Smart proteolysis samplers for pre-lab bottom-up protein analysis - performance of on-paper digestion compared to conventional digestion.

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List of abbreviations:

ABC: Ammonium bicarbonate

ALB: Alumine

APOA1: apolipoprotein 1

A1AT: alpha-1-antitrypsin

CLUS : clusterin

CXCL7: platelet basic protein

DBS: Dried blood spot

DDA-PASEF: Data dependent acquisition-parallel accumulation–serial fragmentation

DTT: Dithiothreitol

FDR: False discovery rate

hCG: Human chorionic gonadotropin

HEMA-VDM: 2-Hydroxyethyl methacrylate-co-2-vinyl-4,4-dimethyl azlactone

IAA: Iodoacetic acid

IGKC: immunoglobulin kappa constant

IGLC2: immunoglobulin lambda constant

ITIH4: inter-alpha trypsin inhibitor heavy chain

KIO_4: Potassium periodate

NAPI: National Network of Advanced Proteomics Infrastructure

MRM: Multiple reaction monitoring

m/z: mass-to-charge ratio

ProGRP: Progastrin-releasing peptide

TCEP: Tris (2-carboxyethyl) phosphine

TCPK: L-1-tosylamido-2-phenylethyl chloromethyl ketone

THRB: prothrombin

TTHY: transthyretin

Keywords:

Dried blood spot

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Abstract

Here the relation between digestion of proteins by trypsin covalently bound to paper and trypsin in-solution is investigated. The trypsin acting on-paper is covalently bound. A trypsin concentration of 0.5 % (w/v) results in the highest digestion activity of all concentrations tested. Additionally it can be seen that trypsin on-paper has retained approx. 50 % of its activity.

Unlike trypsin in-solution, the stability of the smart proteolysis samplers was regarded to be stable for at least four months when kept refrigerated. Autolysis was very small for covalently bound trypsin: less than 2 % compared to in-solution trypsin.

Proteomic analysis of diluted human serum showed more protein identifications (214) in-solution digestions than on-paper digestions (76). Also higher coverage for the in-solution digestion was obtained. Those proteins identified after on-paper digestion with no or few disulfide bonds seem to have more simillar sequence coverages compared to those identified after in-solution digestion. Smart samplers allow to determine at least 70-75 proteins without performing the overnight digestion.

All in all, trypsin covalently bound to paper shows to retain high proteolytic activity and is a stable alternative for conventional digestions. In this way smart proteolytic samplers show their feasibility in pre-lab sample preparation.

1. Introduction

Analysis of compounds from blood dried on-paper has a history for more than a century. The Norwegian chemist Ivar Bang was described to be the first to perform chemical analysis of glucose from dried blood spots (DBS) [1, 2]. It was not before the sixties that Susi and Guthrie boosted the widespread attention for DBS through newborn screening of the inborn error phenylketonuria. This screening was based on the determination of the ratio of the essential amino acids phenylalanine and tyrosine from blood obtained from the infant's heel deposited on filter paper cards[3]. Thenceforth, more and more analyses were developed for the determination of compounds from DBS and at the present moment there are numerous publications on the determination of small biologically active compounds from paper integrated into high-end analytical strategies[4].

The development has come less far for the determination of proteins from DBS. Early reports on protein determination are related to both concentration measurement of proteins in general as well as activity measurement of specific enzymes.

One of the first mentioned immunometric methods to determine protein concentration from DBS was that of C-reactive protein using ELISA[5]. Soon other immunometric assays for protein determination followed for other protein analytes; alpha1-antitrypsin, various antibodies[6, 7] and apolipoprotein B and apolipoprotein A-1[8] are only a few of the available examples.

The dihydropteridine reductase assay in neonates using Cobas Bio centrifugal analysers is one of the first described methods for enzyme activity determination in

eluates from DBS[9]. It became part of the routine newborn screening for hyperphenylalaninemia[9]. Later on methods for the activity determination of acid alpha-glucosidase [10], total hexosaminidase, hexosaminidase A and beta-galactosidase[11] and tripeptidyl peptidase and palmitoyl protein thioesterase[12] followed.

Although the list of publications on protein determination from DBS grows, the number of publications with the combination of mass spectrometry is still limited. Mass spectrometry offers the possibility to perform targeted protein analysis as well as shotgun proteomic analysis. For targeted analysis one of the first reports is the MS-based determination of hemoglobin F-Mauritius from DBS [13] which was followed by a long list of other hemoglobin variants determined using MS with bottom-up and top-down approaches[14-31]. Not only hemoglobin was determined in this way, also targeted determination of other proteins like human chorionic gonadotropin (hCG)[32, 33], insulin[34, 35] and apolipoprotein A-I, apolipoprotein B[36] as well as multiplex strategies using Multiple Reaction Monitoring (MRM) were reported[37-40]. Compared to these targeted analyses, shotgun proteomic analyses of DBS are still in its infancy[41]. As for now only few reports have been published on proteomic profiling from DBS [42, 20, 43, 44]. Most of the above mentioned methods (targeted and shotgun proteomic profiling) rely on a bottom-up approach using the time consuming trypsination.

Our group has been working with the concept of smart sampling in combination with MS determination since 2017[45]. This concept is based on the covalent binding of trypsin to cellulose allowing a DBS sampler to start with the digestion step at the moment of sampling[46-49]. This smart sampler does not compromise the ease of sampling as the

format remains practically unchanged. However, due to its chemical modified surface with trypsin, it will shorten and simplify the sample preparation time at the laboratory. For targeted analyses this concept has proven to allow protein determination of ProGRP (a biomarker for small cell lung cancer) down to 500 pg/mL from a dried sample on trypsin modified filter paper. Also its potential in proteomic profiling has been shown[46, 49]. In these cases HEMA-VDM functionalized paper and potassium periodate (KIO_4) functionalized paper were used to covalently bind trypsin. In the long term it is expected that a smart sampler will simplify the sample handling upon arrival at the lab as well as it will improve proteomic profiling.

The novel concept of smart proteolysis sampling needs a more fundamental understanding. We therefore investigate the relation between on-paper digestion and in-solution digestion of proteins was investigated with the aim to get insight in the similarity, and complementarity of the covalently bound trypsin and in-solution trypsin. Knowledge from this comparison will contribute to the development of trypsin based smart samplers. It is anticipated that this can improve the complicated work flow of MS based protein determination from DBS.

2. Experimental section

2.1 Chemicals, reagents and consumables

Potassium periodate (KIO_4), cytochrome C from equine heart (≥ 95 %), bovine serum albumin (BSA), trypsin from bovine pancreas TPCK treated (≥10 000 BAEE units per

mg protein) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). The internal standard peptide for cytochrome C (TGPNLHGLFG[R_$^{13}C_6$_$^{15}N_4$]) was purchased from Innovagen (Lund, Sweden). All other chemicals used were analytical grade or LC-MS grade.

Human serum was obtained from the blood bank at the Oslo University Hospital Ullevål (Oslo, Norway). The general principles outlined in the declaration of Helsinki for investigating human material were followed. The eppendorf vials used in this paper (2.0 mL, 1.5 mL and 0.5 mL) were all eppendorf Lo-Bind vials (Hamburg, Germany).

2.2 Functionalization of paper discs and trypsin immobilization

For the experiments 6 mm paper discs were used. These were punched out of Grade 1 Qualitative Whatman paper from GE Healthcare (VWR, Norway) using a Philip Harris 6 mm Uni-Core puncher (Birmingham, UK).

Functionalization using KIO_4 : the procedure published by Chen et al [50] was followed. In short: the discs were placed in a 30 mM KIO_4 solution at 65 °C for 2 hours. Not all the KIO_4 was dissolved during the functionalization. The discs were washed by dipping them three times in MilliQ water. After the last wash, the discs were blotted on filter paper and left to dry (air dry) at room temperature and then placed in a dessicator for at least one night.

Trypsin immobilization was carried out on both KIO_4 functionalized paper and untreated paper using the same protocol: 10 µL trypsin at the desired concentration (dissolved in 1 mM HCl) was dripped on the disc. The disc was placed in a 96 well plate (U96 PP 0.5 ml from Agilent, Santa Clara, CA, USA) which was sealed right afterwards.

Immobilization was carried out overnight at room temperature. After the immobilization, the excess of solvent was removed by blotting.

After blotting the discs were washed 10 times. In the first cycle the discs were placed in 2 mL eppendorf vials containing 1.8 mL 50 mM TRIS-HCl (pH approx. 9). The vials were mixed in the HulaMixer (Thermofisher Scientific, Oslo, Norway) set on orbital (35 rpm, 5 seconds) and reciprocal (60°, 5 seconds). After 5 min the discs were transferred to a fresh washing solution and the wash was repeated. In total 8 washes using 50 mM TRIS HCl were carried out followed by a 9th wash in MilliQ water. The 10th wash was in 50 mM ammonium bicarbonate pH 8 (ABC) solution(placed in a ThermoMixer (Fisher Scientific, Oslo, Norway) for 5 min at 600 rpm at room temperature). After this wash the discs were blotted and left to dry (air dry). The dried discs were placed in a closed eppendorf vial at 4 °C vial until use.

2.3 Digestion conditions for activity measurement of trypsin

2.3.1 On-paper activity

A paper disc (with varying amounts of trypsin immobilized on it) was placed in an eppendorf vial. Digestion was started by dripping 10 µL of cytochrome C (1 mg/mL in freshly prepared 50 mM ABC) on the disc. The digestion reaction was stopped after exactly 10 min through extraction in 110 µL 1 % formic acid. The eppendorf vial (containing the disc and the 1 % formic acid) was placed on a ThermoMixer (600 rpm) at room temperature for five minutes. After this the discs were removed and discarded. Ten µL of the extract was diluted 10 times in 0.1 % formic acid (depending on the

purpose with or without internal standard for cytochrome C) before being injected on the LC-MS/MS.

2.3.2 In-solution activity

Ten µL trypsin solution (with concentrations varying from 0.05 % to 1.0 % - depending on the experiment) was pipetted into an eppendorf vial. To this vial, 10 µL 1 mg/mL cytochrome C was added (in freshly prepared 50 mM ABC). The digestion was carried out for exactly 10 min. The reaction was stopped by adding 100 µL 1 % formic acid. Ten µL of this solution was diluted 10 times using 0.1% formic acid (depending on the purpose with or without internal standard for cytochrome C) before being injected on the LC-MS/MS.

2.4 Digestion conditions for timed experiments

In the following experiments various amounts of trypsin were used. The comparison between on-paper digestions and in-solution digestions will be based on the ratio (w/w) between trypsin and protein. In other words; a paired comparison is made between the on-paper digestion and the in-solution digestion having the same trypsin : protein ratio (w/w).

2.4.1 On-paper

A paper disc with immobilized trypsin (immobilization using 0.5 % trypsin) was placed in an eppendorf vial. Digestion was started when 10 µL of a BSA/cytochrome C mixture (5 mg/mL BSA/1 mg/mL cytochrome C in freshly prepared 50 mM ABC) was dripped on the disc. The digestion reaction was stopped after a specific time through extraction in 110 µL 0.1 % formic acid. The eppendorf vial (containing the disc and the 0.1 % formic

218 acid) was placed on a ThermoMixer (600 rpm) at room temperature for five minutes.
219 After this the discs were removed and discarded.
220 *2.4.2 In-solution*
221 To 10 µL of a BSA/cytochrome C mixture (5 mg/mL BSA/1 mg/mL cytochrome C in
222 freshly prepared 50 mM ABC) 90 µL 50 mM ABC was added. The digestion was started
223 through addition of 10 µL 0.5 % trypsin (in freshly prepared 50 mM ABC). The digestion
224 reaction was stopped after a specific time by addition of 10 µL 1 % formic acid.
225 *Reduction and alkylation for both on-paper and in-solution digestions*
226 To the stopped digest mixture three µL 50 mM TCEP (Tris (2-carboxyethyl) phosphine)
227 was added and the vial placed back on the ThermoMixer for 15 min (800 rpm, room
228 temperature). This was followed by adding three µL 250 mM IAA (Iodoacetic acid)
229 before the vial was placed back on the ThermoMixer for 15 min (800 rpm, room
230 temperature). A volume of 3.3 µL of this reduced and alkylated mixture was diluted 30
231 times in 0.1 % formic acid before being injected on the LC-MS/MS.
232
233 2.5 Digestion of complex serum samples
234 *2.5.1 On-paper*
235 Ten µL of a 1:10 diluted serum sample (diluted with freshly prepared 50 mM ABC) was
236 dripped on the disc. The disc was placed in an eppendorf vial followed by closing to
237 prevent evaporation. Depending on the experiment the lid was re-opened after 1 hour or
238 after overnight to air dry the paper discs. Reduction and alkylation was carried out post
239 digestion as follows: to each vial containing a dried disc 100 µL 5 mM dithiothreitol
240 (DTT) (in freshly prepared ABC) was added. The vial was placed at 60 °C and 800 rpm

for 15 minutes (ThermoMixer). After cooling to room temperature 10 µL 250 mM IAA was added followed by shaking 800 rpm at room temperature (ThermoMixer). The disc was removed and 1 µL concentrated formic acid was added to the remaining solution to prevent potential unbound trypsin from further digestion of the proteins.

2.5.2 In-solution

To 10 µL 1:10 diluted serum 80 µL freshly prepared 50 mM ABC and 10 µL 5 mM DTT (in freshly prepared 50 mM ABC) were added. The vial was placed at 60 °C and 800 rpm for 15 min (ThermoMixer). After cooling down the mixture to room temperature 10 µL 250 mM IAA (in freshly prepared 50 mM ABC) was added and the vial placed at room temperature and 800 rpm (ThermoMixer). To this mixture two µL 1 mg/mL trypsin (in freshly prepared 50 mM ABC) was added to start the trypsination. The overnight digestion was stopped by adding 1 µL concentrated formic acid.

For both on-disc and in-solution digestions clean-up was carried out on 100 µL of the sample using in-house made solid phase extraction as described by [46]. After drying, the residue was reconstituted using 100 µL 0.1 % formic acid and transferred to the injection vial for LC-MS/MS analysis.

2.6 LC-MS/MS analysis

2.6.1 MRM analysis for cytochrome C and BSA

The LC-MS/MS system for the determination of tryptic peptides originating from cytochrome C and BSA consisted of the following components: a low pressure gradient pump (LPG-3400) equipped with a degasser, a WPS-3000TRS autosampler and a

FLM-3000 flow manager (all Dionex, Sunnyvale, CA, USA) and a triple quadrupole mass spectrometer (TSQ Quantum Access, Thermo Scientific, Waltham, MA, USA). Separations were carried out on a BioBasic C8 reversed phase column (5 µm, 300 Å, 50 x 1 mm - ThermoScientific). A flow rate of 50 µL/min was employed for gradient elution with a starting point of 0 % B for 3 minutes. From 3 to 23 minutes the content of solvent B increased from 0 to 50 %. After this the content of solvent B was set to 100 % within 0.1 min and kept there for 5 minutes. Then it sharply decreased to 0 % B within 0.1 min and was kept here for 7 minutes to re-equilibrate the column. Solvent A was 95 % 20 mM formic / 5 % acetonitrile and solvent B was 5 % 20 mM formic acid / 95 % acetonitrile. The injection volume was set at 10 µL. The first 5 minutes of the chromatographic run was diverted to waste. The mass spectrometer was operated in the positive mode using electrospray ionization. The voltage was set to 4000 V and the capillary temperature at 270 °C. Collision gas used was Ar at a pressure of 1.7 mTorr. Nitrogen was used as both nebulizing gas (10 units) and drying gas (2 units). Isolation width was set to 0.7 Da (Q1 and Q3). Table 1 shows the peptides, parent masses, fragment masses and collision energies used. The system was operated and the data were analysed using XCalibur version 2.2 SP 1.48.

2.6.2 Shotgun proteome analysis for serum samples

The samples were analyzed by LC-MS using a timsTOF Pro (Bruker Daltonik, Bremen, Germany) which was coupled online to a nanoElute nanoflow liquid chromatography system (Bruker Daltonik, Bremen, Germany) via a CaptiveSpray nanoelectrospray ion source. The peptides were separated on a reversed phase C18 column (25 cm x 75 µm,

1.6 µm, IonOpticks (Fitzroy, VIC, Australia)). Mobile phase A contained water with 0.1 % (v/v) formic acid, and acetonitrile with 0.1 % (v/v) formic acid was used as mobile phase B. The peptides were separated by a gradient from 0-35 % mobile phase B over 54 min at a flow rate of 300 nL/min at a column temperature of 50°C. MS acquisition was performed in DDA-PASEF mode.

Database searches were performed using Mascot in-house version 2.7.0.1 to search the SwissProt database (Human, 20,411 proteins) assuming the digestion enzyme trypsin, at maximum four missed cleavage sites, fragment ion mass tolerance of 0.03 Da, parent ion tolerance of 15 ppm, carboxymethylation of cysteines, oxidation of methionines, and acetylation of the protein N-terminus as variable modifications. Scaffold (version Scaffold_4.4.8, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Protein and peptide identifications were accepted if they could be established at FDR < 1 % by the Scaffold Local FDR algorithm.

3. Results and discussion

The proof of concept of binding trypsin to paper has been published previously. With the present study we wanted to get more insight into the action of on-paper bound trypsin compared to conventional trypsin which digests in-solution. This was done in several ways:

a) comparison of trypsin activity,

b) comparison of peptide production over time,

c) comparison of autolysis and stability,

d) comparison of proteomic parameters after digestion of complex samples.

3.1 Activity of trypsin covalently bound to paper compared to activity of trypsin in-solution

The activity of trypsin covalently bound to paper is investigated by comparison with trypsin in-solution. This comparison is done at two levels: activity related to the nature of trypsin (in-solution vs bound) and activity related to the trypsin concentration used.

3.1.1 Activity related to the nature of trypsin (in-solution vs bound)

A question which arises is the place where the tryptic action takes place. It is clear for in-solution digests but less obvious for the on-paper digestion. At the moment trypsin desorbs from the paper during sample application, its action would be in-solution. Therefore, the following activities were measured to get insight in this: the activity of i) trypsin adsorbed to untreated paper (non-covalent binding), ii) trypsin bound to KIO_4 treated paper (covalent binding) and iii) trypsin in-solution. This was done after a thorough wash of both untreated and KIO_4 treated paper to which trypsin was bound. It was expected that this would minimize the presence of easily desorbed non-covalent bound trypsin. Figure 1A shows the trypsin activity measured on-paper in relation to in-solution at various trypsin concentrations. After thorough washing of the KIO_4 treated paper approximately 50-60 % of the trypsin activity compared to in-solution digestion is

retained on the paper during the fabrication (at trypsin concentrations of 0.05 %, 0.1 % and 0.5 % (w/v)). This drops down to 30 % at a concentration of 1 % trypsin. Trypsin concentrations of 0.1 % and 0.5 % (w/v) on-paper resulted in the highest activity compared to the same trypsin concentrations in-solution: From figure 1B it can be seen that absolutely more trypsin activity is retained on the paper using 0.5 % compared to 0.1 % trypsin concentration on paper.

The activity of the untreated paper (with adsorbed trypsin) was less than 5 % compared to in solution at all trypsin concentrations. The hypothesis that the majority of the activity measured is attributed to the covalently bound trypsin and not to locally desorbed trypsin which acts in-solution is thus supported. The comparison between in-solution digestion and on-paper digestion is thus a comparison between the action of free trypsin and bound trypsin.

3.1.2 Activity related to the trypsin concentration

As shown above, trypsin concentrations varying from 0.05 % to 1.0 % were investigated (Figure 1). By measuring the production of tryptic peptides from cytochrome C produced in exactly 10 minutes, an indication of the proteolytic activity is obtained. Figure 1B shows the production of MIFAGIK at these varying trypsin concentrations. First of all, trypsin in-solution has an activity which is higher than that of the covalently bound trypsin. The activity of adsorbed trypsin is negligibly low.

It shows that for the trypsin in-solution and trypsin covalently bound to paper the activity is the highest at 0.5 %. A similar trend is seen for the production of the peptides TGPNLHGLFGR, TGQAPGFTYTDANK and EETLMEYLENPK (see figures S1 in the

supporting information). There is no clear reason for why the activity of bound trypsin decreases after 0.5 % while the activity of the in-solution trypsin stays similar. A possible explanation can be that during fabrication at 1.0 % relatively more trypsin degradation takes place compared to 0.5 %. As a result, in fabricating the smart samplers, a concentration of 0.5 % trypsin is considered the best choice as this gives the highest activity. It should be stressed that this possible explanation is not tested and can therefore not be supported by experimental data.

3.2 Proteolytic peptide production

Knowledge about where trypsin acts and its activity does not give insight into how proteins are digested. To address this cytochrome C and BSA were subjected to proteolysis on-paper and in-solution to monitor peptide production over time. For the cytochrome C peptides monitored, it appears that there is no clear difference between on-paper and in-solution digestion (see Figure 2A). Peptide production on-paper is slightly faster for MIFAGIK, but slightly slower for TGQAPGFTYTDANK and EETLMEYLENPK. Additionally it can be seen that the absolute intensities for these peptides are well within the same order of magnitude comparing proteolysis on-paper with in-solution. The only exception is TGQAPGFTYTDANK at 18 hours where much more of this peptide is present in the in-solution digest. For cytochrome C it can be stated that within 6 hours, the on-paper digestion acts similar as the in-solution digestion. For BSA larger differences were found for the monitored peptides. The curve shape of LVNELTEFAK production over time is similar for the on-paper and in-solution

digestion, however the intensities for the on-paper digestions are, depending on the time point, only up to 40 % of the in-solution digestion (see Figure 2B).

For HLVDEPQNLIK and SLHTLFGDELCK the signal intensities for all time points the on-paper digestion are much less compared to the in-solution digestion and only small amounts of these peptides were produced on-paper.

3.3 Autolysis and stability of trypsin in-solution and on-paper

Trypsin is known to digest itself (autolysis) and it was reported that the in-solution stability of unmodified trypsin is within the span of some hours [51, 52]. Autolysis not only causes loss of trypsin activity, it also can promote chymotryptic action [53] leading to other proteolytic peptides than with trypsin.

Although this is known for trypsin in-solution, there is no data on the autolysis of trypsin covalently attached to paper. However, it is expected that immobilizing trypsin leads to less autolysis as is seen for other applications [54-56]. The autolysis of trypsin was monitored over time through two autolysis products (*m/z* 577.1 - AA 146-156 in bovine trypsin, and *m/z* 758.7 - AA 90-109) for both trypsin in-solution and trypsin attached to paper. Figure 3 shows the ratios (in percentage) between on-paper and in-solution of signal intensities of the autolysis products m/z 577.1 and m/z 758.7. A considerable difference in autolysis product formation between on-paper and in-solution digestion can be seen right from the start and up to 18 hours. The signal intensity of the autolysis products formed by trypsin covalently bound to paper is at all time points less than 2 % of the autolysis products of trypsin in-solution. It can be assumed that the covalently

400 bound trypsin prevents itself from autolysis and therewith is more stable than trypsin in-
401 solution. This is in agreement with other publications on immobilized trypsin [54-56].
402 The actual stability of the covalently bound trypsin was tested as well. This was
403 performed by measuring the trypsin activity in digesting cytochrome C after up to 4
404 months of storage at room temperature and at 4 °C. Figure 4 shows that the trypsin had
405 no significant loss of activity when stored at 4 °C. For the paper stored at room
406 temperature, the activity loss of trypsin was measured to be 76 % (based on MIFAGIK
407 only) and 54 % on average for all peptides after four months compared to newly
408 prepared paper.
409 It can be concluded that the paper with covalently bound trypsin can be stored at 4 °C
410 for at least four months without losing its activity.
411 That the bound trypsin exhibits a fairly good stability as well as minimal autolysis are
412 promising findings with regard to further development and use of smart samplers.
413

414 ***3.4 Shotgun proteomics on-paper vs in-solution***

415 As already known [46, 49] less proteins are identified for on-paper compared to in-
416 solution digestions. Through evaluating various typical proteomic parameters obtained
417 from on-paper and in-solution digests it is expected to get more clarity about the
418 reasons, which is important for improvement of the smart samplers.
419 Three different digestion settings were compared: on-paper digestion where the
420 sampler was allowed to air dry right after sample application, on-paper digestion where
421 the sampler was sealed for overnight digestion and a conventional in-solution digestion

carried out overnight. Table 2 shows the number of peptides, number of proteins and the number of missed cleavages of these three conditions.

The number of proteins found on-paper regardless of the digestion time is lower than the number of proteins found in the in-solution digestion. In concordance with this, significantly (student t-test, $p<0.0001$) more peptides were identified in the in-solution digestion. Comparing the short digestion on-paper (air dry) with the overnight digestion on-paper, no significant differences were found for the average number of peptides and combined number of proteins identified. This suggests that most of the digestion is done during the first hour after sample application and that there is no need to extend digestion times beyond the drying time.

Interesting is the observation of the number of missed cleavages: approximately 75 % of the peptides did not contain any cleavage site (C-terminal peptides and 0 missed cleaved peptides), around 20 % contained one missed cleavage and less than 5 % contained more missed cleavages. This suggests that digestion occurs to the same degree of completeness for these three conditions.

A factor which might impact the number of identified peptides and proteins is the reduction and alkylation step. Since the workflow of digestion on-paper is reversed compared to in-solution (reduction and alkylation is carried out after the digestion on-paper), proteins might be less accessible for tryptic cleavage during the digestion step. The workflow reflects how the smart sampler will be used in real life: the digestion will start instantly when the sample is applied to the paper, thus prior to reduction and alkylation. It is expected that this will have a negative impact on the coverage of the identified proteins. A selection of 22 proteins observed for all three conditions was

investigated closer. An important observation made was that all the identified cysteine containing tryptic peptides originating from the 22 proteins were modified with carboxymethyl. As the database search was carried out with carboxymethyl as variable modification, also unmodified cysteine containing peptides could potentially be identified. However, none of the cysteine containing peptides were found in the unmodified form in any of the samples and conditions analyzed. This is a strong indication of complete carboxymethylation by iodoacetic acid after reduction. It is therefore assumed that the carboxymethylation step does not impact the number of protein and peptide identifications.

The average protein coverages per condition for four of the selected proteins are shown in figure 5 (complement C3 - 27 disulfides, plasminogen - 24 disulfides, apolipoprotein A1 - no disulfides, immunoglobulin kappa constant - no disulfides) and for all the 22 proteins in figure S2 (supporting information). From these figures it can be seen that there is a clear tendency: on-paper digestions yield lower protein coverages compared to in-solution digestions. Additionally proteins digested overnight on-paper have a higher coverage than those digested short (air dry).

Comparing both overnight digestions (on-paper with in-solution) only a nine proteins had comparable coverage (less than 10 % difference): albumin (ALB), apolipoprotein 1 (APOA1), immunoglobulin kappa constant (IGKC), apolipoprotein 4 (APOA4), prothrombin (THRB), platelet basic protein (CXCL7), clusterin (CLUS), immunoglobulin lambda constant (IGLC2) and transthyretin (TTHY). Of these APOA, IGKC, APOA4, CXCL7, IGCL2 and TTHY had 2 disulfide bonds or less (resp. 0, 0, 2, 2 and 0 disulfide

bonds). It is tempting to ascribe good protein coverage on-paper to good accessibility for trypsin, however, from Figure S2 in the supporting information it can be seen that alpha-1-antitrypsin (A1AT) and inter-alpha trypsin inhibitor heavy chain (ITIH4) - both proteins with zero disulfide bonds - have considerably higher coverage in-solution compared to on-paper. The latter does not outrule the possibility of good accessibility when few disulfide bonds are present, however, on the other hand it is not given that absence of disulfide bonds result in good protein coverages for on-paper digests. In accordance with previously published results [57] this is probably dependent on additional protein properties.

4. Conclusions

In this paper the activity of immobilized trypsin coupled to paper through KIO_4 functionalized cellulose is compared to that of trypsin in-solution. It is proven that the enzymatic action measured for the covalently bound trypsin can be ascribed to the attached trypsin and not to randomly desorbing, thus in-solution acting trypsin. Immobilizing 0.5 % trypsin results in smart samplers with a trypsin activity approx. 50 % compared to that in-solution.

The smart sampler shows to exhibit the same trypsin activity for at least 4 months when refrigerated. This is the first report on the stability of these smart samplers. In accordance with other reports on immobilized trypsin, minimal autolysis was observed in the smart samplers. Both good stability and low degree of autolysis are superior to trypsin in-solution.

Monitoring the production of multiple peptides for cytochrome C and BSA over time reveals that differences between on-paper digestions and in-solution digestions are protein dependent: digestion of a simple protein like cytochrome C produces equal amounts of peptide regardless if it is on-paper or in-solution digestion while more BSA peptides are produced in-solution.

The smart samplers can be used for shotgun proteomic analysis although less proteins were qualified compared to in-solution digestions. The presence of disulfide bridges seems to hamper the on-paper digestion compared to the in-solution digestions: lower coverage is seen using the smart sampler for proteins with three or more disulfide bridges.

All in all, trypsin covalently bound to paper shows to retain high proteolytic activity and is a stable alternative for conventional digestions. In this way smart proteolytic samplers show their feasibility in pre-lab sample preparation.

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6. Conflict of interest statement

The authors declare no conflict of interest.

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Legend to the figures:

Figure 1.

A) Trypsin activity on-paper related to in-solution for MIFAGIK (m/z=390.2). The ratio of the signal intensities for on-paper and in-solution digestions is shown. Dark grey: KIO_4-treated paper with trypsin. Light grey: untreated paper with adsorbed trypsin.

B) Signal intensity of MIFAGIK for four different trypsin concentrations in-solution (black), on-paper with covalently bound trypsin (dark grey) and on-paper (untreated) with adsorbed trypsin (light grey). Signal intensities were corrected with the IS (see table 1)

For all experiments n=3. The error-bars in figure 1B represent the standard deviations of the measurements (+/- 1 SD). No error bars are given in figure 1A since these represent ratios of average numbers.

Figure 2.

A) Production of peptides from cytochrome C. Upper: MIFAGIK, middle: TGQAPGFTYTDANK and bottom: EETLMEYLENPK. B) Production of peptides from BSA. Upper: LVNELTEFAK, middle SLHTLFGDELCK and bottom: HLVDEPQNLIK. Black: in-solution trypsin digestion. Grey: on-paper trypsin digestion.

Figure 3.

Ratio of the autolysis products m/z 577.1 and 758.1 over time (up to overnight): signal intensity on-paper / signal intensity in-solution. The ratio is expressed in percentage and does not exceed 2 %.

Figure 4.

Signal intensity ratio of MIFAGIK (m/z 390.2), TGPNLHGLFGR (m/z 584.8), TGQAPGFTYTDANK (735.8) and EETLMEYLENPK (748.4) compared to internal standard after 10 minutes digestions for paper stored up to 4 months refrigerated (upper) and at room temperature (lower). From 0 days (black), 30 days (dark grey), 60 days (grey), 90 days (light grey) and 120 days (white)

Figure 5.

Average protein coverage (n=3) for proteins digested on-paper (air dry), on-paper (overnight) and in-solution (overnight). The two figures on the left represent proteins containing disulfide bridges, the figures on the right represent proteins with few disulfide bridges (2 or less). Figures S2A-C show similar plots for all 22 proteins.