

Fast-Response Proteomics by Accelerated In-Gel Digestion of Proteins

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Kinetics of in-gel digestion of proteins by modified and native trypsins was studied by MALDI TOF mass spectrometry using ¹⁸O-labeled peptides as internal standards. The effect of the temperature, enzyme concentration, digestion time, and surface area of gel pieces on the yield of digestion products was characterized. Based on the kinetic data, we developed a protocol that enabled the identification of gel-separated proteins with 30-min digestion time without compromising the peptide yield and the sensitivity compared to conventional protocols that typically rely upon overnight enzymatic cleavage. The accelerated digestion protocol was tested in identification of more than 120 proteins from budding and fission yeasts at the subpicomole level.

The major goal of today's proteomics is to decipher the physical organization and quantitatively monitor the dynamics of proteomes of eukaryotic organisms, including humans. High-throughput identification of proteins by mass spectrometry has been generally recognized as a leading technology in this endeavor.^{1–5} In expression proteomics, which is focused on quantitative mapping of protein expression in cells or tissues, high-throughput identification is typically achieved through automated parallel processing and analysis of a large number of protein bands (spots) excised from one-dimensional or two-dimensional polyacrylamide gels.^{6–8} Functional proteomics, which is focused on mapping of protein–protein interactions within the cell, relies on the isolation of protein complexes and requires rapid and confident identification of a relatively small number of proteins produced in a single purification experiment.^{9–11}

Therefore, in functional proteomics, faster protein identification and a shorter turnaround time would provide a practical and cost-efficient alternative to large-scale parallel processing of samples.

To identify gel-separated proteins, the excised spots (bands) are usually digested in-gel with proteolytic enzymes (typically, trypsin) (see ref 12 for the tutorial) and an aliquot of the recovered pool of peptides is analyzed by peptide mass fingerprinting on a MALDI TOF instrument (see refs 13 and 14 for tutorial and review). If required, the peptide mixture can be further characterized by tandem mass spectrometry using a variety of instruments equipped with MALDI or ESI ion sources (reviewed in refs 2, 6, 15, and 16). MALDI mass fingerprints and/or MALDI Q(q)TOF¹⁷ or MALDI TOF/TOF¹⁸ tandem mass spectra can be acquired and searched against a database very rapidly. On the contrary, sample preparation and in-gel digestion are considerably slower and limit the speed of protein identification.

Trypsin is a most commonly used enzyme in in-gel digestion of proteins. It is an aggressive protease with high primary and low secondary specificity, which is capable of cleaving protein substrates at the low-femtomole level. Tryptic digestion repro-

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ducibly yields a relatively small number of autolysis products whose masses can be subtracted from peptide mass fingerprints prior to database searching.^{19,20} Tryptic digestion is usually specific and complete and leaves only a few peptides with miscleaved sites (mostly within sequences with two or more arginine or lysine residues positioned successively), which do not complicate the interpretation of peptide mass maps.²¹ Masses of tryptic peptides are typically within the range of 500–2500 Da and are convenient for detection by major types of mass spectrometers. Furthermore, arginine or lysine residues located at the C-terminus help to localize the positive charge and, by enhancing the abundance of y-ions, to simplify de novo interpretation of tandem mass spectra.¹²

Usually in-gel digestion with trypsin is performed at 37 °C overnight (12–16 h).²² It is conceivable that an elevated temperature might increase the hydrolysis rate and might improve the completeness and the yield of digestion products.^{23,24} Although a higher reaction temperature might also destabilize the enzyme and increase autolysis, these effects are less pronounced in enzymes with surface-directed modifications.²⁵ We therefore reasoned that protein cleavage by a modified trypsin might be performed at a higher temperature and enzyme concentration, compared to a native enzyme, and therefore similar (or higher) yield of peptide digestion products might be achieved faster.

We studied the kinetics of in-gel digestion by MALDI TOF MS and employed ¹⁸O-labeled peptide internal standards for quantifying the yield of digestion products.²⁶ We evaluated how the reaction temperature, enzyme concentration, digestion time, and predigestion preparation of protein bands affected the yield of peptide digestion products and used kinetic data to optimize the digestion conditions, depending on the available amount of proteins and on the desired speed of the analysis. An accelerated in-gel digestion protocol (ADP) was applied to identify more than 120 members of protein complexes, isolated from budding and fission yeasts, by immunoaffinity chromatography. We demonstrated that ADP dramatically simplifies and accelerates the sample preparation routine without compromising the yield of digestion products, sensitivity of peptide detection, and certainty of protein identification.

EXPERIMENTAL SECTION

Materials and Reagents. Unless otherwise noted, all chemicals were purchased from Sigma (Sigma Chemicals, St. Louis, MO) and were of analytical grade. H₂¹⁸O (Cambridge Isotopic Laboratories, Cambridge, MA) was purified by microdistillation as described.¹² Unmodified porcine trypsin was purchased from Fluka (trypsin from porcine pancreas, Catalog No. 93614, Buchs, Switzerland), unmodified bovine trypsin from Roche Diagnostics (trypsin sequencing grade, Catalog No. 1418475, Basel, Switzerland), and modified by reductive methylation porcine trypsin from

Promega (sequencing grade modified trypsin, Catalog No. V5111, Mannheim, Germany). 1-Cyano-4-hydroxycinnamic acid (a matrix for preparing MALDI probes) was purchased from Bruker Daltonik GmbH (Bremen, Germany).

Instrument and Software. Mass spectrometry was performed on a Reflex IV (Bruker Daltonik GmbH) MALDI TOF mass spectrometer, equipped with Scout 384 ion source. Spectra were processed by XMass 5.1.1 and BioTools 2.1 software (Bruker Daltonik GmbH). Proteins were identified using the MASCOT 1.8 (Matrix Science) program²⁷ installed on a local server; database searches were performed against a nonredundant protein database MSDB downloaded from European Bioinformatics Institute (EBI).

Catalytic Activity of Trypsin. The activity was determined as described²⁸ using a chromogenic substrate *p*-benzoyl-DL-arginine-4-nitroanilide (BAPNA). The reaction mixture containing 1.9 mL of buffer, 40 µL of water, and 10 µL of a trypsin sample (2 mg/mL) was incubated at 37 °C in a spectrophotometric cuvette. After 50 µL of BAPNA (10 mM stock solution freshly prepared in DMSO) was added, the reaction was monitored by increased absorbance at 408 nm, extinction coefficient 8800 M⁻¹ cm⁻¹. Thermostability of trypsin preparations was evaluated by measuring the activity after incubating the enzymes in 80 mM Tris-HCl buffer, pH 8.0, at 37, 50, and 65 °C for 30 min. The residual activity was measured with BAPNA after fast cooling of enzyme aliquots in a water bath. *T*₅₀ was defined as the temperature at which 50% of the activity is retained after 30 min. Protein content was determined using a modified Lowry's method.²⁹

In-Gel Digestion Protocols. Bovine serum albumin (BSA) was used as a model protein to study the in-gel digestion kinetics. One-dimensional SDS–polyacrylamide gel electrophoresis was performed using standard methods on the Bio-Rad Mini-Protein II system (7 cm × 10 cm minigels). The 12% polyacrylamide gels of 1-mm thickness were used in model experiments with BSA. BSA (0.5 pmol) was loaded onto each lane, and upon electrophoresis, protein bands were visualized by staining with Coomassie Brilliant Blue R250 (Serva Electrophoresis GmbH, Heidelberg, Germany). The bands were excised from the gel slab, cut into pieces, and put into 0.65-mL PCR microtubes (Roth, Karlsruhe, Germany). The following in-gel digestion protocols were applied:

Conventional Digestion Protocol (CDP).²² Proteins were in-gel reduced by 10 mM dithiothreitol and alkylated by 55 mM iodoacetamide, followed by a washing step, including dehydrating gel pieces with acetonitrile and rehydrating with ammonium bicarbonate buffer. Gel pieces were again dehydrated by acetonitrile and dried in a vacuum centrifuge. Gel pieces were further rehydrated for 40 min at 4 °C in a minithermostat (ThermoStat Plus, Eppendorf, Hamburg, Germany) in a 0.5 µM solution of unmodified bovine trypsin in 50 mM ammonium bicarbonate buffer containing 250 µM calcium chloride. After the rehydration step, the excess of trypsin solution was aspirated from the test tube and samples were digested overnight at 37 °C. For the reference, the same procedure was performed with the modified trypsin.

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Accelerated Digestion Protocol. Proteins were digested in-gel with the modified trypsin, without prior destaining, reduction, and alkylation.³⁰ Gel pieces were washed with water for 5 min and dehydrated in neat acetonitrile for 20 min. Acetonitrile was aspirated, and gel pieces were dried in a vacuum centrifuge and rehydrated for 60 min in 1.5 μ M solution of the modified trypsin in 50 mM ammonium bicarbonate buffer at 4 °C. The excess of the enzyme solution was aspirated, and the digestion was carried out for 30 min at 58 °C. The reaction was stopped by adding 1 μ L of 5% formic acid.

Kinetics of Tryptic Digestion. In-gel digestion was performed as described above with the following modifications:

Effect of Digestion Temperature. Dried gel pieces were rehydrated in 0.5 μ M solution of modified and unmodified trypsins for 20 min followed by 30-min digestion at 37, 50, 65, 75, and 90 °C.

Effect of Enzyme Concentration. Dried gel pieces were rehydrated in 0.5, 1.5, 3.5, and 5.0 μ M solution of the modified trypsin for 20 min followed by 30-min digestion at 58 °C.

Time Curve of In-Gel Digestion. Dried gel pieces were rehydrated in a 0.5 μ M solution of the modified trypsin for 20 min followed by 10-, 30-, 60-, 120-, and 720-min incubation at 58 °C. After digestion, the yield of tryptic peptides was quantified by mass spectrometry.

Kinetics of Rehydration of Gel Pieces. Pieces of 12% polyacrylamide gel (approximately 1 mm \times 1 mm \times 1 mm) were put into a test tube, dehydrated by acetonitrile for 20 min, dried in a vacuum centrifuge, and weighed on the analytical balance (Sartorius BP121S, Göttingen, Germany). Approximately two volumes of water was added (\sim 170 μ L), and the test tube was incubated for 10 min in the thermostat. Water was carefully aspirated from the tube, and the tube was weighed. The procedure was repeated several times with 10-min intervals once the constant weight of fully swollen gel pieces was reached. To check for possible losses of gel pieces during the experiment, fully swollen pieces were again dehydrated by acetonitrile and the tube was weighed.

Saturating Gel Pieces with Trypsin. *Effect of the Incubation Time.* Gel pieces, prepared and dehydrated as above, were rehydrated at 4 °C with the 0.5 μ M solution of modified trypsin, for 0, 10, 30, and 60 min. Digestion was carried out for 30 min at 58 °C, and the yield of digestion products was quantified.

Effect of the Surface Area. Eight identical bands were divided into two equal groups. In both groups, individual bands were cut into a different number of pieces: 1, 2, 4, and 8. Before adding the modified trypsin, gel pieces in the first group were dehydrated by acetonitrile for 20 min, while in the second group, gel pieces were not dehydrated. Gel pieces in both groups were soaked in the 0.5 μ M solution of the modified trypsin at 4 °C. The digestion was then carried out for 30 min at 58 °C, and digestion products were quantified.

Extraction of Digestion Products. The extraction was performed using 5% formic acid and acetonitrile as described.^{12,22} The recovered extracts were pooled together and dried in a vacuum centrifuge.

Probe Preparation and Acquisition of MALDI TOF Spectra. On the surface of an AnchorChip 600/384 target (Bruker Daltonik GmbH), 0.6 μ L of the sample was mixed with 0.6 μ L of matrix solution (2 mg/mL 1-cyano-4-hydroxycinnamic acid in 2.5% trifluoroacetic acid/acetonitrile, 1:2 v/v), allowed to dry down at ambient temperature, and washed with 5% formic acid. Each quantification experiment was performed in duplicate, with three probes prepared in parallel from each sample. Not less than 100 single-pulse spectra were summed and smoothed by Savitzky–Golay filter with the width set to seven data points. Peaks were labeled manually using a centroid peak picking algorithm, and peak areas were recorded. The areas of respective tryptic peptide peaks detected in all acquired spectra (see below) were averaged. Standard deviation of the mean was calculated for each data set.

¹⁸O-Labeled Internal Standards for Quantification. The standards were prepared as described.²⁶ A 0.5 mg/mL BSA solution in 50 mM ammonium bicarbonate buffer in H₂¹⁸O was digested overnight at 37 °C by unmodified trypsin at an enzyme/substrate ratio of 1:10 (w/w). The obtained stock solution of tryptic peptides with ¹⁸O-labeled C-terminal carboxyl groups was diluted 30 times, and aliquots were added as the internal standard to experimental digests. The concentration of individual ¹⁸O-labeled peptide internal standards was determined in a separate experiment by comparison of intensities of their peaks with intensities of peaks of synthetic peptides having the same sequence. Peptides were synthesized by Eurogentec (Seraing, Belgium), and the concentration of their stock solutions was determined by amino acid analysis performed in the Protein Analysis Unit headed by Dr. P. Hunziker in the University of Zürich. The measured yield of the in-solution digestion was close to 100% within the experimental error of amino acid analysis and quantification by mass spectrometry.

Quantification of Digestion Products. Tryptic peptides were recovered from gel pieces and dried as described above. Samples were redissolved in 10 μ L of the 5.8 nM solution of ¹⁸O-labeled tryptic peptides (internal standards) and analyzed by mass spectrometry as described above. The yield of the following three arginine-containing BSA tryptic peptides was determined: YLYEIAR, *m/z* 927.49; LGEYGFQNALIVR, *m/z* 1479.79; and DAFLGSFLYEYSR, *m/z* 1567.74.

The amount of an individual digestion product (m_{PEP}) was calculated from the amount of the internal ¹⁸O-labeled standard (m_{STD}) and the ratio of the area of the monoisotopic peak of the unlabeled peptide (S_{PEP}) and of the sum of deconvoluted areas of monoisotopic peaks of singly and doubly ¹⁸O-labeled forms of the internal standard:^{31,32}

$$m_{\text{PEP}} = \frac{S_{\text{PEP}}}{S_{\text{DOUBLE}} + S_{\text{SINGLE}} \cdot (1 - f_3) + S_{\text{PEP}} \cdot (f_3^2 - f_5 - f_3)} m_{\text{STD}}$$

where S_{SINGLE} and S_{DOUBLE} are the areas of the isotopic peaks spaced from the monoisotopic peak of the unlabeled peptide by 2 and 4 Da, respectively. Coefficients f_3 and f_5 are the calculated

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ratios of the intensity of, respectively, third (+2 Da) and fifth (+4 Da) isotopic peaks to the intensity of the monoisotopic peak of the unlabeled peptide; f_3 and f_5 were calculated for each peptide using the MS-Isotope program from ProteinProspector software developed in the UCSF Mass Spectrometry facility (<http://prospector.ucsf.edu>).

The yield of digestion (Y) was calculated by averaging the amount of n (typically, of three) peptides measured in N (typically, in six), parallel runs:

$$Y = \frac{\sum_{j=1}^N \left(\sum_{i=1}^n (m_{\text{PEP}})_i / n \right)_j}{N}$$

RESULTS AND DISCUSSION

Quantification Method. Optimization of in-gel digestion efficiency should rely on quantifying the yield of digestion products rather than on the qualitative assessment of peptide mass maps (although protein identification remains the ultimate goal of peptide mass fingerprinting). To this end, we employed internal standards: ^{18}O -labeled peptides, produced in a separate digestion of the same model protein in H_2^{18}O and quantified digestion products by MALDI MS.^{26,31–33} It is important to use the same mass spectrometric method for the quantification of peptides and for the identification of proteins, because spectra acquired from the same digest by MALDI and ESI demonstrated remarkably different peptide profiles.^{34,35}

MALDI peptide mass fingerprints of tryptic digests are dominated by peptides containing arginine residues at the C-terminus.³⁶ If produced by digesting the protein in H_2^{18}O water, these peptides incorporate one or, mostly, two ^{18}O atoms into their C-terminal carboxyl groups,^{26,33} and therefore, their molecular mass is increased by 2 and 4 Da, respectively. We found that ~10% of the total amount of any arginine-containing peptide incorporated only one ^{18}O atom. The profile of the isotopic cluster that contains peaks of unlabeled and singly and doubly ^{18}O -labeled peptides is rather complex but can be deconvoluted using isotopic ratios calculated from the peptide elemental composition.^{31,32,37} The deconvolution procedure might also help to account for Lys-containing peptides, which become predominantly singly ^{18}O -labeled upon digestion of the protein in H_2^{18}O . However, those peptides are in general less abundant than Arg-containing peptides. Since chemical noise strongly affects the accuracy of deconvolution, accounting for Lys-containing peptides resulted in a 2-fold increase in the error of measurements (data not shown) and was not employed in this study.

To test linearity of the method, several bands containing 0.5 pmol of BSA were digested in parallel and the aliquots were diluted in the range from 1:1 to 1:16. The subsequent analysis of samples spiked with an equal amount of ^{18}O -labeled internal standards

Table 1. Catalytic Activity and Thermostability of Different Types of Trypsin Determined Using BAPNA Substrate

| trypsin | activity ^a (nkat mg ⁻¹) | residual activity ^b (%) | | | T_{50} ^c (°C) |
|--------------------|---|------------------------------------|-------|-------|-------------------------------|
| | | 37 °C | 50 °C | 65 °C | |
| unmodified porcine | 25 | 68 | 21 | <1 | 41.0 |
| unmodified bovine | 9 | 66 | 18 | <1 | 40.5 |
| modified porcine | 8 | 92 | 42 | <2 | 48.0 |

^a The calculated specific activity values are related to the initial activity of freshly dissolved enzymes. ^b Residual activity after 30-min incubation at the specified temperature. The initial activity of freshly dissolved enzyme assumed as 100%. ^c The temperature at which the enzyme retains 50% of its activity upon incubating for 30 min.

confirmed linearity of the curve (correlation coefficient $r = 0.9989$, data not shown).

Activity and Thermostability of Modified and Native Trypsins. We determined the specific activity of a few commercially available trypsins using the BAPNA substrate (Table 1). No further characterization or purification of enzyme preparations was performed at this point, and the reported activity was considered solely as a reference.

To determine how reductive methylation of the porcine trypsin affects its thermal stability, we incubated 0.5 μM enzyme solution for 30 min at various temperatures, determined the percentage of residual catalytic activity, and calculated the temperature T_{50} at which 50% of the enzyme activity is retained. The enzyme concentration was lower than 10% of a typical value for K_m (see discussion below) and slowed enzyme autolysis. The modified enzyme did not lose its activity at 37 °C and retained ~40% of the specific activity at 50 °C. On the contrary, both unmodified enzymes lost their activity substantially at all temperatures tested. All tested enzymes were inactivated by 30-min incubation at 65 °C. We also noted that the preparation of native bovine and porcine trypsins showed a similar pattern of thermal inactivation, although their catalytic activity differed by a factor of 3. This observation suggests that autolysis might not play a major role in the inactivation of trypsin during a short period of incubation.

Peptide Yield and Digestion Temperature. We further investigated whether digestion of proteins at elevated temperatures accelerated the protein cleavage. We reasoned that since the enzyme is added in a relatively high concentration, partial thermal inactivation might not impact the completeness of cleavage significantly over a short period of time. The peptide yield of CDP digestion (37 °C, overnight) served as a reference.

The average yield of a 30-min digestion by the modified enzyme had a broad temperature optimum between 50 and 65 °C, corresponding to ~50% of the yield of the CDP (37 °C, overnight) (Figure 1A). As anticipated, the yield dropped considerably at temperatures higher than 60 °C and was ~40% lower if the digestion was carried out at 37 °C. For comparison, a 30-min digestion was performed using unmodified trypsin (Figure 1B). The peptide yield was lower than 20% compared to the yield of the overnight digestion and decreased with increasing reaction temperature. Thus, modification of trypsin by reductive methyl-

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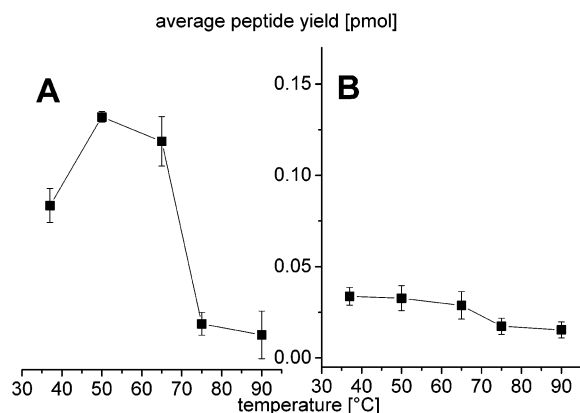


Figure 1. Effect of digestion temperature on the peptide yield: (A) modified trypsin; (B) native (unmodified) trypsin. The enzyme concentration was $0.5 \mu\text{M}$ and digestion time 30 min. Average peptide yield of control digestion (CDP) was 0.25 pmol.

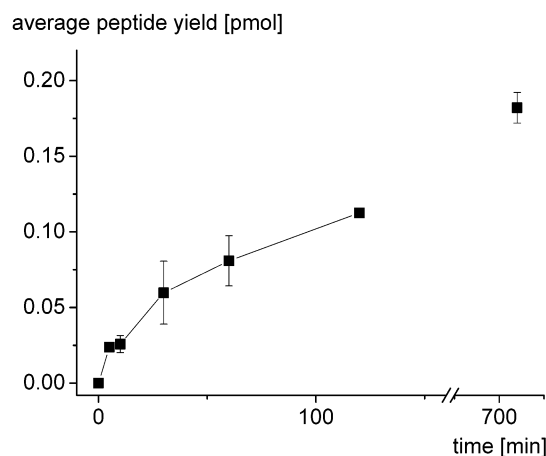


Figure 2. Time curve of digestion by the modified trypsin. The enzyme concentration was $0.5 \mu\text{M}$ and digestion temperature 58°C . The peptide yield in the control experiment (CDP) was 0.17 pmol.

ation not only reduced the autolysis³⁸ but also shifted its working optimum to higher temperatures.

Within a much shorter digestion time (30–60 min), the modified enzyme at the elevated temperature (58°C) provided a substantially better peptide yield compared to the conventional method. However, our results cautioned against using elevated temperatures for digesting proteins overnight since the digests appeared to be heavily contaminated with trypsin autolysis products, which severely complicated the interpretation of peptide mass fingerprints.

Peptide Yield and Digestion Time. We monitored the time course of digestion at the elevated temperature with the modified enzyme (Figure 2). Approximately 50% of the yield of CDP was reached in 30 min at 58°C with $0.5 \mu\text{M}$ concentration of the modified trypsin and allowed confident protein identification upon database searching. Overnight cleavage under the same conditions still gained an extra 20% of the peptide yield over CDP.

Peptide Yield and Concentration of Trypsin. We set out to investigate further whether the relatively modest yield (50% of the yield of CDP) achieved after a 30-min digestion could be

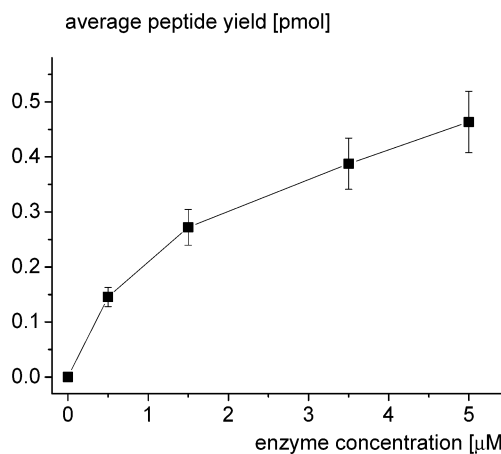


Figure 3. Effect of trypsin concentration on the peptide yield. Digestion was performed with the modified trypsin at 58°C for 30 min. Average peptide yield of the control digestion (CDP) was 0.21 pmol.

improved by increasing the concentration of trypsin. Modified trypsin is more stable toward autolysis compared to the native enzyme, and we anticipated its rapid inactivation would not occur (Table 1).

The yield of digestion rapidly increased with increased concentrations of trypsin (Figure 3). Typical K_m values for trypsin-catalyzed cleavage of low molecular weight chromogenic substrates are in the range of $100\text{--}200 \mu\text{M}$.²⁸ Venkatesh and Sundaram reported $K_m = 240 \mu\text{M}$ for the in-solution cleavage of BSA by bovine trypsin.³⁹ A typical band of 12% polyacrylamide gel containing 0.5 pmol of BSA with an approximate size of $0.8 \text{ mm} \times 0.8 \text{ mm} \times 6.4 \text{ mm}$ absorbs $4 \mu\text{L}$ of digestion buffer (see below). Assuming that it reasonably represents the internal void volume of the excised gel plug, the initial protein concentration would be $\sim 0.13 \mu\text{M}$, if the reaction occurs in-solution. Release of partially cleaved products temporarily increases the total substrate concentration, which may at some point exceed the molar concentration of trypsin, but it will still remain well below K_m . Consequently, trypsin is not saturated with substrates and the rate and completeness of cleavage will benefit from its higher concentration (Figure 2). In 30-min time period, a 3-fold higher concentration of the modified trypsin ($1.5 \mu\text{M}$) at 58°C helped to achieve the same yield as overnight digestion with the unmodified $0.5 \mu\text{M}$ enzyme at 37°C . However, we found it is not practical to apply trypsin at concentrations higher than $1.5 \mu\text{M}$ because of increased background of autolysis products. We note here that if another technology, with a higher dynamic range of detection and a higher ability to dissect protein mixtures, is employed (e.g., LC MS/MS), even higher concentrations of trypsin could be applied to further accelerate the digestion and improve the peptide yield.

Saturating Gel Pieces with Trypsin. In-gel digestion occurs in the milieu that restricts free diffusion and accessibility of enzymes and substrates. The enzyme is introduced by rehydrating dried gel pieces in a digestion buffer containing trypsin. It is therefore conceivable that the actual starting concentration of trypsin in the gel pieces is controlled by its diffusion during rehydration since a short exposure time (30 min) and a low temperature (4°C) minimize its inactivation by autolysis. We

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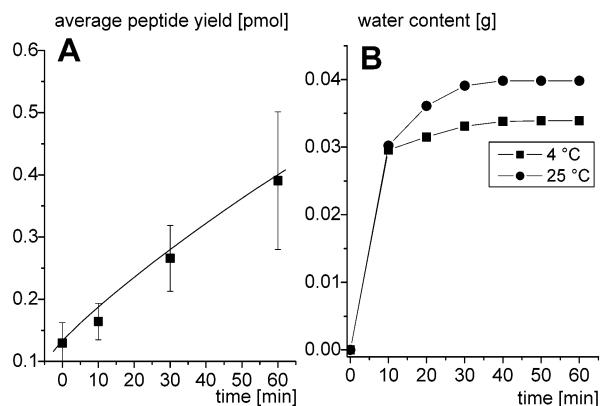


Figure 4. Saturation of dehydrated gel pieces with the trypsin solution and its effect on the peptide yield. (A) Peptide yield versus on rehydration time. (B) Amount of digestion buffer absorbed by gel pieces at 4 and 25 °C. Total weight of dry gel pieces was 5.2 mg.

monitored the time course of rehydrating pieces of 12% gel with the digestion buffer (Figure 4B). After ~30 min (regardless of the temperature), gel pieces did not absorb more buffer solution. Nevertheless, the yield of in-gel digestion was strongly dependent on the rehydration time and reached the limit neither after 30 nor after 60 min (Figure 4A). Thus, we concluded that penetration of trypsin inside the gel matrix (and, consequently, its actual concentration) is controlled by diffusion and proceeds much slower than swelling of dehydrated polyacrylamide matrix. The diffusion control might explain why proteases with relatively high molecular weights (e.g., thermolysin) are less efficient in in-gel (data not shown) compared to in-solution²⁴ digestion. Thus, despite the necessity of speeding up the analysis, the saturating step should not be shortened.

Further, we investigated how the surface area of gel pieces influenced the peptide yield. We observed that smaller sizes of gel pieces (and larger surface areas) positively affected the peptide yield (Figure 5A). Increasing the surface area by 40% improved the peptide yield by more than 50%. However, cutting gel pieces into pieces of even smaller sizes (less than 0.5 mm³) resulted in lower gains in the peptide yield and complicated handling of samples because of frequent clogging of the pipet tips and massive losses of gel material.

Smaller sizes of gel pieces enhanced rehydration (Figure 5B). The peptide yield was almost 50% higher for small (0.5-mm³) gel pieces dehydrated by acetonitrile. However, dehydration by acetonitrile did not enhance the digestion of uncut bands. Thus, in further experiments, gel slabs were cut into cubes of ~0.5 mm³, dehydrated with acetonitrile, and rehydrated with trypsin solution in the digestion buffer for at least 60 min.

Relative Performance of Conventional and Accelerated Digestion Protocols. We directly compared the performance of conventional (CDP) and accelerated (ADP) protocols by identifying members of a protein complex isolated from the budding yeast. A protein Set1 was epitope-tagged in the strain with deleted *eaf3* gene and was immunoaffinity purified using the tandem affinity purification (TAP) method.^{11,40} TAP isolation resulted in nine Coomassie-stained bands in the range of 15–130 kDa that

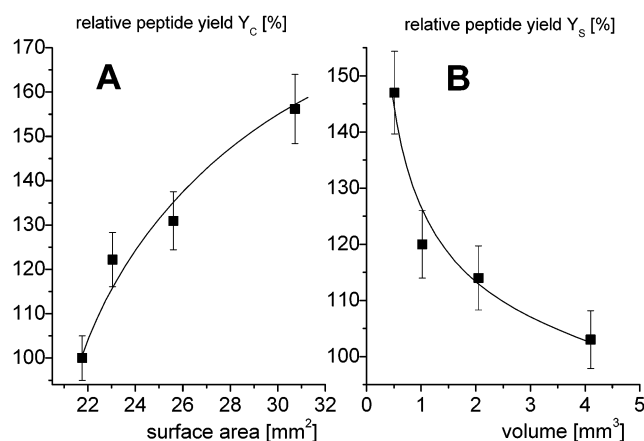


Figure 5. Effect of gel cutting (A) and gel dehydration (B) on the digestion yield. (A) Relative peptide yield versus the surface area of gel pieces, $Y_C = (Y_X/Y_1) \times 100\%$, where Y_X is the yield from the band cut in X equal pieces; Y_1 is the yield from the uncut band (reference). (B) Relative peptide yield from dehydrated gel pieces, compared to the yield from nondehydrated pieces of the same size, $Y_S = (Y_D/Y_{ND}) \times 100\%$, where Y_D is the yield from the gel pieces dehydrated prior to soaking in trypsin solution and Y_{ND} is the yield from nondehydrated gel pieces of the same size (reference). Total internal volume of gel pieces was ~4 mm³.

contained, by comparison with the staining intensity of BSA standards, 0.2–1.0 pmol of the protein material. Each band was cut into two parts, which were digested in parallel following CDP and ADP and were analyzed by MALDI TOF. The quality of peptide mass fingerprints and the certainty of protein identification were evaluated by MOWSE scores (a merit of statistical significance provided by MASCOT database searching software), by the number of peaks manually picked from the spectrum, and by the number of peaks matched to the sequence of identified proteins. Up to two sites of miscleavage were allowed in database searches. All proteins were positively identified by using ADP; CDP failed to identify both components of the protein mixture in the band 04 (Table 2). Peptide maps generated with CDP contained more incompletely digested peptides compared to ADP. Thus, being much faster (30-min digestion instead of overnight), ADP produced peptide mass maps of superior quality.

ADP demonstrated a similar performance in the characterization of other protein complexes isolated from budding and fission yeasts by TAP. Purification of interaction partners of 6 epitope-tagged bait proteins resulted in the successful identification of 112 unique proteins from Coomassie-detectable bands (data not shown).

CONCLUSION AND PERSPECTIVES

Reductive methylation of trypsin decreases autolysis and shifts the optimum of its catalytic activity to 50–60 °C, thus effectively transforming the enzyme into a thermophile-like protease. However, such modification does not alter its stringent substrate specificity and results in almost no increase in its molecular weight (and, consequently, its diffusion mobility). Therefore, the enzymatic cleavage can be carried out at higher temperature and higher enzyme concentration and complete digestion of protein substrates can be achieved much faster. It is also conceivable that targeted covalent modification by reactive oligomers could further

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Table 2. Comparison of Conventional and Accelerated Digestion Protocols in Protein Identification

| band | identification | MW | conventional digestion | | | accelerated digestion | | |
|------|----------------|---------|------------------------|----------------------|----------------------|-----------------------|---------|-------------------|
| | | | peaks | | score ^{b,c} | peaks | | score |
| | | | total ^a | matched ^d | | total | matched | |
| 01 | SET1 | 124 000 | 28 | 14 | <u>119</u> | 32 | 23 | <u>250</u> |
| 02 | BRE2 | 58 000 | 31 | 16 | <u>193</u> | 33 | 15 | <u>170</u> |
| 03 | SWD1 | 49 000 | 44 | 7 | <u>45</u> | 46 | 8 | <u>70</u> |
| 04a | CDC12 | 47 000 | 25 | na | na | 53 | 9 | <u>67</u> |
| 04b | TEF1 | 50 000 | 25 | na | na | 53 | 7 | <u>51</u> |
| 05 | SWD2 | 37 000 | 14 | 7 | <u>96</u> | 20 | 8 | <u>99</u> |
| 06 | SDC1 | 19 000 | 26 | 6 | <u>79</u> | 13 | 6 | <u>98</u> |
| 07 | RPS5 | 25 000 | 11 | 5 | <u>74</u> | 15 | 8 | <u>124</u> |
| 08 | RPS7 | 22 000 | 29 | 6 | <u>67</u> | 10 | 8 | <u>151</u> |
| 09 | RPS19 | 16 000 | 28 | 5 | <u>63</u> | 33 | 8 | <u>106</u> |

^a Total number of peaks picked. ^b MOWSE score. ^c Statistically significant scores are underlined; bold underlined: best score for compared experiments; na, not available; hits were not reported. ^d Number of peaks matching the sequence of identified proteins.

increase stability and catalytic activity of trypsin at elevated temperature by enhancing the rigidity of its secondary structure.

The quantitative study of in-gel digestion kinetics enables intelligent adjustment of the reaction conditions, depending on the available amount of proteins and specific goals of the experiment, either aimed at rapid protein identification or at the best possible quality of peptide mass fingerprints.

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