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Quantitative analysis of immobilized proteins and protein mixtures by amino acid analysis

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Abstract

Biomolecular surface engineering of materials often requires precise, versatile and efficient quantification of immobilized proteins at solid surfaces. Acidic hydrolysis of surface-bound proteins and subsequent HPLC analysis of fluorescence-derivatized amino acids were adapted and critically evaluated for that purpose. Contaminations and concentration-dependent amino acid retrieval during HPLC were found to influence the accuracy of the method. In addition to the choice of adequate conditions for hydrolysis, derivatization and chromatographic separation extensions of the data evaluation were suggested to improve the accuracy of the approach when applied to single protein systems: comparing the experimentally obtained amino acid ratio to the protein constitution enabled to identify the properly separated and detected amino acids. Those amino acids were selected for a more precise calculation of the amount of immobilized protein. To further increase the accuracy of the method, the retrieval of amino acids corresponding to protein amounts in the range between 0.5 and 4.0 μg was analyzed for a variety of proteins of interest to derive protein-specific correction factors. The evaluation of amino acid data was furthermore applied to quantify binary protein mixtures at similar settings. This method was proven useful to detect the composition of protein mixtures throughout a wide range of absolute and relative concentrations.

Keywords: Numerical analysis; Immobilized proteins; Proteins; Amino acids

1. Introduction

The immobilization of biopolymers to solid surfaces is utilized to support various advanced biomedical and biotechnological applications. Examples comprise the coating of implant materials by proteins [1], the binding of adhesion proteins [2] and growth factors [3] to cell culture carriers or the coupling of enzymes to solid supports for diagnostic assays [4]. In all of these cases information on the amount and distribution of the immobilized proteins is required to optimize the immobilization procedure and to check for the stability of the protein layers. Since immobilized proteins, either covalently bound or adsorbed, cannot be easily removed from solids the applicability of classical biochemical methods for the quantification of proteins in solution such as Lowry et al. [5], the Coomassie blue assay according to Bradford [6] and the bicinchoninic acid (BCA) method [7] is rather limited. As a main reason the

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sensitivity of these methods allows for the detection of surface-bound proteins only at higher protein amounts as with large surface areas or with extended protein layers.

Methods recently suggested for the quantification of surface-bound proteins include ellipsometry [8,9], reflectometry [10], surface plasmon resonance [11], optical waveguide light mode spectroscopy [12], X-ray photoelectron spectroscopy [13], mass spectrometry [13,14], quartz crystal microbalance measurements [15], radioimmunoassays [16] and enzyme-linked immunosorbent assays (ELISA) [11,17]. Although all these methods permit valuable insights about proteins at solid surfaces some of the techniques require sample characteristics that restrict the analysis to model substrates. Also, some of the techniques provide only semi-quantitative results and many techniques cannot distinguish between different protein types.

A very versatile method for the quantification of proteins is the total acidic [18], alkaline [19], or microwave supported [20] hydrolysis followed by HPLC of the released amino acids which can be also applied to proteins at interfaces. This technique is frequently applied for the qualitative [21] and quantitative [22] analysis of dissolved proteins and protein mixtures but was also suggested for the compositional analysis of proteins blotted on polyvinylidene difluoride (PVDF) membranes after separation by 2D polyacrylamide gel electrophoresis [23,24]. The method is advantageous since it can be applied to samples of irregular shapes and absolute amounts of proteins can be determined. Therefore, the method was selected for the quantification of proteins in the context of the biomolecular surface engineering of materials.

Accordingly, experimental conditions for the application of the technique and evaluation procedures were adapted and critically evaluated in this work.

Major problems in HPLC analysis are the degradation of amino acids during hydrolysis and the different sensitivity of peptide bonds against the hydrolyzing agents leading to deviations in the detected amino acid patterns from the actual protein composition. Furthermore, protein samples and their hydrolyzates, respectively, often include impurities either by the original protein preparation or by the solid support.

As a result of this study, a method for the determination of immobilized amounts of proteins at interfaces is suggested which provides a substantially improved accuracy when applied to single protein systems. For that aim a numerical fit of the measured amino acid distribution to the known sequence of the protein was applied. Utilizing this method for the analysis of standard protein solutions a strong correlation between known protein quantities and the detected amounts was obtained and used to derive protein-specific correction factors addressing inaccuracies caused by incomplete hydrolysis or chromatographic separation. Examples of the quantification of fibronectin and albumin layers immobilized to solid supports are given to illustrate the potentialities of the approach.

Very often the analysis of immobilized protein layers requires the simultaneous quantification of different protein components. Therefore, the chromatographic technique established for the characterization of single protein systems was further applied to analyze binary systems consisting of albumin and chymotrypsin. The results obtained in experiments with dissolved protein samples of known composition demonstrate the reliability of this extended use of the method.

2. Experimental

2.1. Chemicals and reagents

All aqueous solutions were prepared with Milli-Q water (Millipore, Molsheim, France). Hydrochloric acid solution for amino acid analysis, triethylamine for amino acid analysis, phenol and tetrahydrofurane were purchased from Fluka (Buchs, Switzerland). PBS (phosphate-buffered saline, pH 7.4) tablets, *o*-phthalaldehyde (OPA), 2-mercaptoethanol and the amino acid standard solution for fluorescence detection were obtained from Sigma–Aldrich (Steinheim, Germany). Methyl alcohol for HPLC was purchased from Acros Organics (Geel, Belgium).

2.2. Proteins and sample preparation

Human serum albumin (HSA, albumin), α-chymo-

trypsin (CT) from bovine pancreas, ribonuclease A (RNAse) from bovine pancreas, and superoxide dismutase (SOD) from bovine erythrocytes were purchased from Sigma–Aldrich. Collagen type I from calf skin was purchased from Fluka and fibronectin from human plasma was purchased from Roche Diagnostics (Mannheim, Germany). The proteins were used without further purification taking into account the specifications of the suppliers. Protein solutions were prepared by dissolving 50 mg of the protein in 50 ml PBS. Further dilution resulted in concentrations of 100 μg/ml. Collagen I was dissolved and diluted in 0.01 *M* acetic acid. For the quantification of pure proteins 40, 20, 10 and 5 μl, respectively, of each solution were placed in glass tubes and stored at −18 °C prior to hydrolysis. Protein mixtures consisting of two components were prepared by mixing protein solutions.

2.3. Adsorption/Immobilization

The adsorption and immobilization of proteins was performed in home-built immobilization chambers, where well-defined surface areas of glass slides or silicon wafer are in contact with the protein solution.

Prior to the adsorption of proteins glass slides and wafers were cleaned in 50% ethanol and subsequently rinsed in Milli-Q water in an ultrasonic bath for at least 30 min and afterwards exposed to Caro's acid (an oxidizing solution consisting of sulfuric acid and potassium peroxodisulfate) for 1 h. Surfaces were thoroughly rinsed with Milli-Q water and dried under a nitrogen stream. Immediately after the cleaning procedure the slides were placed in the chambers and treated with protein solutions of different concentrations.

For the covalent immobilization of proteins glass coverslips were coated with poly(octadecene *alt* maleic anhydride) (Polysciences, Warrington, PA). Films of the reactive polymer were produced by spin coating (RC5, Suess Microtec, Garching, Germany) of a 0.1% polymer solution in tetrahydrofuran (Fluka, Deisenhofen, Gemany) on top of coverslips which had been cleaned in Caro's acid and thereafter surface-modified with 3-aminopropyl-dimethylethoxysilane (ABCR, Karlsruhe, Germany). Stable covalent binding of the polymer films to the glass carriers and the reconstitution of the anhydride moiety were achieved by tempering at 120 °C for 2 h. The slides were placed in the chambers and covalent immobilization was achieved by the subsequent immersion of the reactive surface with protein solutions.

After 2 h the protein solutions were replaced and the surfaces with adsorbed or covalently immobilized proteins were washed three times with PBS and three times with water. The glass slides were put into appropriate glass vials and stored prior to hydrolysis at −18 °C.

2.4. Hydrolysis

All glassware used in hydrolysis and subsequent derivatization steps was treated with chromosulfuric acid (Merck, Darmstadt, Germany) overnight, rinsed with Milli-Q water and dried at 80 °C. For gas-phase hydrolysis the glass vials containing the samples were placed in a 400-ml glass vessel. Together with the samples at least two glass tubes without probes were checked in parallel to give a blank value. The samples were dried under reduced pressure in the sealed vessel at room temperature for at least 1 h. Subsequently, 4 ml of 6 *M* HCl (containing 1% phenol, w/v) were added. The hydrolysis vessel was sealed again and the samples were exposed to vacuum for 15 s and afterwards the vessel was rinsed with nitrogen for 12 s. The last two steps were repeated twice. Finally, the nitrogen was removed from the vessel and vacuum was applied to the samples. The hydrolysis vessels were kept at 110 °C for 24 h. Afterwards, the HCl was removed and 30 μl of redrying reagent (water–ethanol–triethylamine, 2:2:1) were added to each vial for the neutralization of the samples. The samples were dried under vacuum and stored prior to analysis at −18 °C.

2.5. HPLC analysis

Chromatographic separation and analysis were performed with an Agilent 1100 capillary LC system (Agilent Technologies Deutschland, Böblingen, Germany) equipped with a vacuum degasser, a quaternary pump, an autosampler with thermostated sample rack, a thermostated column compartment and a fluorescence detector. A Zorbax SB-C_{18} (4.6×150 mm, 3.5 μm, Agilent Technologies Deutschland)

was utilized coupled to a security guard holder (Phenomenex Aschaffenburg, Germany) with a C_{18} (ODS) cartridge.

Derivatization of the hydrolyzates and standards with *o*-phthalaldehyde was achieved by an automated procedure. In general, hydrolyzates were solved in 200 μl of 50 m*M* sodium acetate buffer at pH 6.8, whereas hydrolyzates on glass slides were removed from the surface by a repeated rinsing with the buffer. A total of 25.2 mg OPA was dissolved in 500 μl methanol, 20 μl 2-mercaptoethanol and 4.5 ml 0.2 *M* borate buffer at pH 10.2 were added. One-third of the reagent was placed in the autosampler and the remaining mixture was stored at 4 °C in the dark. The reagent in the autosampler was replaced after 24 h by addition of 20 μl 2-mercaptoethanol. Derivatization of the amino acid samples was performed by mixing 30 μl of the OPA reagent with 10 μl of the sample using an autosampler program. Five μl of the derivatized samples were than injected into the system.

Separation of the derivatized amino acids was carried out using a binary gradient. Eluent A was 50 m*M* sodium acetate, pH 6.8–methanol–tetrahydrofurane (80:19:1) and eluent B was methanol–50 m*M* sodium acetate at pH 6.8 (80:20). The flow-rate was kept constant at 0.8 ml/min and the column was maintained at 30 °C. The gradient was established starting with 0% eluent B and continued linearly to 100% eluent B within 30 min, eluent B was kept at 100% for 3 min and within 1 min the gradient was switched to 100% eluent A.

The excitation wavelength of the fluorescence detector was λ_{ex}=335 nm. The emission was measured at λ_{em}=455 nm. Other settings of the fluorescence detector were optimized and maintained during all separations.

Amino acid standards, representing 166, 83, 42 and 21 pmol were analyzed with the samples. Each sample had to pass two runs and each standard had to pass at least four runs and only runs with fully consistent chromatograms were accepted for analysis. The control of the components of the chromatographic system and the analysis of the chromatograms with identification and quantification of the amino acids was performed with the Chemstation software Rev. 08.01 (Agilent Technologies Deutschland).

2.6. Numerical analysis of the measured amino acid distribution

Numerical analysis of the measured amino acid distribution was performed with MATLAB 6 (The MathWorks, Natick, MA, USA). A short routine was written to solve the linear equation system $Ax = B$ which results from assigning the amino acid ratios of the proteins of interest to the vector A and the measured amino acid ratios to the vector B. The amino acid ratios were calculated by normalizing the portion of the amino acid residues to the molecular mass of the protein. The numerical solution of the linear equation system for the amino acid ratio analysis was already utilized by Walsh and Brown [25]. The vectors A and B are of length 15 corresponding to the 15 amino acids aspartic acid, glutamic acid, serine, histidine, glycine, threonine, arginine, alanine, tyrosine, methionine, valine, phenylalanine, isoleucine, leucine and lysine which can be detected in the HPLC analysis. The solution x of the overdetermined linear equation system is calculated in the least-square sense. To improve the result of the calculation up to three amino acids are eliminated from the calculation when they differ more than 50% from the exact solution of $Ax = B$*. Lysine, serine and glycine were often eliminated according to this criterion probably due to problems in HPLC detection or contaminations containing those amino acids.

3. Results and discussion

3.1. Samples containing one type of protein

Due to the conditions of hydrolysis and derivatization the amounts of only 15 of the 20 proteinogenic amino acids were available for the quantification of proteins in solution and at interfaces. Glutamine and asparagine are known to be hydrolyzed to glutamic acid and aspartic acid, respectively, tryptophan is fully destroyed under the conditions of acid hydrolysis [18]. Proline cannot be detected since it possesses no primary amine function that is necessary for the derivatization with OPA. Cystein and cystin are susceptible to oxidation during hydrolysis and the cysteine sulfhydryl group competes

with the thiol component of the fluorescence derivatization reagent becoming a part of the product [26]. Thus, the quantification of proteins had to be accomplished with the detectable amino acids using the known protein sequences excluding the mentioned components.

The simplest approach to obtain protein amounts would be the addition of the detected quantities of the different amino acids. Subsequently, the sum had to be multiplied with a factor according to the percentage of the 15 amino acids in the protein. Direct implementation of this procedure may cause deviations in the calculated amount of the protein due to contaminations and analytical problems for certain amino acids. Since all deviations of the amino acids are added an even greater deviation in the calculated amount of the protein may occur. Using numerical analysis as outlined in Section 2, the composition of all 15 detectable amino acids can be used to calculate the amount of the protein of interest. Due to the solution of the linear equation system in the least-square sense the calculated amount of the protein has a strong correlation to the distribution in all 15 amino acids. This method allows a much better calculation of the amount of the protein because deviations in the single amino acids are not summed in the final result. Instead, the impact of the deviations is diminished due to the algebraic solution algorithm.

Furthermore, strong deviations of single amino acids from the expected distribution can be detected and the amino acids can be eliminated from the calculation accordingly. In Fig. 1 a typical example of the analysis by the numerical procedure is given. In Fig. 1a the amino acid distribution of 2.0 μg HSA experimentally obtained after HPLC is compared to the expected amounts of amino acids according to the protein sequence and the deployed amount of protein. Fig. 1b shows the recalculation of the amino acid distribution after elimination of tyrosine in the solution of the linear equation system of the amino acid ratios. The recalculated amounts are shown for all amino acids including tyrosine in order to indicate the expected amount of the amino acids for the calculated amount of the protein. The initial calculation reveals a total protein amount of 1.91 μg and the recalculation results in a total protein amount of 1.96 μg. Comparing these results with the calculation according to the simple addition of amino acids yielding 1.90 μg, a marked difference of the calculated amounts is only observed if the deviations of single amino acids were considered by the numerical analysis. Elimination of tyrosine provided a higher protein amount since the recalculation was performed without the underestimating value of tyrosine.

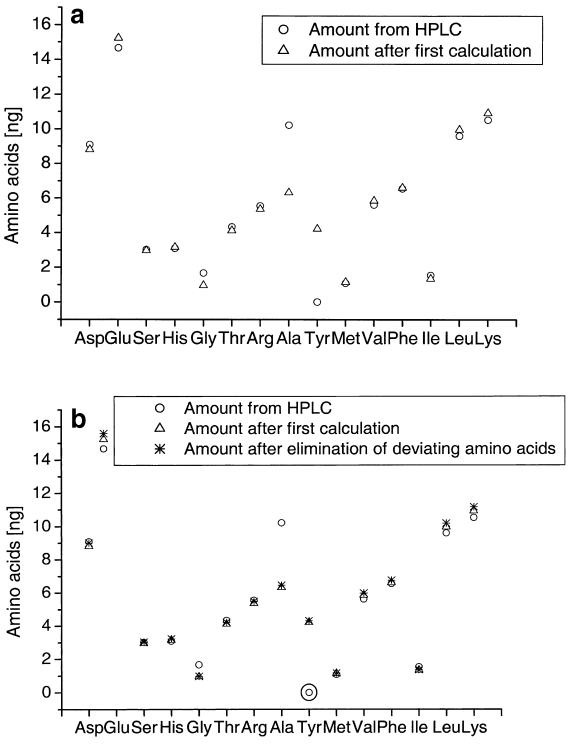

Fig. 1. (a) Amino acid quantities obtained from 2.0 μg HSA after hydrolysis/HPLC and compared to the amino acid composition of HSA. (b) Recalculation of the amino acid composition of 2.0 μg HSA after elimination of tyrosine. Data points marked with ○ are eliminated before recalculation.

Furthermore, HPLC was utilized for the calculation of surface-bound protein amounts. By studying the adsorption of HSA to a variety of surfaces distinct differences in the distribution of the detected amino acids (Fig. 2) were observed. Experiments were performed in duplicate and a good self-consistency of the data could be shown for the separate surfaces. Since only one type of protein was applied in those experiments one would expect similar amino acid ratios for each sample only differing by a

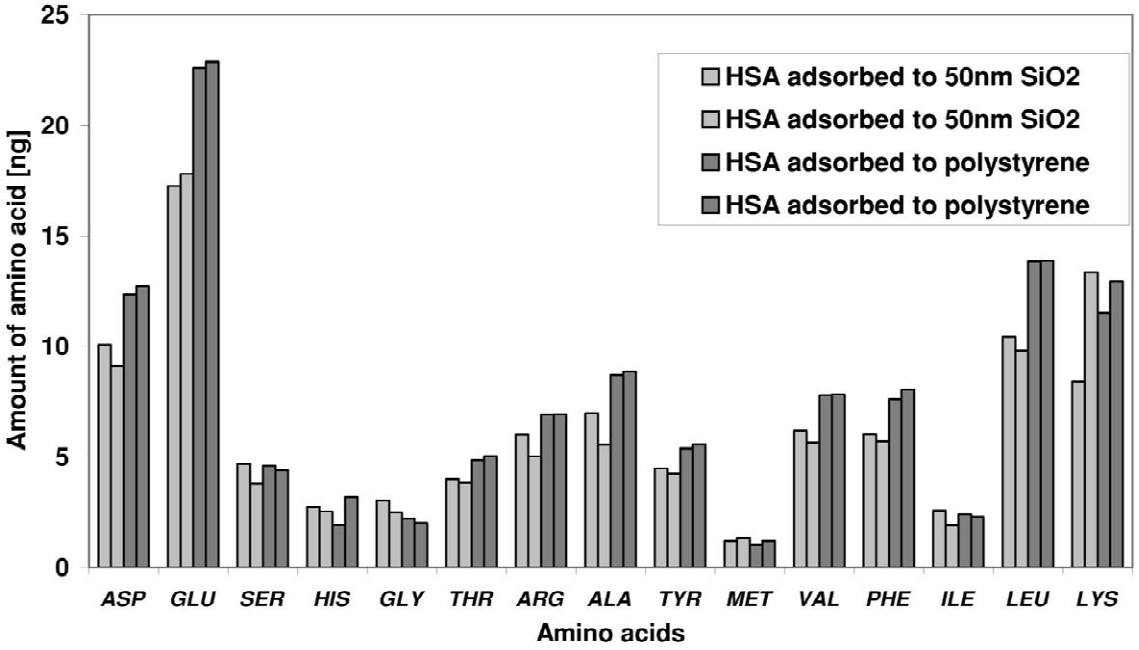

Fig. 2. Amino acid compositional analysis of HSA adsorbed to different surfaces. Adsorption of HSA to the different surfaces was performed in duplicate.

constant factor for all amino acids. However, the amino acids displayed a broader deviation and the deviation seemed to depend on the type of surface examined. The numerical analysis indicated higher amounts of adsorbed HSA on the hydrophobic polystyrene (653 ± 12 ng/cm^2) as compared to the silica substrates (508 ± 12 ng/cm^2) confirming earlier findings [27]. The example impressively demonstrates that the quantification of immobilized proteins is accompanied by broader difficulties as the quantification of proteins in solution. During the immobilization procedure glass slides or wafers as well as the protein solutions themselves were in contact with a couple of other materials including the equipment of the cleaning and coating process and the parts of the immobilization chambers. After hydrolysis the displacement of the released amino acids by repeated rinsing demanded the permanent contact to other materials. Therefore, impurities covering the surfaces of the external materials may additionally contribute to deviations of the amino acid compositions as well as the cleanness and the properties of the surfaces themselves influence the amino acid composition of the hydrolyzed proteins.

In addition to the substrate-related deviations observed for HSA the hydrolyzates of different surface-bound proteins often exhibit deviations in the amino acid distribution for other reasons. Such effects can be caused by impurities during the preparation and analysis or result from degradation during hydrolysis. To check for the cause of these problems detailed investigations were carried out. Blank values were used to indicate whether irregularities appeared during hydrolysis or chromatographic analysis. The blank values that were kept together with the samples during hydrolysis served as a control for the procedure and the protein amounts that could be determined in blank tubes were in the range of 20 and 50 ng. In addition, the solvents and the eluents of HPLC were run with the samples to test their purity.

Also, for the precise determination of protein quantities a direct comparison of the known amount of protein solutions with the amount calculated after HPLC analysis was established.

For that aim, well-defined amounts of HSA were prepared as indicated in Section 2, hydrolyzed and separated by HPLC thereafter. Following numerical

determination of the amount of the protein, mean values were calculated from at least four independent experiments. In the examined range between 0.2 and 4.0 μg for HSA a linear correlation between the amount of the deployed protein and the amount after hydrolysis and HPLC as shown in Fig. 3 was found. For samples containing less than 0.8 μg of HSA higher amounts of protein were determined. Contaminations and the noise of the baseline notably affected the result of the calculated amount. Around 1.0 μg a good correlation between the deployed and the calculated amount of HSA was observed. Initial weights above 1.0 μg of protein provided decreased values after hydrolysis and HPLC. The retrieval of the deployed amount of 4.0 μg HSA could be attained only with 87.5%. In the corresponding chromatograms the shapes as well as the separation of the peaks provided no evidence for an overload of the column. The pronounced linearity of the ratio of the deployed and experimentally determined protein amount permits the recalculation of the actual amount.

In addition to HSA we further analyzed a number of other proteins with respect to the applicability of the HPLC-based quantification. Referring to their molecular mass, their shape and their structural features these proteins are often used in model studies to analyze fundamental aspects of adsorption and displacement processes. The data combined in Fig. 4 confirmed our previous observations about the dependency of the retrieval of a protein on the deployed amount. The calculated values for the lowest protein amount of 0.5 μg are rather similar for all studied proteins. However, with increasing amounts a marked protein-dependent divergence was observed. For all proteins the ratio between detected and deployed amount decreased again in a linear dependence on the amount of protein detected as observed for HSA.

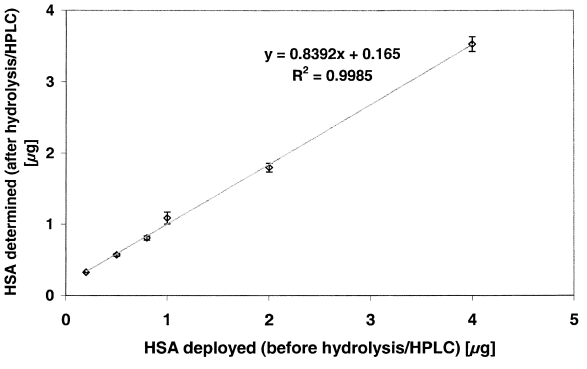

Fig. 3. Correlation of the amount of deployed HSA and the determined amount after hydrolysis and HPLC. Mean values were calculated from four independent measurements.

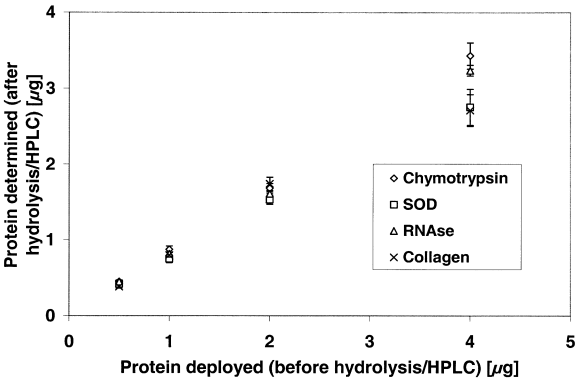

Fig. 4. Correlation of the amount of the proteins chymotrypsin, superoxide dismutase, ribonuclease and collagen before and after hydrolysis/HPLC. Amounts were calculated from at least four independent measurements.

An example for the numerical analysis of a covalently bound protein is given in Fig. 5. Here, the hydrolyzates of surface-bound fibronectin attached to thin films of poly(octadecene *alt* maleic anhydride) were examined. Starting concentrations of 2 and 100 μg/ml were chosen to adjust different surface concentrations of immobilized fibronectin. For comparison, the total amounts of surface-bound fibronectin were calculated by the simple addition method to 0.148 and 0.467 $\mu g/cm^2$, respectively. The numerical analysis provided 0.110 and 0.468 $\mu g/cm^2$. For the determination of the lower amount glycine, valine and lysine were excluded by numerical analysis. These results reflect the general observation that low amounts of proteins at interfaces are more affected by contaminations of the surface or contaminations during HPLC than higher amounts. The exclusion of the strong deviating amino acids by numerical analysis permitted the recalculation of the total protein amount on the base of the remaining amino acids according to the sequence of the protein examined.

Analyzing all data collected from different pro-

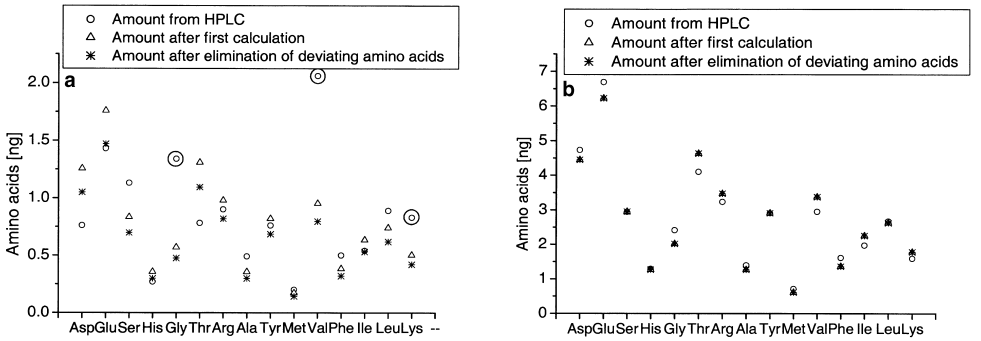

Fig. 5. Detection of single amino acids in samples of surface-bound fibronectin. Data points marked with ○ are eliminated before recalculation. Impact of low (a) and high (b) total protein amount on the deviation of single amino acids.

teins in solution and from proteins at interfaces we acquired a set of amino acids that were more affected with detection problems than others. Such deviations were already described by Hobohm et al. [21], who compared amino acid composition of hydrolyzed proteins with protein compositions in a database and acquired amino acid-specific errors that could be also applied to our system.

Because of these known problems the suggested numerical analysis of the whole amino acid distribution as well as the comparison of deployed and calculated protein amounts allowed a significantly improved determination of proteins due to comparison of the amino acid ratio in the protein sequence to the measured amino acid ratio and additionally allowing for the exclusion of falsely detected amino acids.

3.2. Samples containing protein mixtures

In addition to pure proteins in solution and at interfaces mixtures of proteins were examined with respect to the quantification of the single proteins after hydrolysis and HPLC. The compositions contained well-defined amounts of two proteins, namely HSA and chymotrypsin. Amounts of 0.5–3.0 μg of the proteins were mixed and analyzed subsequently.

Numerical analysis of the measured amino acid distributions was carried out in analogy to the method described for single protein systems. In case of protein mixtures the linear equation system $Ax = B$ consists of a matrix A of format $(m \times n)$ with $m = 15$ and n being the number of different proteins and x being a vector of length n. B is again the vector of length m resulting from the measured amino acid ratio. Like vector A for the single protein systems resulting from the amino acid ratio of the protein of interest matrix A in the analysis of protein mixtures is arranged by the amino acid ratios of the different proteins in the columns n of the matrix. Thus, vector x is again calculated from the overdetermined linear equation system in a least-square sense containing the proportional factors of the different proteins. From the procedure of the amino acid analysis, it can be concluded that it is more difficult to reveal the exact portions of the proteins in the mixture, because only the mixture of amino acids is analyzed, which no longer contains the information on the origin of the amino acid from any protein.

Despite of the mentioned difficulty the numerical analysis allows a good determination of the protein mixtures. As an example different mixtures of HSA and chymotrypsin were analyzed. The results in Table 1 shows that the analysis reveals the exact amount of the proteins for varying concentrations of the deployed proteins. This example demonstrates the reliability and usefulness of the numerical analysis approach for analyzing mixtures of proteins and is currently applied to competitive protein adsorption phenomena at polymer surfaces in our group.

Table 1
Analysis of mixtures of proteins

Mixture	HSA (deployed) (μg)	HSA (analyzed) (μg)	Chymotrypsin (deployed) (μg)	Chymotrypsin (analyzed) (μg)	DEV (μg)
1	0.5	0.48	3.0	2.99	±0.1
2	0.5	0.55	2.0	2.12	±0.07
3	2.0	1.90	0.5	0.52	±0.06
4	3.0	3.08	0.5	0.47	±0.1

The deployed amount and the analyzed amount by HPLC and numerical analysis is given.
DEV is the deviation of the numerical analysis calculated as the sum of the deviations of the calculated amount from the measured amount of each amino acid.

4. Conclusions

Numerical analysis of the amino acid ratios obtained from hydrolyzed proteins and evaluation of the retrieval of precise protein amounts for different proteins were found to enhance HPLC-based protein quantification. The suggested method can be applied to samples containing simple proteins and protein mixtures. The numerical analysis improved the accuracy of protein quantification by comparing the sequence of the examined protein to the experimentally determined ratio of the amino acids. Based on that method, the retrieval of well-defined protein quantities after acidic hydrolysis and subsequent HPLC separation could be evaluated to further improve protein quantification: examining different proteins a strong correlation between deployed and analyzed protein amount in the range between 0.5 and 4.0 μg was obtained providing protein-specific correlation factors. These correlation factors enabled a more exact quantification of both dissolved and immobilized proteins. The determination of surface-bound proteins was found to be particularly improved since deviations resulting from the preparation of immobilized protein layers could be corrected.

Furthermore, numerical analysis was successfully applied to analyze amino acid patterns obtained from hydrolysis of protein mixtures containing two components at different ratios. Advantageously, the approach allows for the simultaneous quantification of proteins out of mixed samples without use of labels or secondary binding reactions. Ongoing studies will further scrutinize this method for unraveling protein adsorption and displacement at solid surfaces.

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References

[1] P. Ducheyne, Q. Qiu, Biomaterials 20 (1999) 2287.
[2] L.G. Griffith, G. Naughton, Science 295 (2002) 1009.
[3] M.E. Nimni, Biomaterials 18 (1997) 1201.
[4] W. Tischer, F. Wedekind, Top. Curr. Chem. 200 (1999) 95.
[5] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265.
[6] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[7] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Anal. Biochem. 150 (1985) 76.
[8] H. Elwing, Biomaterials 19 (1998) 397.
[9] C. Werner, K.-J. Eichhorn, K. Grundke, F. Simon, W. Grählert, H.-J. Jakobasch, Colloids Surf. A 156 (1999) 3.
[10] G. Ladam, P. Schaaf, G. Decher, J. Voegel, F. Cuisinier, Biomol. Eng. 19 (2002) 273.
[11] R. Green, R.A. Frazier, K.M. Shakesheff, M.C. Davies, C.J. Roberts, S.J.B. Tendler, Biomaterials 21 (2000) 1823.
[12] C. Calonder, Y. Tie, P.R. Van Tassel, Proc. Natl. Acad. Sci. USA 98 (2001) 10664.
[13] M.S. Wagner, S.L. McArthur, M. Shen, T.A. Horbett, D.G. Castner, J. Biomater. Sci. Polym. Edn. 13 (2002) 407.

[14] P. Kingshott, S. McArthur, H. Thissen, D. Castner, H. Griesser, Biomaterials 23 (2002) 4775.
[15] Y.C. Liu, C.M. Wang, K.P. Hsiung, Anal. Biochem. 299 (2001) 130.
[16] M.J. Danilich, K. Kottke-Marchant, J.M. Anderson, R.E. Marchant, J. Biomater. Sci. Polym. Edn. 3 (1992) 195.
[17] H.M. Kowalczynska, M. Nowak-Wyrzykowska, J. Dobkowski, R. Kolos, J. Kaminski, A. Makowska-Cynka, E. Marciniak, J. Biomed. Mater. Res. 61 (2002) 260.
[18] M. Fountolakis, H.-W. Lahm, J. Chromatogr. A 826 (1998) 109.
[19] J.-C. Huet, J.-C. Pernollet, J. Chromatogr. 355 (1986) 451.
[20] L. Joergensen, H.N. Thestrup, J. Chromatogr. A 706 (1995) 421.
[21] U. Hobohm, T. Houthaeve, C. Sander, Anal. Biochem. 222 (1994) 202.
[22] G.A. Eberlein, J. Pharm. Biomed. Anal. 13 (1995) 1263.
[23] M. Weiss, M. Manneberg, J.F. Juranville, H.-W. Lahm, M. Fountoulakis, J. Chromatogr. A 795 (1998) 263.
[24] O. Golaz, M.R. Wilkins, J.-C. Sanchez, R.D. Appel, D.F. Hochstrasser, K.L. Williams, Electrophoresis 17 (1996) 573.
[25] M.K. Walsh, R.J. Brown, J. Chromatogr. A 891 (2000) 355.
[26] V. Barkholt, A.L. Jensen, Anal. Biochem. 177 (1989) 318.
[27] P. van Dulm, W. Norde, J. Colloid Interface Sci. 91 (1983) 248.