B buffer) corresponding to the on-line system set-up. The results are reported in Table 1. A large peak area, i.e., a large UV signal from the peptide mixture, indicates that the trapping column either retains the peptides efficiently or that many peptides elute early. Observe that some peptides may elute in the front and these peak areas were not considered. This was the case for both the Poros material and Kromasil. The materials μRPC and Grom-Sil showed, apart from the Poros material, the best performance in this test. Kromasil showed to be a poor trapping column for these analytes and buffers. To avoid losses during buffer exchange or salt removal, the trapping of the peptides should, according to published data, be performed in 100% aqueous solvent [19]. This is however not possible, as the enzyme reactor demands some organic modifier in the digestion buffer and so do the trapping column material used in this work. A similar study was made where the peptide sequence coverage from LC–MS runs was used as a quality measure of the trapping column. The results show not only the ability for the different trapping column materials to retain the peptides but also their ability to handle high amounts of salts. Peptide coverage, or recovery, was calculated from theoretical fragments from trypsin digestion of hog muscle LDH. The amino acid sequence for hog muscle LDH was taken from SwissProt [20] and the theoretical resulting peptides from the different LDHs were calculated in the software GPMAW (Lighthouse, Denmark). The corresponding peptide recovery for the trapping columns can be seen in Table 1. A peptide recovery of 20% denotes that 20% of the theoretical peptides were found from the resulting experimental peptides. The Poros material showed very promising results in the breakthrough experiments but gave a poor recovery in the LC–MS experiments. This could imply that the Poros material could not handle the high amounts of salts used in the on-line experiments. In conclusion, μRPC and Grom-Sil were the materials that worked best in the peptide release study using the on-line system. The peptide recovery with these materials were 50% and

Table 1
Peptide elution from different trapping columns to a UV detector in 10% and 80% methanol

| | Peptide release from trapping column[a] | | Peptide recovery[b] (%) |
|---|---|---|---|
| | 10% | 80% | |
| μRPC | 12 | 3 | 50 |
| Grom-Sil | 12 | 2 | 55 |
| Kromasil | 0.23 | 0.4 | 15 |
| Poros | 23 | 4 | 16 |
| Vydac | – | – | 35 |

[a] Peak area (UV signal from peptide standard $\cdot 10^{-6}$).
[b] The peptide recovery is the peptide sequence coverage from LC–MS.

more which indicates that this on-line system may be used for studies where a high coverage is needed, e.g., studying post-translational modifications of a certain protein. The recovery obtained in the different parts of the system, e.g., the digestion step and the trapping step, will need to be studied in more detail. This is especially important for the analysis of polar peptides, e.g., phosphorylated peptides.

The Bull and Breese index (B&B, GPMAW) is a measure of the hydrophobicity of the theoretical peptides; the more negative the value is the more hydrophobic is the peptide. The peptides that were found in the total ion chromatograms using the different kind of trapping columns were correlated to their total hydrophobicity, i.e., the sum of their B&B indexes. These values showed that μRPC and Grom-Sil retained peptides with the highest total hydrophobicity (B&B~−35000), the Vydac trapping column had a B&B index of about −20000 and Poros and Kromasil −10000.

It is important to note that even though the system has been developed to be able to remove high amounts of salts there are still some remaining salts and digestion buffer left during the LC–MS runs. One consequence is that the peptides forms adducts with ammonium (+45) and guanidine (+95) apart from the usual sodium adducts in the ESI process, which would lead to more difficult interpretation of the resulting data and loss in sensitivity of the method. This problem is presently studied in further detail with the neutral urea as a denaturing agent to overcome adducts formation.

### 3.2. System performance

Compared to trypsin digestion in solution of LDH the peptide sequence coverage has increased from about 25 to 55% when using the trypsin reactor in the on-line system presented in this study. This is illustrated in Fig. 3 and shows the impact of the much higher enzyme to substrate ratio when the enzyme is immobilized onto a solid support. The LC conditions were not the same in A and B in Fig. 3 as the dead volume in the system was minimized in the B run, explaining the differences in retention time of the analysis. A flow-rate of 1 μl/min through the trypsin reactor, corresponding to a residence time of 10 min in the reactor, gave excellent digestion efficiency. This can be compared to the reaction time of 4 h that was used in the earlier used digestion protocol. Observe that the amount of LDH injected into the enzyme reactor in the on-line system and the amount injected on the LC column for the classical digestion procedure is exactly the same, 1.6 μg.

The temperature chosen for digestion was 25 °C. The digestion efficiency is usually increased when increasing the temperature (up to 40 °C) according to the manufacturer of the immobilized enzyme material but the lower temperature was chosen to increase the lifetime of the enzyme reactor. Lower digestion temperatures, i.e., 25 °C [16] and 5 °C [21], has also been reported to increase the repeatability of the peptide map, It is important to emphasise that the effect of digestion temperature may be dependent on the type of protein and the other digestion conditions which all need to be optimised. The repeatability of the system, measured as the relative standard deviation in retention time of one selected peptide, was about 3% ($n$=7). The peptide had a retention time of 21 min and was centred in the chromatogram. Considering the fact that the method was evaluated using manual valve controls, the repeatability is well within the acceptance limit. The repeatability in peak area was not as good, probably due to the facts that no internal standard was used and that some of the parameters, like regeneration of the enzyme reactor, were not optimised. Despite this, PCA modelling of the derived mass spectral representations of the samples separated the two LDH variants studied. This indicates that the reported on-line method can be used to differentiate between similar proteins

## 4. Conclusions

An on-line system with proteolysis, desalting and separation coupled to MS detection has been developed for the analysis of LDH. The main considerations were: enzyme digestion, the trapping column and the applicability of protein analysis with electrospray ionisation. The loading time of the peptides onto the trapping column needed to be optimised to ensure that Gua–HCl was not transferred to the LC–MS system and thereby obstruct the detection of the peptides. Different trapping column materials were also evaluated. Grom-Sil and μRPC

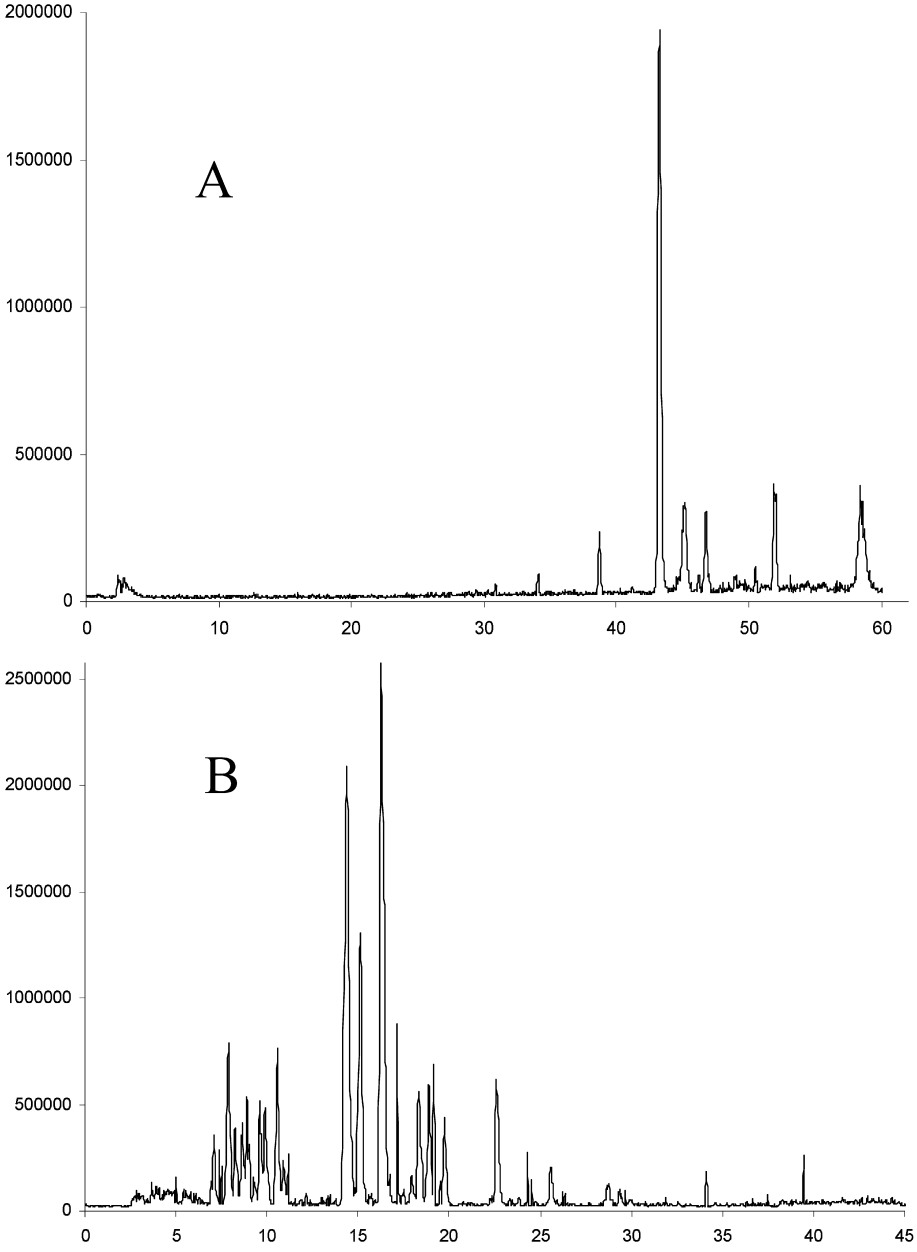


Fig. 3. Base peak chromatograms of a tryptic digest of LDH from hog muscle using (A) digestion off-line in solution, (B) digestion in an on-line trypsin reactor. Conditions: (A) trypsin digestion during 4 h, desalting on ZipTip, SpeedVac and reconstituted in mobile phase A. The separation was performed on a PepMap $C_{18}$ column using gradient elution that started at 7% B and increased to 93% B over 40 min. (B) LDH was injected onto a trypsin reactor and digested for 10 min, the peptides were thereafter trapped on a Grom-Sil trapping column and eluted onto the LC system under the same conditions as in (A). The detection was made in positive ion mode ESI-MS. Time scale in min.

showed the best properties for these kinds of peptides and buffers.

In the on-line system herein reported there was no manual handling of the protein solution and the total analysis time for this method was about one-eighth compared to the earlier reported method.

The options regarding buffers are more limited when a mass spectrometer is used as detector. Adduct formation of the peptides in ESI is highly dependent on the salts and buffers used in the system and will need further studies.

Although the developed analysis is mainly aimed towards quality control of biopharmaceuticals, the encouraging results indicate that the method also could be used for proteomics approaches where fast and reliable protein analysis is required.

## Acknowledgements

This work was financed by the Swedish Natural Research Council, contract No. K 5104-706/2001. Rudolf Kaiser at AstraZeneca, Södertälje is gratefully acknowledged for helpful discussions and for providing material. Ardeshir Amirkhani is acknowledged for assisting in the construction of the trapping columns.

## References

[1] R.L. Garnick, N.J. Solli, P.A. Papa, Anal. Chem. 60 (1988) 2546.
[2] M.S. Denton, W.D. Bostick, S.R. Dinsmore, J.E. Mrochek, Clin. Chem. 24 (8) (1978) 1408.
[3] S. Ekström, P. Önnerfjord, J. Nilsson, M. Bengtsson, T. Laurell, G. Marko-Varga, Anal. Chem. 72 (2000) 286.
[4] Y.L.F. Hsieh, H.-Q. Wang, C. Elicone, J. Mark, S.A. Martin, F. Regnier, Anal. Chem. 68 (1996) 455.
[5] T. Nadler, C. Blackburn, J. Mark, N. Gordon, F.E. Regnier, G. Vella, J. Chromtogr. A 743 (1996) 91.
[6] S. Wang, F.E. Regnier, J. Chromatogr. A 913 (2001) 429.
[7] K.A. Cobb, M. Novotny, Anal. Chem. 61 (1989) 2226.
[8] J. Lippincott, E. Hess, I. Apostol, Anal. Biochem. 252 (1997) 314.
[9] L.N. Amankwa, W.G. Kuhr, Anal. Chem. 64 (1992) 1610.
[10] J. Gao, J. Xu, L.E. Locascio, C.S. Lee, Anal. Chem. 73 (2001) 2648.
[11] K.R. Blackburn, R.J. Anderegg, J. Am. Soc. Mass Spectrom. 8 (1997) 483.
[12] G. Marie, L. Serani, O. Laprévote, Anal. Chem. 72 (2000) 5423.
[13] I.M. Lazar, R.S. Ramsey, J.M. Ramsey, Anal. Chem. 73 (2001) 1733.
[14] L.N. Amankwa, K. Harder, F. Jirik, R. Aebersold, Protein Sci. 4 (1995) 113.
[15] D. Bylund, J. Samskog, S.P. Jacobsson, K.E. Markides, J. Am. Soc. Mass Spectrom. (2003) in press.
[16] M.D. Jones, L.A. Merewether, C.L. Clogston, H.S. Lu, Anal. Biochem. 216 (1994) 135.
[17] J.P.C. Vissers, W.P. Hulst, J.-P. Chervet, H.M.J. Snijders, C.A. Cramers, J. Chromatogr. B 686 (1996) 119.
[18] B.H. Forngren, J. Samskog, S.-Å. Gustavsson, N. Tyrefors, K.E. Markides, B. Långström, J. Chromatogr. A 854 (1999) 155.
[19] H.D. Meiring, E. van der Heeft, G.J. ten Hove, A.P.J.M. de Jong, J. Sep. Sci. 25 (2002) 557.
[20] 2002. http://www.expasy.org/sprot/sprot-top.html.
[21] J.J. Dougherty Jr., L.M. Snyder, R.L. Sinclair, R.H. Robins, Anal. Biochem. 190 (1990) 7.