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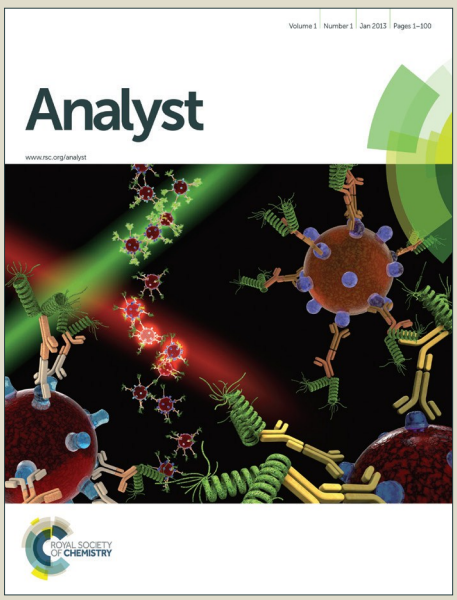

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Mechanistic investigation of the on-surface enzymatic digestion (oSED) protein adsorption detection method using targeted mass spectrometry

Torgny Undin[1],Andreas Dahlin[2], Katarina Hörnaeus[1], Jonas Bergquist[1], Sara Bergström Lind[1*]

1. Department of Chemistry-BMC, Analytical Chemistry and Science for Life Laboratory, Uppsala University, PO Box 599, SE-751 24 Uppsala, Sweden

2. Department of Engineering Sciences, Uppsala University, PO Box 534, SE-751 21 Uppsala, Sweden.

* Correspondence to:

Sara Bergström Lind, Department of Chemistry-BMC, Uppsala University, PO Box 599, SE-751 24 Uppsala, Sweden

Email: sara.lind@kemi.uu.se

Telephone: +46 18 471 3673

Fax: +46 18 471 3682

Abstract

This paper describes an effort to study some of the mechanistic aspects of the earlier established on-surface enzymatic digestion (oSED) method. In a multitude of application areas, it has become important to be able to fully characterize and understand the selective protein adsorption to biomaterial surfaces. Those applications can be in for example biomedicine (implants etc.), nanotechnology (microchip surfaces and sensors) and material sciences. Here the investigation of the mechanistic aspects was based on microdialysis catheter tubes that were flushed with controlled protein solutions mimicking the extracellular fluid of the brain. The protein adsorption properties were monitored by high resolution liquid chromatography tandem mass spectrometry (LC-MS/MS) using a targeted method. The temporally resolved results show that most proteins stay adsorbed onto the surface during the entire digestion process, only cut away piece by piece while smaller proteins and peptides seem to desorb rather easily from the surface. This information will simplify the interpretation of data generated with the oSED method, but could also be used for the characterization of the physicochemical properties controlling adsorption of individual proteins to specific surfaces.

1. Introduction

A general problem for biomaterial in contact with biological matrices is the formation of an adsorbed protein layer[1]. This can be problematic since it can cause rejection and encapsulation of implants and other foreign materials. As another example, microdialysis has been used for sampling and characterization of peptides and proteins in several publications from our group[2-6]. The problem with protein adsorption has in these studies been observed and surface deactivations have been used in order to reduce the binding of proteins to the surface[7, 8]. There are several methods for the investigation of adsorption layer of proteins, e.g. surface plasmon resonance (SPR) [9, 10] and attenuated total reflection (ATR) Fourier transform infrared (FTIR) spectroscopy[11] to mention a few. These methods do, however, not identify what proteins that are adsorbed. Recently, several approaches using mass spectrometry (MS) for characterization of adsorbed proteins on biomaterial surfaces have been reported[6-8]. The idea is to use a method where on-surface enzymatic digestion (oSED) of proteins is performed. The extracted peptides are further characterized by liquid chromatography (LC) coupled to tandem MS detection (LC-MS/MS) to provide identification of adsorbed proteins. Somewhat similar approaches, where proteins adsorbed to liquid chromatography stationary phases are enzymatically digested, have been reported[12], but with the purpose of investigating specific interaction sites on a studied protein. The oSED method we have developed is a more versatile method for characterization of any surfaces, no matter the material or its physical and chemical properties. Identification of adsorbed proteins is possible in an unbiased manner without any prior knowledge needed.

The oSED method has been used for surface characterization studies in experiments where a static liquid surrounds a membrane with adsorbed proteins[6-8]. Reagents for reduction of disulfide bonds and alkylation of the cysteines to prevent the reversed reaction, dithiothreitol (DTT) and iodoacetamide (IAA), were added and incubation was performed as for in-solution

digestion. Thereafter trypsin was added and the proteins were enzymatically cleaved into peptides. The peptides were further desalted using C18-based material solid phase extraction before LC-MS/MS analysis. Which steps that influence the release of adsorbed proteins (desorption) have to our knowledge, however, not been investigated. To investigate the details of the oSED mechanisms, a dynamic system where peptides/proteins can be extracted and quantified after each step, is hereby suggested.

The field of quantitative proteomics using MS detection is constantly growing. Approaches for relative comparisons of protein amounts between different studied groups are implemented via both labeled-based e.g. stable isotope labeling in cell culture, dimethyl labeling, isobaric tags for relative and absolute quantification (iTRAQ) and label-free approaches as reviewed by Elliott and Zhang et al. [13, 14]. Furthermore, targeted MS approaches i.e. multiple reaction monitoring (MRM), selected reaction monitoring (SRM), and parallel reaction monitoring (PRM) to selectively monitor and quantify peptides and proteins with MS [15, 16]become more and more important in order to perform rapid quantifications. These methods allow for shorter LC-gradients to be used. Generally, MS instruments as triple quadrupoles have been used for targeted approaches, for example in MRM where the precursor of interest is selected in the first quadrupole, fragmented in the second and the fragments are monitored in the third quadrupole. Nowadays, also high resolving instruments such as linear ion trap (LTQ) Orbitrap have been reported to be useful in targeted approaches [17]. Methods implemented can then combine the high resolution in the Orbitrap with fast tandem mass spectrometry (MS/MS) of selected peptides in the LTQ to assure the peptide sequence [18]. The method is a modified single ion monitoring (SIM) approach and has been used for e.g. monitoring the relative abundance of selected proteins [19], but can also be used for absolute quantification, as in this study, where the model system is well characterized in terms of protein concentrations.

In this study we use a targeted LC-MS/MS method for investigation of the mechanisms of the oSED approach using a well-defined but dynamic system. Polyurethane tubes, used in clinical microdialysis sampling catheters, were filled and incubated with a standard protein solution of known protein concentrations resembling artificial cerebrospinal fluid (CSF). The CSF proteome includes more than 2500 proteins [20] and in this study a selected fraction was used for mechanistic studies. The solution was pushed through the tubing and tubes were stepwise filled and incubated with solutions of reduction, alkylating and digesting reagents, with washes in between. Pushed through solutions were collected and treated in parallel with the same reagents as for the proteins that were digested inside the tube. By quantifying the proteins in the model system, collected after different incubations, with a rapid targeted LC-MS/MS method, it was possible to reveal what steps in the oSED procedure that had the largest and smallest impact, respectively, on protein desorption.

2. Experimental

2.1. *Chemicals and reagents*

Acetonitrile (ACN) and acetic acid (HAc) were obtained from Merck (Darmstadt, Germany). All other chemicals were from Sigma Aldrich (St.Louis, MO, USA) if not otherwise stated. Ultrapure water was produced by a Milli-Q+ system, Millipore Corp (Marlborough, MA, USA). Artificial CSF was used as standard solution to study the adsorption and was composed of an aqueous solution of 147 mM NaCl, 2.7 mM KCl, 1.2 mM $CaCl_2$ and 0.85 mM $MgCl_2$ in acetate buffered solution (pH 7.4) (Ringer's acetate solution) and a protein mixture, mimicking the composition of the most abundant proteins and neuropeptides in human CSF, according to Table 1. The total protein content was 0.407 mg/mL and the percentages of the different proteins are listed in Table 1.

2.2. Protein adsorption procedure and sample preparation

The sample setup was comprised of four individual tubes (polyurethane, inner diameter 150 µm, length 1000 mm, surface area of 4.71 cm^2 and a volume of 17.7 µL) connected to syringes in a syringe pump. Between different steps the syringes where changed to ones specific for the step (Table 2). The flow rate of all sequences was 10 µL/min, except in the fourth step where the protein standard had a flow of 0.5 µL/min. This generated a laminar flow over the surface and the total protein incubation time was 82 minutes. The monitored liquid was collected and further handled in vials with low adsorption of peptides and proteins (Protein LoBind tubes, Eppendorf, Hamburg, Germany). At each step the vials where weighed on a microbalance to control the volumes in the experiment. The steps included the generally used procedures in enzymatic protein digestion before MS detection; reduction and alkylation using DTT and IAA followed by tryptic digestion. After the final step of eluting the peptides with buffer, the trypsination was quenched with 100µL 1% HAc where after all vials

were dried in a speed vac at 40°C. Non-adsorbed proteins, e.g. in protein rinse, tube reaction and tube rinse were reduced, alkylated and digested in the same way but in a vial. Re-suspension of the peptides was conducted in 1% formic acid (FA) and the solution was thereafter desalted on 50 mg C_{18}Isolute SPE-columns (Biotage, Uppsala, Sweden) according to the manufacturer guidelines. After the final drying step, the samples were re-suspended in 0.1% FA for the following LC-MS/MS analysis.

2.3. Nano LC-MS/MS analysis

Mass data were aquired using an LTQ Orbitrap Velos Pro Mass spectrometer (ThermoFisher Scientific, Bremen, Germany) fitted with a nano-electrospray ionization (ESI) ion source. The on-line reversed phase liquid chromatography separation was performed using a Thermo Easy-nano Liquid Chromatography instrument (ThermoFisher Scientific, Bremen, Germany). A sample volume of 5 µL was injected onto the column. For separation of the peptides, a 10 cm × 75 µm, C_{18}-A2 column (Easy column, ThermoFisher Scientific, Bremen, Germany) with a particle size of 3 µm and an H_2O:ACN:acetic acid solvent system (H_2O, 0.1% FA mobile phase [A]; ACN, 0.1% FA mobile phase [B]) were used. A flow rate of 300nL/min was applied, starting with isocratic elution at 2% B for 2 min, followed by gradient elution from 3% to 45% B during 15 min, and then from 45% to 80% B within 3 min, and finally 80% B for 15 min. The mass spectrometer was operated in positive ionization mode. First, a modified single ion monitoring (SIM) scan was performed in the Fourier Transform (FT) cell, recording a window between 400 and 650 m/z with a resolution of 30000 to detect the peptide ions. Secondly, a CID MS/MS experiment of the most intense ion in the mass range occuring on a global inclusion list, was detected in the ion trap. The following parameters were used: mass with: 2 m/z, resolution: 7500, normalized collision energy: 35 and activation time 10 ms. These two steps where repeated for massranges 650-850 m/z and 850-1070

m/z.

Acquired data (.raw-files) was imported into Proteome Discoverer (version: 1.4.0.288) where protein identification was performed using the internal Sequest-HT search engine against a FASTA-file containing the proteins in the protein mix. The search parameters were set to enzyme: Trypsin, fixed modifications: carbamidomethylation (C), variable modifications: oxidation (M), deamidation (N, Q), peptide mass tolerance: ± 0.02 Da, fragment mass tolerance: ± 0.7 Da, maximum missed cleavages = 0. Proteins were only considered to be positively matched if they passed the scoring ($p \leq 0.05$) of the target decoy PSM validator.

2.5. Data analysis

Two softwares for evaluation of targeted quantification were used: PinPoint (ThermoFisher Scientific) and Skyline[21]. The quantification was, in both softwares, based on the chromatographic peak areas recorded in the SIM scans of selected peptides from the proteins of interest. Final quantification at the protein level was performed using 1-4 selected peptides per protein (Table 3). The responses of the peptides were related to concentrations using a calibration curve based on a dilution series of the analyzed standard. The averaged corresponding concentrations observed for the peptides were reported as the protein concentration.

3. Results and discussion

3.1. Implementation of a targeted LC-MS/MS method for proteins in CSF

The implemented targeted LC-MS/MS method was advantageous due to its highly selective and rapid analysis of the targeted proteins. The parameters optimized to tailor the method were the number of *m/z*-windows for parental ion detection, the LC gradient and the collision energy for peptide fragmentation. Three *m/z*-windows were concluded enough for the peptides of interest in this study. Further windows would have made the acquisition slower and were in this case not necessary since the peptides of interest were covered by the method. The LC gradient was optimized to evenly spread out the retention times of the peptides of interest. The inclusion list of peptides to be fragmented at each time point was adjusted with the optimal gradient settings. The normalized collision energy was set to 35 for all peptides. Initial experiments included a screening for suitable peptides by analyzing a protein standard with LC-MS/MS, using a data dependent acquisition method where the top-ten peaks of highest intensity in a full scan were fragmented in MS/MS mode. From these experiments, five to ten peptides per protein were selected. In the following processing step the method was optimized to focus on two to three peptides per protein. The included neuropeptides were, however, analyzed in their original state as they appear as single peptides. The final inclusion list of peptides to analyze for each protein was limited to only include peptides with an interpretable response in the evaluation of the calibration curve of analyzed standard dilutions. The peptides are listed in Table 3. Also, peptides with post translational modifications were avoided, especially peptides with multiple modifications for example oxidations and deamidations. Using the developed method it was possible to evaluate the absolute amounts of proteins in each sample fraction. Protein LoBind were used in all steps to avoid unspecific binding of proteins and peptides to vial surfaces, which otherwise could affect the results.

3.2. Sample composition evaluation

In this mechanistic study of the oSED principle on-surface, a protein model system of known concentrations of each protein was used (see Table 1 for exact composition). The reasons for not using a real CSF sample were that *i)* it would have been too complex to evaluate and *ii)* there is no *a priori* knowledge of the individual protein concentrations in such sample. The proteins in the standard solution were chosen to mimic the composition of CSF and as a first step the sample composition in different fractions was evaluated. The events in the adsorption studies are presented in Table 2. Using this dynamic system it was possible to collect samples both after each step of incubation with reagents and after washes. As presented in Figure 1, the theoretical composition was very similar to the analyzed standard. This result further proved that the targeted LC-MS/MS method worked properly. The following three fractions of Protein rinse, Tube reaction and Tube rinse did also have a similar protein percentage distribution as the analyzed standard. The most pronounced difference in composition was observed for the alpha-1-acid glycoprotein, which showed an increased portion in these three fractions compared to the analyzed standard. The experiments thus state that this protein is relatively less adsorbed to the surface compared to the other proteins. The relative changes in protein composition were most pronounced in the peptide elution fraction, where peptides had been produced via oSED digestion inside the polyurethane tube. The largest relative change is the reduced relative presence of serum albumin in this fraction.

3.3. Protein adsorption behavior

To further evaluate the protein adsorption behavior for each protein and neuropeptide in the model system, the amounts of specific proteins in different fractions were evaluated. The data

is presented in Figure 2 and 3. For the neuropeptides bradykinin and vasopressin (Figure 2A and 2B), the adsorption was, as expected, not as pronounced as for the larger proteins. The highest amounts of these peptides were detected in the Protein rinse fraction, i.e. in the first wash with buffer fraction after the protein standard had been in contact with the polyurethane tube. For all proteins (transthyretin, alpha-1-acid-glycoprotein, serum albumin, serotransferrin, IgG and alpha-2-macroglobulin, Figure 2C-D and Figure 3A-D) the highest amounts were detected in the Peptide elution fraction after trypsin digestion. The low amounts of proteins in the Tube rinse and Tube reaction fractions demonstrate that proteins are not released in these steps. A possible theory that has been considered is that the reduction with DTT could cause a conformational change of the proteins and thereby cause desorption from the surface. The data from this study reject this hypothesis. Instead, tryptic digestion was needed to desorb the proteins from the surface. This is an important observation that concludes that the digestion in the oSED approach actually takes place at the membrane surface as first foreseen by Dahlin et al [6]. The protocol followed for reduction, alkylation and digestion for adsorbed proteins was adopted from what is generally used for enzymatic digestions of proteins in in-solution and in-gel based approaches[22]. The incubation times for reduction and alkylation (minutes) are therefore relatively short compared to the digestion time (hours). Reduction and alkylation are, however, performed with excess reagents and the kinetics for these events are considered faster compared to digestion so the time for desorption has been considered long enough. Therefore, the observed results strongly support enzymatic digestion to be the key to protein desorption.

4. Conclusions

The oSED method for characterization of protein adsorption to biomaterial surfaces has been investigated for mechanistic aspects. The optimization of a targeted LC-MS/MS method

provided a rapid, sensitive and selective quantification of the selected CSF-proteins in a standard solution. The dynamic system for investigation of the mechanistic aspects, based on microdialysis tubes flushed with protein solution as well as reagents for reduction, alkylation and digestion, was a key for sampling of peptide and protein fractions that revealed the oSED mechanism. The oSED method was concluded a very versatile and useful tool for investigation of protein adsorption. During the progression of the oSED method, proteins to the most extent stayed adsorbed onto the surface and did not desorb in any higher extent into the solution prior to the step of adding trypsin. As anticipated, smaller neuropeptides did not behave like the larger proteins and were more readily desorbed from the surface upon washes and addition of different reagents. The oSED method is a versatile tool for investigation of protein adsorption to biomaterials and futures studies will reveal the mechanistic aspects in presence of a complex sample and on a variety of surfaces.

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References

1. J. M. Anderson, A. Rodriguez and D. T. Chang, *Semin Immunol*, 2008, **20**, 86-100.
2. A. P. Dahlin, K. Purins, F. Clausen, J. Chu, A. Sedigh, T. Lorant, P. Enblad, A. Lewen and L. Hillered, *Anal Chem*, 2014, **86**, 8671-8679.
3. A. P. Dahlin, M. Wetterhall, K. D. Caldwell, A. Larsson, J. Bergquist, L. Hillered and K. Hjort, *Anal Chem*, 2010, **82**, 4376-4385.
4. L. Hillered, A. P. Dahlin, F. Clausen, J. Chu, J. Bergquist, K. Hjort, P. Enblad and A. Lewen, *Front Neurol*, 2014, **5**, 245.
5. M. Wetterhall, J. Bergquist, L. Hillered, K. Hjort and A. P. Dahlin, *Eur J Pharm Sci*, 2013, **57**, 34-40.
6. A. P. Dahlin, K. Hjort, L. Hillered, M. O. Sjodin, J. Bergquist and M. Wetterhall, *Anal Bioanal Chem*, 2012, **402**, 2057-2067.
7. T. Undin, S. Bergstrom Lind and A. P. Dahlin, *Future Science OA*, 2015, **FSO1532**.
8. T. Undin, S. Bergstrom Lind, J. Bergquist and A. P. Dahlin, *manuscript in doctoral thesis from Uppsala University* 2015.
9. N. Aldred, T. Ekblad, O. Andersson, B. Liedberg and A. S. Clare, *ACS Appl Mater Interfaces*, 2011, **3**, 2085-2091.
10. C. T. Campbell and G. Kim, *Biomaterials*, 2007, **28**, 2380-2392.
11. S. E. Glassford, B. Byrne and S. G. Kazarian, *Biochim Biophys Acta*, 2013, **1834**, 2849-2858.
12. M. I. Aguilar, D. J. Clayton, P. Holt, V. Kronina, R. I. Boysen, A. W. Purcell and M. T. Hearn, *Anal Chem*, 1998, **70**, 5010-5018.
13. M. H. Elliott, D. S. Smith, C. E. Parker and C. Borchers, *J Mass Spectrom*, 2009, **44**, 1637-1660.

14. Y. Zhang, B. R. Fonslow, B. Shan, M. C. Baek and J. R. Yates, 3rd, *Chem Rev*, 2013, **113**, 2343-2394.

15. S. Gallien, A. Bourmaud, S. Y. Kim and B. Domon, *J Proteomics*, 2014, **100**, 147-159.

16. V. C. Wasinger, M. Zeng and Y. Yau, *Int J Proteomics*, 2013, **2013**, 180605.

17. N. Tanaka, K. Nagasaka and Y. Komatsu, *Biol Pharm Bull*, 2011, **34**, 135-141.

18. R. Kiyonami, M. Zeller and V. Zabrouskov, *Application note : 557 Thermo Scientific*, 2011.

19. J. M. Bergman, M. Wrande and D. Hughes, *PLoS One*, 2014, **9**, e109255.

20. S. E. Schutzer, T. Liu, B. H. Natelson, T. E. Angel, A. A. Schepmoes, S. O. Purvine, K. K. Hixson, M. S. Lipton, D. G. Camp, P. K. Coyle, R. D. Smith and J. Bergquist, *PLoS One*, 2010, **5**, e10980.

21. B. MacLean, D. M. Tomazela, N. Shulman, M. Chambers, G. L. Finney, B. Frewen, R. Kern, D. L. Tabb, D. C. Liebler and M. J. MacCoss, *Bioinformatics*, 2010, **26**, 966-968.

22. M. Kinter, Sherman, N.E. , *John Wiley & Sons ISBN-13: 978-0-471-32249-8*, 2000.

Figure legends

Figure 1. Sample composition in percentages for the analyzed fractions. For specification, see Table 2.

Figure 2: Amounts of neuropeptides and proteins detected with LC-MS/MS analysis in the different experimental fractions specified in Table 2. Panels are: a) Bradykinin; b) [Arg8] Vasopressin; c) Transthyretin; d) Alpha-1-acid-glycoprotein. Statistical test is a two-tailed Welch t-test.*P<0.05, **P<0.01.

Figure 3: Amounts of proteins detected with LC-MS/MS analysis in the different experimental fractions specified in Table 2. Panels are: a) Serum albumin; b) Serotransferrin; c) IgG; d) Alpha-2-macroglobulin. Statistical test is a two-tailed Welch t-test. *P<0.05, **P<0.01, ***P<0.001.

Table 1.The proteins in the artificial CSF standard used for adsorption studies.

Protein	**Fraction [%]**
Serum albumin	76.9
Serotransferrin	8.8
IgG	8.8
Alpha-1-acid glycoprotein	2.8
Alpha-2-macroglobulin	1.4
Transthyretin	0.7
Bradykinin	0.3
Vasopressin	0.3
Sum	**100**

Table 2. The experimental setup for protein adsorption studies

Step	In syringe	Volume [µL]	Flow rate [µL/min]	Vial
1	Air	40	10	
2	Buffer (0.1M NH_4HCO_3)	40	10	
3	Protein standard	20	10	Analyzed standard
4	Protein standard	40	0.5	Analyzed standard
5	Air	40	10	Analyzed standard
6	Buffer (0.1M NH_4HCO_3)	40	10	Protein rinse
7	Air	40	10	Protein rinse
8	DTT (45mM in 0.1M NH_4HCO_3))	40	10	Tube rinse
9	Air	40	10	Tube reaction
10	IAA (100mM in 0.1M NH_4HCO_3)	40	10	Tube rinse
11	Air	40	10	Tube reaction
12	Trypsin	40	10	Tube rinse
13	Air	40	10	Peptide elution
14	Buffer (0.1M NH_4HCO_3)	40	10	Peptide elution
15	Air	40	10	Peptide elution

Table 3. Unique peptides used for quantification of the proteins.

Protein	Peptide sequence
Bradykinin	RPPGFSPFR
[Arg 8] Vasopressin	CYFQNCPR
Alpha-1-acid glycoprotein	NWGLSVYADKPETTK
	TLMFGSYLDDEK
Serum albumin	AVMDDFAAFVEK
	CCAAADPHECYAK
	LVAASQAALGL
	VHTECCHGDLLECADDR
	VPQVSTPTLVEVSR
Transthyretin	AADDTWEPFASGK
IgG	VDNALQSGNSQESVTEQDSK
	VYACEVTHQGLSSPVTK
	ALPAPIEK
	GPSVFPLAPSSK
	STSGGTAALGCLVK
	GLPAPIEK
	AAPSVTLFPPSSEELQANK
	AGVETTTPSK
	YAASSYLSLTPEQWK
Alpha-2-macroglobulin	DTVIKPLLVEPEGLEK
	TEHPFTVEEFVLPK
	YGAATFTR
Serotransferrin	ASYLDCIR
	EDPQTFYYAVAVVK
	EGTCPEAPTDECKPVK
	MYLGYEYVTAIR
	NPDPWAK

Figure 1.

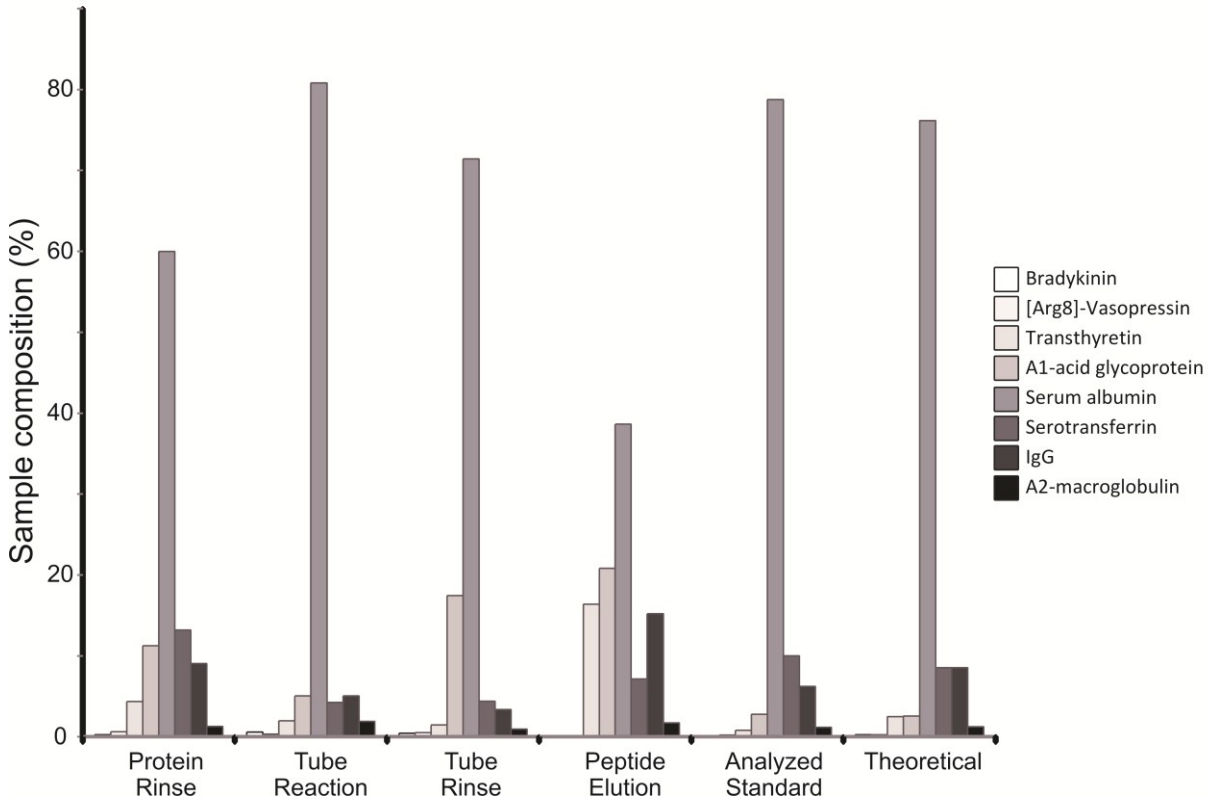

Figure 2

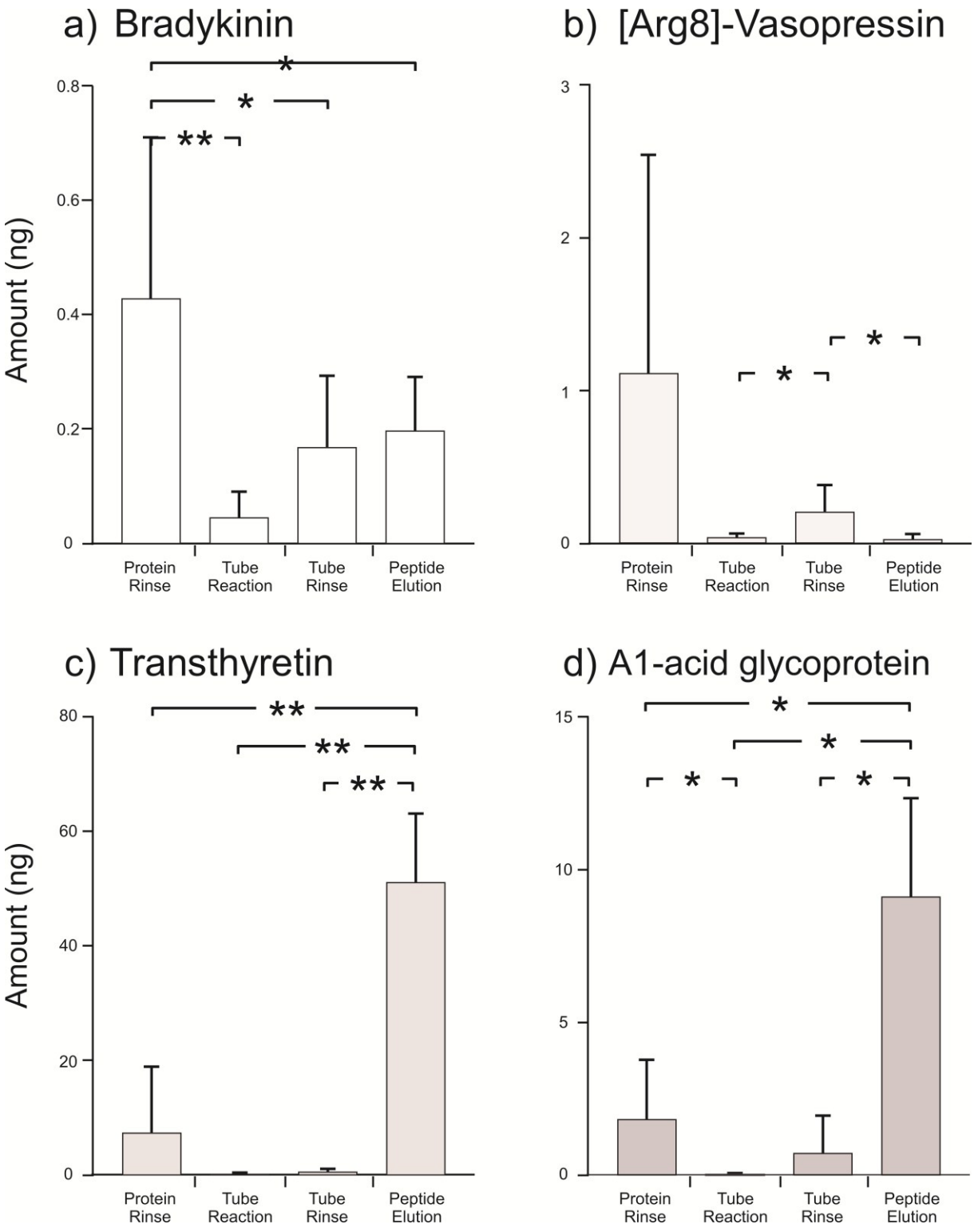

Figure 3