Development of an integrated system for activity-based profiling of matrix metallo-proteases

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A chemically modified, immobilized trypsin reactor for improved digestion reactor efficiency

Tryptic digestion followed by identification using mass spectrometry is an important step in many proteomic studies. Here, we describe the preparation of immobilized, acetylated trypsin for enhanced digestion efficacy in integrated protein analysis platforms. Complete digestion of cytochrome c was obtained with two types of modified-trypsin beads with a contact time of only 4 sec, while corresponding unmodified-trypsin beads gave only incomplete digestion. The digestion rate of myoglobin, a protein known to be rather resistant to proteolysis, was not altered by acetylating trypsin and requires a buffer containing 35% acetoniitrile to obtain complete digestion. The use of acetylated-trypsin beads led to fewer interfering tryptic autolysis products, indicating an increased stability of this modified enzyme. Importantly, the modification did not affect trypsin’s substrate specificity, as the peptide map of myoglobin was not altered upon acetylation of immobilized trypsin. Kinetic digestion experiments in solution with low-molecular-weight substrates and with cytochrome c confirmed the increased
catalytic efficiency (lower $K_M$ and higher $k_{cat}$) and increased resistance to autolysis of trypsin upon acetylation. Because of the increased trypsin activity, the digestion rate is enhanced, which facilitates on-line digestion of low abundance proteins with higher yields and in less time. These are favourable properties of the new chemically modified trypsin reactor and should make it a valuable tool in automated protein analysis systems.

## 4.1 Introduction

Protein digestion is one of the key elements in most proteomic studies. The most widely used proteomic method is separation by 2-dimensional gel electrophoresis [1] followed by staining and in-gel digestion of excised protein spots with specific proteases. Since selected proteases exhibit high cleavage specificity, each protein yields a unique peptide map, of which the masses can be determined by mass spectrometry (MS) to identify the protein by database search methods (peptide mass fingerprinting) [2–6]. Tandem mass spectra from individual peptides of a digested protein can also be used for identification [7, 8]. Another approach, less used but more automated, starts with digestion of the sample, followed by separation of the generated peptides by multidimensional chromatography and identification by tandem MS (shotgun proteomics) [9]. The pancreatic serine endoprotease trypsin is used mostly for digestion and has a well-defined substrate specificity. Digestion with trypsin is generally performed in solution, which presents a number of drawbacks that may limit the progress in high-throughput protein identification technology. Long digestion times (typically >5h) are needed because the trypsin-to-substrate ratio has to be kept low to avoid the appearance of interfering autolysis peptides. At low-micromolar substrate concentrations, the production of sufficient peptides to obtain positive protein identification becomes problematic with standard in-solution protocols, since the digestion rate is limited by substrate concentration [10]. The use of long incubation times and elevated temperatures inevitably leads to more digestion artifacts like transpeptidation and non-specific cleavage [11], deamidation and oxidation [12, 13], and trypsin autolysis products [14]. Another drawback is the manual sample handling and the extra steps that are required for the solution-based methods, which can lead to the loss of peptides and the introduction of contaminants like human keratins [12]. An approach to increase the speed, yield and robustness of the digestion process has involved the immobilization of proteolytic enzymes on solid supports. Digestion times are strongly reduced, because the effective protease concentration on the solid support is very high. Immobilized protease reactors based on
packed beads can also be incorporated into multidimensional separation systems for automated proteomics. Such an advanced degree of integration cannot be accomplished with in-solution digestions short of using robotic liquid handling systems.

Several papers have reported the implementation of digestion cartridges packed with Poroszyme immobilized-trypsin beads into integrated systems including mass spectrometric detection for automated peptide mapping [15–20]. The most advanced integration effort in this regard was achieved by Hsieh et al. [16], who demonstrated the implementation of the Poroszyme cartridge in an automated chromatography system including 5 columns. The stability of the immobilized trypsin cartridges was shown, with over 1000 injections resulting in no apparent decrease in performance [16,17].

Other applications involving digestion reactors include trypsin-coated agarose beads in a chip [21] and trypsin immobilized on porous silicon layers in a chip [22]. Trypsin immobilization on monolithic supports has resulted in fast digestions, caused by the almost complete lack of diffusional transport limitations [23–25]. A very effective, monolithic trypsin reactor has been described by Palm and Novotny, who were able to identify model proteins at a concentration of 80 nM [26]. Immobilization of trypsin by means of adsorption onto a membrane, which is then integrated into a chip or a capillary, resulted in sensitive protein identification (low fmol amounts) [27–29]. Another approach is the encapsulation of trypsin in a gel network on the surface of various supports [30–32].

The digestion efficiency of (proteolytically resistant) proteins using immobilized trypsin can be enhanced by the use of organic solvents during digestion [33,34]. This is because proteins tend to denature in the presence of organic solvents like acetonitrile, thereby increasing cleavage-site accessibility. This does, however, also increase trypsin autolysis to a point that the most intense peaks resulted from autolysis at low protein concentrations [33]. Chemical modification has been successfully used to enhance the stability of enzymes towards thermal denaturation and autolytic inactivation in solution. However, one attempt to reduce autolysis of immobilized trypsin by reductive methylation of the primary amine groups met with limited success, because reduced autodigestion was accompanied by a decrease in activity [35]. Other modifications to stabilize trypsin in solution included conjugation with methoxypolyethylene glycols (PEGylation), intra-molecular cross-linking, conjugation with β-cyclodextrin derivatives, transformation of tyrosines into aminotyrosines, and acetylation [36–41]. The effect of most of these modifications was assessed with low-molecular-weight substrates. It therefore remains to be seen how they affect the digestion of proteins.
These examples clearly illustrate the great interest in immobilized trypsin and the rapid developments in this field. However, a number of limitations remain, notably with respect to catalytic activity and autolysis. As part of our interest in developing automated proteome analysis platforms using integrated on-line digestion, we describe here that acetylation of immobilized trypsin results in enhanced stability and catalytic activity. We show that the use of chemically modified, immobilized trypsin results in preserved or even improved digestion efficiencies (depending on the protein substrate) while reducing trypsin autolysis.

4.2 Material and methods

4.2.1 Chemicals and materials

Trypsin (TCPK-treated (tosyl phenylalanyl chloromethylketone), bovine pancreas, 10,000-15,000 BAEE units/mg protein), cytochrome c (bovine heart, >95% pure), myoglobin (horse heart, >90% pure), benzamidine (>98% pure), calcium chloride (>99% pure), ethanolamine (>98% pure), trifluoroacetic acid (>99% pure), tris(hydroxymethyl)aminomethane (Tris, >99.5% pure) and NaN₃ (>99.5% pure) were purchased from Sigma. Formic acid (>98% pure) was obtained from Merck. Acetic acid N-hydroxysuccinimide (NHS) ester (AANHS), Brij-35 was from ICN Biomedicals while NHS-activated Sepharose 4 fast flow was obtained from Amersham. Benzyloxycarbonyl-Phe-Arg-7-amino-4-methylcoumarin (Z-Phe-Arg-AMC) was purchased from Bachem, and Z-Leu-Arg-AMC was from Novabiochem. Poroszyme immobilized-trypsin beads were from Applied Biosystems, while immobilized-trypsin agarose beads were supplied by Pierce. Acetonitrile was from Biosolve, Ultra-pure water, produced by an Elga purification system, was used for all buffer and mobile phase preparations.

4.2.2 Trypsin immobilization and modification

The following solutions were used for trypsin immobilization: wash solution, 1 mM HCl; wash buffer, 0.1 M K₂HPO₄, pH 7.8; coupling buffer, 0.1 M K₂HPO₄, 5 mM ethanolamine, with or without 4 mM benzamidine, pH 7.8; modification buffer, 0.1 M K₂HPO₄, pH 7.8, 22 mM AANHS; blocking buffer, 0.5 M ethanolamine, pH 8.0.

Prior to trypsin immobilization, NHS-activated Sepharose beads were washed
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with 10 volumes of wash solution and wash buffer at 4°C. An equal volume of 20 mg/ml trypsin (dissolved at 0°C) in coupling buffer was added to the beads and incubated for 25 min at 25 °C with rotary shaking at 1100 rpm. After immobilization, the supernatant was removed and trypsin beads were modified by the addition of an equal volume of modification buffer (20 min incubation at 25 °C and 1100 rpm). Excess reactive NHS groups were blocked by the addition of 5 volumes of blocking buffer (incubated for 10 min as described above). Pierce and Poroszyme trypsin beads were modified after washing with wash buffer, using the same modification procedure. All trypsin beads were stored at 4 °C in 50 mM Tris, pH 8.2, 1 mM CaCl₂, 0.02% NaN₃ (storage buffer).

4.2.3 HPLC analysis

Samples from the trypsin solution before and after immobilization were diluted 80 times with 0.1% TFA in water and analyzed for their trypsin content by HPLC (Merck-Hitachi) on a C₄ column (Vydac, 250 mm, 2.1 mm i.d., 5 μm, 300 Å pore size). Detection was at 214 nm, and a 20 μl injection volume was used. Elution was performed using a linear gradient with a mobile phase composition ranging from 25% to 55% acetonitrile (in water + 0.1% trifluoroacetic acid) in 25 min at a flow rate of 0.25 ml/min.

4.2.4 Digestion experiments

The different trypsin beads were slurry-packed with μm pore size) from Spark-Holland (Emmen, NL). Samples were pumped through the cartridge (housed in a clamp from Spark-Holland) with a syringe pump (KD Scientific). The trypsin cartridges were washed with 20 cartridge volumes of a mixture of 50 mM Tris, pH 8.2, and acetonitrile (1:1), followed by 20 cartridge volumes of digestion buffer (50 mM Tris, pH 8.2) before sample loading. Digestion of protein samples was performed at room temperature unless indicated otherwise. Cytochrome c was digested at a concentration of 4 μM in 1 mm cartridges at a flow rate of 40 μl/min (residence time of 4 s). Myoglobin was digested at a concentration of 4 μM in 2 mm cartridges at 8 μl/min (residence time of 1.4 min) and varying percentages of acetonitrile in the digestion buffer. Trypsin autolysis experiments were performed with 2 mm cartridges packed with trypsin immobilized on Sepharose fast flow. Directly after washing with a solution containing digestion buffer and acetonitrile (1:1), 120 μl of digestion buffer (4
cartridge volumes) was pumped through the cartridges at 4 µl/min and analysed by LC-MS.

4.2.5 LC-MS analysis

All protein digest analyses were performed on an Agilent 1100 capillary HPLC system equipped with a Vydac C8 column (250 mm, 1 mm i.d., 5 µm, 300 Å pore size), coupled on-line to an SL ion trap mass spectrometer (Agilent). 16 pmol (4 µl) of total protein digest were injected for each run. For the autolysis experiment, 8 µl were injected. Peptides were eluted in a linear gradient (0.75% acetonitrile/min) from 3 to 47% acetonitrile with 0.1% formic acid at a flow rate of 65 µl/min.

4.2.6 Differential modification of trypsin in solution

Trypsin was acetylated in solution by gradual, stepwise addition of 1 M AANHS (dissolved in acetonitrile) to a 0.5 mM trypsin solution in 20 mM K2HPO4, 5 mM benzamidine, pH 8.0 at 25°C and shaking at 900 rpm. Increasing volumes of AANHS solution were added at 0, 7, 14 and 21 min, resulting in final concentrations of respectively 5, 15, 30 and 50 mM of added total AANHS.

The reaction was terminated by dilution of the samples, taken from the reaction mixture at different time points, with 50 mM Tris, pH 8.5 (to a final trypsin concentration of 1.25 µM). Diluted samples were stored on ice for kinetic experiments.

The extent of modification and the level of heterogeneity introduced by the modification reaction was monitored by direct infusion of the solutions into the mass spectrometer (Agilent, SL ion trap) at a flow rate of 5 µl/min with a KD Scientific syringe pump. For this analysis, samples were taken from the reaction mixture at different time points and diluted 100 times to 5 µM trypsin with acetonitrile/water 2:3 (v/v) and 0.1% formic acid.

4.2.7 Kinetic measurements

The rate of cytochrome c digestion in solution was determined as a function of the degree of trypsin modification. The digestion reaction was performed with 500 µg/ml cytochrome c and 20 µg/ml trypsin in 50 mM Tris, pH 8.5 at 37°C and 900
rpm (rotary shaking). The digestion reaction was monitored by LC-MS analysis as described earlier (after 10-fold dilution of the samples with 0.25% formic acid in water).

Proteolysis rates of Z-Leu-Arg-AMC and Z-Phe-Arg-AMC were measured in duplicate at 12.5, 16.7, 25 and 50 µM substrate concentrations, with 10 nM differentially modified acetyl-trypsin species in 50 mM Tris buffer (pH 8.5) containing 10 mM CaCl$_2$ and 0.01% Brij-35 (w/v). The assay was carried out at 25°C in 96-well plates (Costar-white) and monitored over 4 min with a Fluorostar Optima plate reader (BMG Labtech) with $\lambda_{ex,em} = 390, 440$ nm. $K_M$ and $k_{cat}$ values were obtained from Lineweaver-Burk plots.

4.3 Results and Discussion

4.3.1 Trypsin immobilization

Trypsin was immobilized in a one-step reaction to N-hydroxysuccinimide (NHS)-activated Sepharose. Since trypsin is often immobilized at slightly basic pH, there is a considerable risk of autodigestion before and during immobilization. This would not only reduce the amount of active immobilized trypsin but could also lead to immobilized autodigestion products. This in turn may result in reduced activity, altered substrate specificity and increased non-specific adsorption. Figure 4.1 shows that inclusion of the reversible trypsin inhibitor, benzamidine, prevents trypsin autodigestion before and during immobilization, as indicated by a reduced number of earlier eluting peaks (trypsin autolysis peptides). Determination of the immobilized trypsin concentration was done indirectly by measuring the decrease in height of the major trypsin peak (15-16 min) in the supernatant before and after immobilization. The immobilized trypsin concentrations in the absence and presence of benzamidine were calculated to be 0.6 and 0.7 mM, respectively. When comparing the traces in Fig. 4.1A before and after immobilization, it becomes obvious that most of the autodigestion products are also immobilized. Furthermore, the digestion of immobilized active trypsin caused by remaining trypsin in solution during immobilization is likely decreased by benzamidine. For these reasons, the real concentration of intact trypsin immobilized in the absence of benzamidine is probably lower than the indirectly determined concentration.
4.3.2 The effect of acetylation on digestion efficiency

There are many papers describing the chemical modification of enzymes in solution to enhance their stability, for application in different areas such as the food industry, proteomics and use as biopharmaceuticals [42]. In proteomics and protein analysis, it is recognized that immobilization itself may have a strong stabilizing effect, reducing denaturation and autolysis. There is only one report that describes an attempt to modify immobilized trypsin by reductive methylation to further increase its stability [35]. In this case, modification resulted in reduced autolysis but was also accompanied by a decrease in trypsin activity. In order to investigate this further, we selected a mild, single-step modification of the primary amino groups of lysine and the N-terminus of trypsin with acetic acid N-hydroxysuccinimide-ester (AANHS). This modification is known to stabilize trypsin with respect to autolysis and thermal denaturation, with the additional advantage that the steric changes introduced to trypsin are only minor in nature [39]. Interestingly, acetylation of immobilized trypsin led to a striking enhancement of the cytochrome c digestion rate, as shown in Figure 4.2. This enhancement was unexpected, given the slight increase in trypsin activity for the low-molecular-weight substrate benzyol arginine p-nitroanilide (BAPNA) upon acetylation, observed by Murphy et al. [39].

![Figure 4.1: HPLC analysis of trypsin in immobilization buffer before and after covalent coupling to NHS-activated Sepharose beads. The immobilization supernatant, before (upper traces) and after (lower traces) immobilization is given, in the absence (A) and presence (B) of 4 mM benzamidine.](image-url)
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Figure 4.2: Effect of acetylation on the trypsin digestion rate of 4 μM cytochrome c, analyzed by LC-MS. Trypsin immobilized on the different solid-supports (A) Sepharose, (B) Agarose (Pierce beads) and (C) Poroszyme (Polystyrene-Divinylbenzene) beads was studied. Digestion was performed in 1 mm cartridges at a flow rate of 40 μL/min (residence time 4 s). Intact cytochrome c peaks are denoted by asterisks.

The absence of a negative effect on trypsin activity resulting from the increased hydrophobicity of the trypsin surface, due to the conversion of positively charged amines into neutral amides, is surprising. In fact, it was anticipated that the charged side chains were important for enzyme activity, either by affecting substrate binding, or indirectly affecting the enzyme’s three-dimensional structure. Also noteworthy is the fact that the increased digestion efficiency is independent of the type of solid support used. Nearly complete digests were obtained for both modified Poroszyme and Sepharose trypsin beads with a residence time of only 4 sec (Fig. 4.2, panels A and C). Although trypsin immobilized on agarose beads
produced a less complete cytochrome c peptide map, the digestion rate was also enhanced upon acetylation (Fig. 4.2, panel B). Peptide fragments 1 and 2 in Figure 4.2B are the result of the first cleavage in cytochrome c, since their masses minus a water molecule add up to the mass of cytochrome c. They also appear as the first digestion products in the cascade of reactions leading to a complete digest (see Fig. 4.8).

Although acetylation-dependent enhancement of digestion efficiency was evident for cytochrome c, it may not be predictive of the digestion efficiency for other proteins. Therefore, we also tested the impact of acetylation using trypsin Poroszyme beads on the digestion efficiency of myoglobin. This protein is known to be difficult to digest due to a high structural stability. A certain percentage of organic solvent in the digestion buffer is required to obtain complete digestion in the absence of other digestion-enhancing denaturing chemicals such as chaotropic agents or surfactants [33]. Acetonitrile is thought to cause (partial) unfolding of myoglobin, resulting in better accessibility of the tryptic cleavage sites.

![Figure 4.3: Effect of acetonitrile on the digestion efficiency of 4 µM myoglobin, performed with regular and acetylated Poroszyme beads (2 mm cartridge at 8 µL/min, residence time 1.4 min) analyzed by LC-MS. (A) 15% acetonitrile, acetylated Poroszyme, (B) 35% acetonitrile, regular Poroszyme, (C) 35% acetonitrile, acetylated Poroszyme (intensity scales are equal for the three traces). Peak labeling: asterisks denote tryptic myoglobin peptides, t denotes trypsin autolysis peptides. The arrow indicates the conversion of the peptide HGTVVLTALGGILKK into the corresponding peptide without the C-terminal lysine (no missed cleavages (MC)) with acetylated, immobilized trypsin.](image-url)
4.3. Results and Discussion

Figure 4.3A shows that acetylation of immobilized trypsin alone did not result in enhanced myoglobin digestion rates at low levels of acetonitrile, as only a large undigested myoglobin peak could be detected. The same chromatogram was obtained with unmodified trypsin cartridges (data not shown). The fact that the modification does not improve the digestion rate at these low acetonitrile levels can be explained by limited cleavage-site accessibility. This is probably also true for other types of protease modifications and when tightly-folded proteins with inaccessible cleavage sites are considered. Hence, the need for unfolding agents in on-line digestion will not be reduced by chemical modification of immobilized proteases. Figures 4.3B and 4.3C show that 35% acetonitrile is sufficient to obtain complete myoglobin digestion with both types of immobilized trypsin at a contact time of 1.4 min. Again, we did not observe an acetylation-dependent enhancement of myoglobin digestion rates under these conditions.

4.3.3 Cleavage site specificity and autolysis

Figures 4.3B and 4.3C show that the myoglobin digestion pattern is not greatly affected by trypsin acetylation, because all the peptides obtained with modified trypsin were also identified in the peptide map obtained with the unmodified beads. Maintenance of cleavage site specificity is of crucial importance for the correct assignment of peptide maps or peptide sequence tags obtained by mass spectrometry. The major difference is the complete conversion of a peptide having 1 missed cleavage (present in the digest obtained with unmodified Poroszyme, Fig. 4.3B, 1 MC) into the corresponding completely digested peptide (indicated by the arrow in Fig. 4.3) when digestion is performed with modified Poroszyme beads. This may be an indication of a slight change in specificity with respect to the P1’ position (C-terminal to the scissile bond). Though it is well known that trypsin prefers to cleave after Arg and Lys, some residues neighboring these bonds (P2 and P1’ positions) are known to have a negative influence on the cleavage [43]. The peptide with 1 missed cleavage is not completely digested, because it has two adjacent Lys residues (P1 and P1’) preceded by a Leu residue (P2 position), which is in agreement with results obtained by Keil [43]. The fact that modified trypsin is able to convert the Lys-Lys bond with higher efficiency is an indication that cleavage efficiency by modified trypsin is less affected by a Lys residue in the P1’ position. This result is in agreement with the work of Elsner et al. [44], who showed that succinylation of trypsin’s lysine residues leads to an altered substrate specificity through better acceptance of basic residues in the P1’
binding pocket. This shifted substrate specificity is explained by the modification of Lys-63 in the S1’ binding pocket of trypsin, leading to conversion of a positive into a negative charge. Although acetylating Lys-63 only neutralizes the positive charge, it probably also leads to better acceptance of basic residues by decreased electrostatic repulsion.

Another difference observed in the chromatogram of myoglobin digested in 35% acetonitrile using regular and acetylated Poroszyme trypsin beads is the reduced intensity of trypsin autolysis peptides after acetylation (Figs. 4.3B and C). Although the autolysis peptides have a relatively low intensity compared with the high concentration of myoglobin-derived peptides at 4 µM, they would interfere strongly with the analysis if protein substrates at lower concentrations were to be analyzed. This has been reported by others when digestion was performed with unmodified trypsin beads at lower substrate concentrations. [33,35] Autolysis also leads to decreasing digestion efficiencies over time and can potentially alter the cleavage specificity [43].

Figure 4.4: Effect of acetylation on the autolysis of trypsin immobilized on Sepharose. Digestion buffer (50 mM Tris, pH 8.2) without protein substrate is slowly pumped through a 2 mm cartridge at 4 µL/min (residence time 2.7 min), to be collected and analyzed by LC-MS. The upper trace is obtained using a regular trypsin cartridge, and the lower trace represents an acetylated trypsin cartridge (both traces are plotted with the same intensity scale). Identified trypsin autolysis peptides are numbered according to their position in the primary structure and given in Table 4.1.
Figure 4 shows that acetylation also reduces autolysis of immobilized trypsin in acetonitrile-free digestion buffer. Decreased autolysis has already been observed for acetylated-solution-phase trypsin, so it was expected that the same holds true for immobilized trypsin [39]. While some autolysis peaks disappeared completely, others were strongly reduced and two new autolysis peptides (peaks 4 and 5 in Fig. 4.4B) appeared after acetylation. The mass increase corresponding to one acetylation, in combination with one missed cleavage after a lysine residue proved acetylation of Lys-89 and Lys-111 in these two peptides (Table 4.1). Although strongly reduced, autolysis peptides 2 and 3 are still present after acetylation, showing that acetylation of Lys-89 is not complete (see Table 4.1). Further information about the acetylation pattern can be derived from other changes in peptide signals, indicating that Lys-63, 145, 156, 169, 190 and 237 are also modified. From the appearance of peptides 4 and 5 it can be concluded that Lys-109 was hardly modified. For the five other lysine residues, located mainly near the C-terminus, no information can be obtained from this experiment, since they were not observed among the autolysis peptides for either unmodified or modified immobilized trypsin. This could also be an indication that this part of the trypsin structure is most autolysis-resistant.

Besides the autolysis peptides given in Table 4.1, there are a number of peaks that could not be identified. Some of them may originate from the fact that the samples were not reduced and alkylated before LC-MS analysis, in order to cleave and block disulfide bridges or prevent disulfide bond shuffling between cysteine-containing peptides. With only one cysteine-containing autolysis peptide identified (out of twelve cysteine residues in trypsin), these are indeed underrepresented.

<table>
<thead>
<tr>
<th>Number</th>
<th>Sequence</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(K)-SGIQVR</td>
<td>64-69</td>
</tr>
<tr>
<td>2</td>
<td>(R)-LGEDNINVVEGNEQFISASK</td>
<td>70-89</td>
</tr>
<tr>
<td>3</td>
<td>(K)-SIVHPSYNSTLNNIDMLIK</td>
<td>90-109</td>
</tr>
<tr>
<td>4</td>
<td>(R)-LGEDNINVVEGNEQFISASK$_{Ac}$ SIVHPSYNSTLNNIDMLIK</td>
<td>70-109</td>
</tr>
<tr>
<td>5</td>
<td>(K)-LK$_{Ac}$SAASLNRS</td>
<td>110-119</td>
</tr>
<tr>
<td>6</td>
<td>(K)-SAASLNRS</td>
<td>112-119</td>
</tr>
<tr>
<td>7</td>
<td>(K)-SSGTSYPDVLK</td>
<td>146-156</td>
</tr>
<tr>
<td>8</td>
<td>(K)-SAYFGQITSNMFACAGYLEGGK</td>
<td>170-190</td>
</tr>
<tr>
<td>9</td>
<td>(K)-QTIASN (C-terminus)</td>
<td>238-243</td>
</tr>
</tbody>
</table>

**Table 4.1:** Identified trypsin autolysis peptides. The numbers correspond to the peaks shown in Fig. 4.4. The amino acid preceding the scissile bond is given in parentheses. Numbering of amino acids is according to entry P00760 in the Swiss-Prot database.
Although trypsin contains only two arginines (Arg69 and 119), it is striking that both are involved in the appearance of the autolysis peptides 4 and 5 after acetylation. This indicates the important role of arginine in autolysis, an observation that is in agreement with earlier results from bovine trypsin autolysis experiments, showing that Arg115-Val116 is an important autolytic cleavage site [45].

4.3.4 Acetylation of trypsin in solution

A more accurate idea about the degree of acetylation and its heterogeneity was obtained by monitoring the reaction using direct infusion ESI-MS of strongly diluted and acidified samples taken during the acetylation reaction of trypsin in solution. Figure 4.5A shows part of the mass spectrum of native trypsin before AANHS addition. Besides some major peaks (labeled with their charge states), several phosphate adducts can be observed, originating from the modification buffer (see also Fig. 4.5B). This was confirmed by infusion experiments with native trypsin in the absence of phosphate, in which these adduct peaks were not observed (data not shown). Figures 4.5B-E show the different degrees of trypsin acetylation based on a shift of the 11+ charge state at increasing reaction times and reagent concentrations. This charge state was used to monitor the reaction, because the higher charge states disappeared at higher degrees of acetylation due the depletion of positive charges. Each acetyl conjugation corresponds to the expected m/z shift of 42/11=3.8 amu. Figure 4.5 reveals heterogeneity of at least 6 different acetyl-conjugated species present at all stages of the reaction. This insight cannot be obtained with the indirect reagent monitoring method, using 2,4,6-trinitrobenzenesulfonic acid (TNBS), which gives only an average number of acetylations [38, 39]. It is noteworthy that even after 32 minutes of reaction with a 6.7-fold molar excess of AANHS (after all reagent additions) over primary trypsin amine groups, the population of acetylated trypsin species has a distribution with the same width as at the beginning of the reaction. This heterogeneity is probably a reflection of differences in primary amine reactivity, which depends on steric factors and the local physico-chemical surrounding.
Figure 4.5: MS analysis of the trypsin acetylation reaction kinetics. A: mass spectrum of trypsin before AANHS addition (peak labels indicate the charge state). B-E: zoomed in at the mass spectra of the 11+ charge state of differentially modified trypsin at 0, 5, 16 and 32 minutes of reaction time (6.7-fold molar excess of AANHS (after all stepwise reagent additions) over primary trypsin amine groups). Labels in C, D and E indicate the number of introduced acetyl groups.
4.3.5 The effect of trypsin acetylation on cytochrome c digestion in solution

To investigate the influence of the degree of acetylation on the catalytic activity of trypsin, cytochrome c was digested in solution with native trypsin and three different acetyl-trypsin species, and the digestion reaction was monitored by LC-MS analysis. The digestion kinetics were determined by plotting the level of intact cytochrome c and that of three fully digested peptides against the digestion time as determined by the peak area in extracted ion chromatograms. Figure 4.6 confirms that the acetylation-dependent increase in trypsin activity that was observed for immobilized trypsin is not related to immobilization as such. While cytochrome c is completely degraded into fragments (regardless of the size) within 40 minutes with all three types of acetylated trypsin, the digestion with native trypsin reached only 20% after 75 minutes. A similar trend is
observed for the three fully digested peptides (without missed cleavages), though on a slightly longer time scale, because these are the end products in the digestion pathway. Nearly complete digestion is reached after about 75 minutes with the three types of acetylated trypsin while the conversion with native trypsin remains around 20%. With respect to the digestion kinetics, differences between different species of acetylated trypsin were less strong but still significant. Trypsin with the highest degree of acetylation is the most active and digests cytochrome c with the highest rate. It is assumed that the different acetylated trypsin species would also exhibit differing rates of digestion if immobilized.

Besides the three peptides given in Figure 4.6, all other fully digested peptides consisting of 5 amino acids or more could be detected. The kinetic profiles of these 6 peptides were generally very similar compared to the profiles given in Figure 6, indicating overall enhanced digestion kinetics of cytochrome c. Altogether, these 9 fully digested peptides represented 79% of the total cytochrome c amino acid sequence.

Also noteworthy is the observation that acetylated trypsin is stabilized against autolysis. After 75 min of cytochrome c digestion, the peak area of the autolysis peptides 2 and 3 (also detected during the autolysis experiment with immobilized trypsin, see Figure 4.4) are reduced by a factor 10 and 18, respectively, for modified trypsin (containing an average of 11 acetylations) compared to native trypsin (data not shown). These features also promote the use of solution-phase acetylated trypsin for improved digestion yields in traditional solution-phase digestion protocols.

4.3.6 Cytochrome c digestion pathway

The detection of many intermediates with 1 or more missed cleavages, allowed the construction of the digestion pathway through which the fully digested peptides are formed. An example of such a digestion pathway is given in Figure 4.7, together with the kinetic profiles of all the peptides in this pathway. Despite the low number of points defining the kinetic profile of the first cytochrome c digestion products, their rapid appearance and rapid further degradation confirms the enhanced digestion kinetics of acetylated trypsin. This pathway also shows that the positive modification effect is not only based on a coincidental enhancement of just one rate limiting step. All pathway intermediates are produced faster and degraded faster with acetylated trypsin, thereby proving that the proteolytic kinetics for all cleavage sites is enhanced.
Figure 4.7: Cytochrome c digestion pathway presented with the kinetic peptide profiles measured by LC-MS. The kinetic profiles were obtained by digestion with native trypsin (Δ) and trypsin with an average of 4 (○) and 11 (×) acetyl conjugations, respectively (see Fig. 4.5). LC-MS signals were normalized with the maximal intensity given above the y-axis. Peptides are indicated by their amino acid positions within intact cytochrome c, and the number of tryptic cleavage sites (missed cleavages) is given between brackets.
Downstream in the pathway, the lifetime of most intermediates increases, probably because of a decreasing number of potential cleavage sites, and the fact that the most favorable cleavage sites have already been hydrolyzed. The two remaining trypsin cleavage sites, ERE and LKK in peptide 89-100, are predicted to be rather resistant to tryptic cleavage, as was investigated in a study by Keil who investigated the influence of the amino acids in the P2 and P1’ positions during tryptic cleavage [43]. After 75 minutes of digestion, 2 intermediates (88-100 and 89-100) are still present at considerable levels (indicated by the high signal intensities), which means that digestion was still incomplete in this part of the pathway. Interestingly, the fully digested peptide 92-99 is degraded further by non-specific cleavage after a tyrosine residue. This non-specific cleavage occurs with both native trypsin and the acetylated trypsin species and may be due to a slight contamination with chymotrypsin.

**Figure 4.8:** Digestion pathway of cytochrome c, showing all detected (by LC-MS analysis) pathway intermediates and end products. Peptides are indicated by their amino acid positions within intact cytochrome c, and the number of tryptic cleavage sites (missed cleavages) is given between brackets.

Figure 4.8 shows the complete tryptic digestion pathway of cytochrome c, in which all detected intermediates with different numbers of missed cleavages, and
end products can be found. The pathway also shows the digestions steps through which the complete digestion into peptides without missed cleavages most likely occurs. Though, the pathway may be incomplete, because some intermediates may be rapidly degraded further and do not reach levels at which they can be detected. All peptides shown in this pathway have been detected in the digestions with native and acetylated trypsin, again indicating that trypsin’s substrate specificity to cleave after Arg and Lys is not affected by the acetylation. The kinetic profiles, which could not be shown for all peptides, generally resemble the profiles given in Figure 4.7. The early intermediates with 3 missed cleavages or more, such as peptides 1-79, 1-55 and 9-27 can only be detected during the very early stages of digestion in the case of acetylated trypsin. When digested with native trypsin they are detected for much longer periods of time, be it at lower levels, due to the slower digestion rate.

Towards the end of the digestion pathway, the differences between the kinetic profiles of different peptide intermediates increased. Most peptides with 1 missed cleavage were processed further into the fully degraded peptides, since there levels first increased and later decreased again. However, four peptides (8-13, 39-53, 56-73 and 73-79) with 1 missed cleavage were rather resistant to trypsin and nearly reached a constant level (for the different modified trypsin species) after 75 min of digestion.

Table 4.2 gives the sequences of all peptides with 1 missed cleavage. In three of

<table>
<thead>
<tr>
<th>Position</th>
<th>Sequence</th>
<th>Trypsin resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-13</td>
<td>KIFVQK</td>
<td>Yes</td>
</tr>
<tr>
<td>9-22</td>
<td>IFVQKCAQCHTVEK</td>
<td>No</td>
</tr>
<tr>
<td>14-25</td>
<td>CAQCHTVEKEKK</td>
<td>No</td>
</tr>
<tr>
<td>26-38</td>
<td>HKTGPNLHGLFGGRK</td>
<td>No</td>
</tr>
<tr>
<td>28-39</td>
<td>TGPNLHGLFGGRK</td>
<td>No</td>
</tr>
<tr>
<td>39-53</td>
<td>KTQAPGFSYTDANK</td>
<td>Yes</td>
</tr>
<tr>
<td>40-55</td>
<td>TQAPGFSYTDANKNKK</td>
<td>No</td>
</tr>
<tr>
<td>56-73</td>
<td>GITWGEETLMEMEYLENPKK</td>
<td>Yes</td>
</tr>
<tr>
<td>73-79</td>
<td>KYIPGTK</td>
<td>Yes</td>
</tr>
<tr>
<td>74-86</td>
<td>YIPGTKMIFAGIHK</td>
<td>No</td>
</tr>
<tr>
<td>80-87</td>
<td>MIFAGIKK</td>
<td>No</td>
</tr>
</tbody>
</table>

Table 4.2: Cytochrome c peptides with with 1 MC. Tryptic cleavage sites (P2-P1') are given in bold letters. Numbering of amino acids is according to entry P00006 in the Swiss-Prot database.

the trypsin resistant peptides, the cleavage sites were located completely at the N-terminus (without any Lys preceding amino acid in the P2 position), indicating the tryptic resistance of these N-terminal cleavage sites. Such N-terminal cleavage
sites were indeed not present in the peptides which were degraded rapidly (see sequences in Table 4.2). Since the digestion with native trypsin proceeded more slowly, a 16 h cytochrome c digestion was performed to be able to compare with the faster digestions with modified trypsin.

The four digestion resistant 1 MC peptides were detected at similar levels after 16 h digestion compared to 75 min digestion with modified trypsin, indicating that native trypsin has the same difficulties to cleave these sites. This type of trypsin cleavage behavior was not described by Keil, who investigated the influence of the amino acid surrounding the Arg- and Lys- cleavage bonds, but did not study N-terminal cleavage sites [43].

Apparently, acetylation does not suppress non-specific cleavage events, since with both native and modified trypsin two peptides which are the result of a non-specific cleavage after aromatic amino acids (phenylalanine in 28-38 and tyrosine in 92-99) are detected. After the 16 h incubation with native trypsin, both peptides were present at about 5- and 10-fold higher levels compared to a 75 min digestion with maximally acetylated trypsin. Thus, because shorter digestion incubation times can be used with acetylated trypsin, the non-specific cleavage can be significantly suppressed.

The effect of chemical modifications on in-solution tryptic digestion has to date been mainly studied with low-molecular-weight substrates, generally resulting in a slight increase in activity. Conversion of synthetic substrates is, however, not predictive of the effect on protein digestion, since steric factors are less critical for the conversion of low-molecular-weight substrates. Inefficient digestion of casein and bovine serum albumin was observed when trypsin conjugated with bulky $\beta$-cyclodextrins [36] or an extremely bulky carbohydrate containing-polymer [46] was used. This indicates the influence that steric factors can have on protein digestion. The steric changes caused by acetylation of immobilized trypsin are apparently too small to affect the digestion of macromolecular substrates such as cytochrome c.

4.3.7 Acetylation of trypsin and catalytic activity in solution

It is difficult to determine biochemical rate constants of protein digestion experimentally, because proteins are essentially a collection of substrates within the same molecule due to the numerous cleavage sites. Another complicating factor is the fact that proteins are folded, resulting in lower digestion rates of cleavage sites.
that are (initially) inaccessible but may become accessible as digestion proceeds. To investigate how the kinetic properties of trypsin are affected by the degree of acetylation, the Michaelis-Menten kinetics of trypsin were determined with the fluorescent substrates Z-Phe-Arg-AMC and Z-Leu-Arg-AMC.

Table 4.3 shows that the increased digestion efficiency that was observed for cytochrome c upon acetylation is also reflected by lower $K_M$ and higher $k_{cat}$ values for the low-molecular-weight substrates. Although $k_{cat}$ only increases slightly, the $K_M$ value decreases strongly upon acetylation for both substrates. These changes in rate constants may also explain the increased overall activity of immobilized trypsin and trypsin in solution with respect to cytochrome c digestion. The increased hydrophobicity of the enzyme surface in general and of the $S_1'$ binding pocket in particular may lead to a higher affinity for the substrates with a hydrophobic fluorophore in the $P_1'$ position. Because the trypsin assay is done under steady-state conditions while the cytochrome c digestion is performed until completion, the two experiments are not fully comparable. However, the digestion rate enhancing effect of acetylation is stronger for cytochrome C than for the low-molecular-weight substrates.

<table>
<thead>
<tr>
<th>Z-Phe-Arg-AMC</th>
<th>Kinetic parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin preparation ((\ast))</td>
<td>$K_M$ ((\mu M))</td>
</tr>
<tr>
<td>Native</td>
<td>55 ± 7</td>
</tr>
<tr>
<td>Trypsin-2.5Ac</td>
<td>41 ± 4</td>
</tr>
<tr>
<td>Trypsin-5Ac</td>
<td>37 ± 2</td>
</tr>
<tr>
<td>Trypsin-8Ac</td>
<td>26 ± 2</td>
</tr>
<tr>
<td>Trypsin-9.5Ac</td>
<td>21 ± 1</td>
</tr>
<tr>
<td>Trypsin-12Ac</td>
<td>18 ± 1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Z-Leu-Arg-AMC</th>
<th>Kinetic parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin preparation ((\ast))</td>
<td>$K_M$ ((\mu M))</td>
</tr>
<tr>
<td>Native</td>
<td>42 ± 1</td>
</tr>
<tr>
<td>Trypsin-2.5Ac</td>
<td>30 ± 1</td>
</tr>
<tr>
<td>Trypsin-5Ac</td>
<td>29 ± 1</td>
</tr>
<tr>
<td>Trypsin-8Ac</td>
<td>23 ± 2</td>
</tr>
<tr>
<td>Trypsin-9.5Ac</td>
<td>20 ± 2</td>
</tr>
<tr>
<td>Trypsin-12Ac</td>
<td>18 ± 2</td>
</tr>
</tbody>
</table>

Table 4.3: Determination of the biochemical rate constants for the conversion of two profluorescent substrates by different forms of acetylated trypsin in solution. Values are obtained from Lineweaver-Burk plots, determined in duplicate at 4 substrate concentrations and presented with standard deviations. \(\ast\) average number of acetylations based on ESI-MS measurements (see Fig. 4.5).
Figure 4.9: Correlation between the catalytic efficiency ($k_{cat}/K_M$) and the degree of acetylation of trypsin in solution determined with Z-Phe-Arg-AMC (Δ) and Z-Leu-Arg-AMC (○). Values are obtained from Lineweaver-Burk plots, determined in duplicate at 4 substrate concentrations and presented with standard deviations.

Figure 4.9 shows that the catalytic efficiency ($k_{cat}/K_M$) increases gradually by a factor of roughly 3 from native trypsin to maximally acetylated trypsin for both substrates. A high catalytic efficiency and a low $K_M$ are important factors for reaching higher digestion yields at low substrate concentrations. At substrate-limiting concentrations (below $K_M$), an increase in catalytic efficiency will translate into almost the same increase of the digestion rate, while this increase will be lower under saturating substrate conditions. In other words, the effect of an increased catalytic efficiency is stronger at lower substrate concentrations. In proteomics applications with real life samples, protein concentrations can be sub-micromolar and lower, and are thus often below the $K_M$. Since online digestion of low-abundance proteins is still difficult, even with immobilized trypsin, the improved biochemical reaction constants of acetylated trypsin may contribute to a higher digestion yield at low protein substrate concentrations.

4.4 Conclusions

The efficiency of an immobilized enzyme reactor is largely determined by two intrinsic factors namely, the catalytic activity of the immobilized enzyme and the mass transfer rate of the substrate between the mobile and the stationary phase. This is illustrated by the fact that the most efficient proteolytic reactors
reported thus far are based on membranes or monoliths, where the strongly reduced diffusional transport limitations can result in protein substrate digestion in less than one minute [24–29]. With these solid supports, the main limitation in terms of speed of digestion is likely to be the catalytic activity of the immobilized enzyme itself. With our post-immobilization modification approach we have demonstrated that chemical modification can augment the efficiency of immobilized enzyme reactors, as the diffusional transport limitations in porous beads are not altered upon modification. Complete digestion of cytochrome c was achieved at a contact time of 4 sec even with stationary phases considered to have slow mass transfer properties (Sepharose). A combination of both highly active trypsin with materials having low diffusional transport limitations may thus lead to ultrafast digestion devices. This represents a significant advance toward the development of chemically modified protease reactors as valuable tools in integrated proteome analysis platforms.

Although the stability of acetylated, immobilized trypsin with respect to storage and thermal or chemical denaturation has not yet been investigated in detail, it is known that hydrophilic modifications have a strong stabilizing effect. Modification of immobilized trypsin with hydrophilic conjugates may therefore result in enhanced thermal and chemical stability, making the use of elevated temperatures or higher concentrations of unfolding agents possible. This opens up a route to extremely efficient tryptic digestion of low-abundant or proteolysis-resistant proteins. A more hydrophilic enzyme surface may also contribute to improved sensitivity by further reducing non-specific adsorption of peptides, especially at low concentrations. Care should be taken, however, not to introduce bulky or rigid groups having adverse effects on protein substrate accessibility.

Acetylation of trypsin as described in this chapter is only one possible modification of a widely used protease. Many other modifications can be envisaged for a range of enzymes immobilized on different stationary phases, making this a very promising approach for proteomics applications.
References


References


