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Protein Species – the Future Challenge for Enzymology

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Abstract

Protein species – this term was originally introduced by Jungblut *et al.,* 1996 [1] – to name protein variants, which differ in their exact chemical composition. The term protein species differentiates between splicing variants, truncated proteins and posttranslational modified proteins. The exact chemical composition critically determines the function of a protein. Phosphorylation can activate or inactivate enzymatic activities. There are already many other posttranslational modifications, which are known to regulate enzymatic activities. Truncations also play an important role in activating enzymes. Therefore the knowledge of the identity comprising 100% sequence coverage and every posttranslational modification at its exact position is fundamental for assigning a function to an individual protein species. However, knowledge about the relationship of the function of a protein and its exact chemical composition is still not yet fully taken into account in many investigations of enzymes. In most of the proteomics approaches protein identification is based on sequence coverage significantly below 100% and posttranslational modifications are more or less ignored. Also in studies investigating single enzymes, a total analysis of the chemical structure of the enzyme of interest is not usually performed. Therefore it is recommended that this issue should be addressed in biochemical and biological investigations. The total analysis of the chemical composition

of an enzyme is quite a big challenge; however it is even more challenging to develop strategies, which allow the validation of the correctness of the function–chemical composition relationship.

Introduction

During the period of the human genome-sequencing project in the nineties of the last century a holistic view developed in life sciences resulting in the ‘‘ome’’ terminology. The expression ‘‘proteome’’, which was introduced in 1995 by Wasinger *et al*.[2], is a hybrid from the words **prote**in and gen**ome**, thus implicating the description of the entire protein complement encoded by the DNA of an organism. There is a huge difference in complexity between the genome and the proteome, because the genome is more or less static in contrast to the proteome, which is continuously changing from the beginning of the life of an individual organism until its death, thereby reflecting the different stages of development as well as the interaction of the organism with its environment. The proteome of an organism is many orders of magnitude larger than its genome. It is estimated that the human genome consists of 20,000 to 25,000 genes, which code more than 500,000 protein species [1]. The large number of proteins arising from the significantly smaller number of genes, is caused by at least 7 protein structure modifying steps following the transcription of a gene and resulting in the individual protein species, a term which was originally defined by Jungblut *et al.* [1] by its exact chemical structure comprising posttranslational modifications. The RNA processing and protein structure modifying steps, thus being multiplicators of the number of products originating by one single gene, are the cause of the huge number of protein species arising from a relatively small quantity of genes. In eukaryotes the RNA-transcript originating from a gene is processed yielding mRNA. Alternative splicing multiplies the number of mRNA coded by one single gene (multiplicator 1) [3]. After protein synthesis at the ribosome translating the information from the mRNA into the protein sequence, the protein will be folded into its 3-dimensional structure. Many proteins are then directed to particular locations in the cell guided by their N-terminal signal sequences. On their journey to their destination two further steps can occur to change the chemical structure of the protein. The N-terminal signal sequence will be removed by proteases (multiplicator 2) and further proteolytical processing may occur to the protein thus activating or inactivating it (multiplicator 3). For example, protease-activated receptors (PAR) are a group of proteins activated by this process. On the path from the protein synthesis at the ribosomes to the mature protein, disulfide bonds may be formed and/or posttranslational modifications such as carbohydrate chains may be added (multiplicator 4). It is estimated that several hundred posttranslational modifications (PTM) exist in eukaryotes [4]. Two basic forms of PTMs can be distinguished. The static PTMs such as oligosaccharides are known to have a key role in protein targeting. Dynamic PTMs like phosphate groups critically determine the activity of a protein. Furthermore, alternative splicing can also occur on the protein level (multiplicator 5). Protein activity is not only determined by PTMs or the action of proteases but also by the interaction of a defined protein species with other biomolecules or ions, which bind non-covalently to the

protein species (multiplicator 6). For example metallo-proteases require adequate metal ion to be active. Many enzymes of energy metabolism need cofactors like NADH. Other enzymes will be activated by forming complexes with defined proteins. Further changes to the structure of a protein happen at the end of its life-time (multiplicator 7). Ubiquitinylation for example starts its degradation [3]. The "record file" shown in Fig. 1 summarizes the parameters which are necessary for a comprehensive description of an individual protein species. Beside all the protein modifying steps listed above being responsible for the exact chemical structure of an individual protein species, the number of copies (the concentration of a protein species), the time of occurrence and the localization of protein species are three further fundamental parameters for fully describing an individual protein species in the context of the proteome (Fig. 1). The localization of a protein species in space in the organism is described exactly by information about its coordinates in the cell, in the tissue and in the organ. Furthermore, each individual protein species is determined by the time interval of its appearance measured on a time scale starting with the beginning of life of the organism and ending with its death. All parameters summed up in Fig. 1 determine the function of a protein species. A change of one of these parameters results in a different protein species and can be associated with an alteration of the function, as will be shown in the following examples. For several decades the phosphorylation and dephosphorylation of proteins have been known to switch enzymatic activities on and off [5]. Nitrosylation radically alters the function of GAPDH. Nitrosylated GAPDH is a switch for apoptosis [6]. Truncations of the amino acid chain can activate proteolytic activities of proteases (Fig. 2) [7]. Binding partners modulate substrate specificity as known from thrombin [7]. Proteins being the product of different splicing on the mRNA level may differ drastically in their functions as reported from the products of the angiotensin-converting enzyme gene. The protein species, which is synthesized e. g. in endothelial cells, is part of the blood pressure regulating system, whereas the splicing product present in the testis is involved in male fertility [8].

Identity: **PROTEIN SPECIES A** coded by **GENE XY**			
Structure	Amino acid sequence	3D structure	PTMs
Interactions	Metal ions	Small organic molecules	proteins
Localisation	intracellular	type of cell	tissue
Concentration			
Time interval of appearance			
Function: Enzymatic properties			

Figure 1. "Record file" of a protein species: Parameters describing all aspects of an individual protein species.

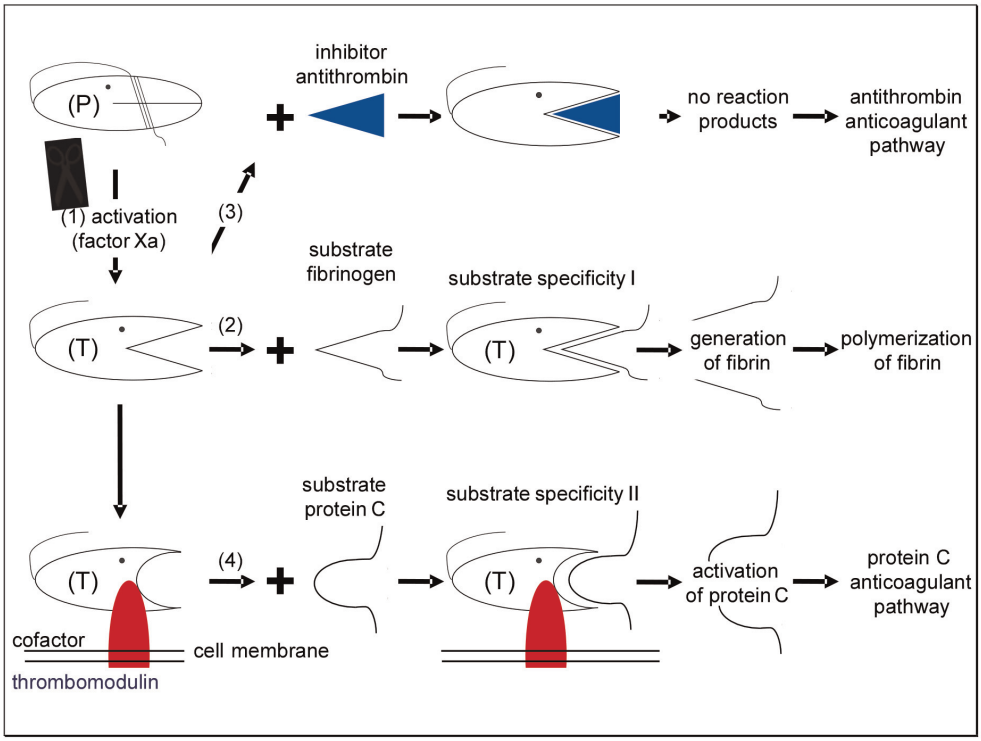

Figure 2. General scheme illustrating the dependency of identity of a protein species, defined by its exact chemical composition, and its functional status exemplified for thrombin. Thrombin (T) is generated from prothrombin (P) by factor Xa (1). Active thrombin cleaves its substrate fibrinogen into the fibrinopeptides (not shown) and fibrin (2) which polymerizes and therefore plays a central role in clog formation. Binding of thrombin to antithrombin inhibits the proteolytic activity of thrombin (3). Interaction of thrombin with the membrane bound cofactor thrombomodulin (4) results in a change of the substrate specificity of thrombin thus being able to activate protein C by proteolysis which leads to the protein C anticoagulant pathway.

In summary the function of a protein species depends on its exact chemical structure, its interactions with other biomolecules or ions (Fig. 2), its concentration as well as time and site of appearance in an organism (Fig. 1). To assign a defined function to a protein species unambiguously, knowledge of all the parameters listed in Fig. 1 is necessary. In this review the following question will be considered, which parameters that describe protein species can be yielded, by applying the strategies and techniques so far available for proteomics.

Access to the Protein Species Level

For the analysis of proteomes the ‘‘2-DE approach’’ and the ‘‘shot-gun approach’’ are the most established analytical procedures (Fig. 3). One of the big advantages of the 2-DE is that the presence of different individual protein species, coded by one single gene, can be resolved because of the very high resolution of the 2-DE and can be recognized (Fig. 3) – in this case these protein species will occur at different positions on the 2-DE gel [9, 10]. In the two-dimensional electrophoresis (2-DE) approach, the proteins are separated first by the two electrophoresis steps by their isoelectric point and their size. Many of the past studies using 2-DE and cataloguing the proteins present on the 2-DE gel, identified the proteins after enzymatic digestion via MALDI-MS peptide mass fingerprint (PMF) analysis followed by database searches [11]. Today the analysis of the enzymatic digests of protein spots cut from the 2-DE gel are performed preferably with PMF combined with tandem mass spectrometry (MS/MS) or pure MS/MS approaches yielding fragment spectra of the individual enzymatic peptides, thus ensuring a much higher grade of confidence concerning the correctness of the identity. In usual proteomics approaches full sequence coverage of the analysed proteins is not achievable with either the PMF approach or with the MS/MS analysis, as those peptides, which are modified by PTMs, are ignored or, even worse, wrongly interpreted in the case of the PMF analysis. Furthermore, some peptides may get lost during the LC steps or may not be resolved by the mass spectrometer, because they do not desorb into the gas phase, are not ionisable, or are too small or too big. As a result it is difficult to yield the identity of the protein species including the exact chemical composition, comprising 100% sequence coverage and all posttranslational modifications. However, recently Okkels *et al.* [12] resolved the complete chemical structure of 6 ESAT-6 protein species from a 2-DE gel of *Mycobacterium tuberculosis* and Myung demonstrated that 100% sequence coverage is achievable [13], if a multi-enzyme digestion strategy using trypsin, AspN, LysC and chymotrypsin is applied.

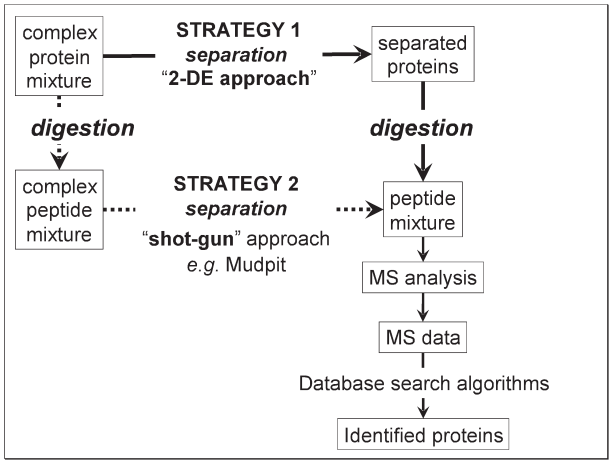

Figure 3. The two main strategies of proteomics.

In contrast to the 2-DE approach the recognition of the presence of different protein species originating from one gene is not possible using the ”shot-gun approach”. Here, many peptides of these protein species resulting from enzymatic digestion have the same amino acid sequence, thus eluting in one single peak (Fig. 4). The 2-DE pattern shows, that there are different protein species stemming from a single gene. In contrast, in the case of the shot-gun approach the origin of the enzymatic cleavage peptides in the LC (liquid chromatography) chromatogram cannot be assigned to the 4 protein species. From the data, recognizing the presence of different protein species is not feasible. As a result no statement about the quantitative relationship of the 4 protein species is possible (Fig. 5). More sophisticated shot-gun approaches such as MudPIT (Multidimensional Protein Identification Technology) [14, 15], also start with the enzymatic digestion of complex protein mixtures, followed by the separation of the generated peptides and have the same limitation.

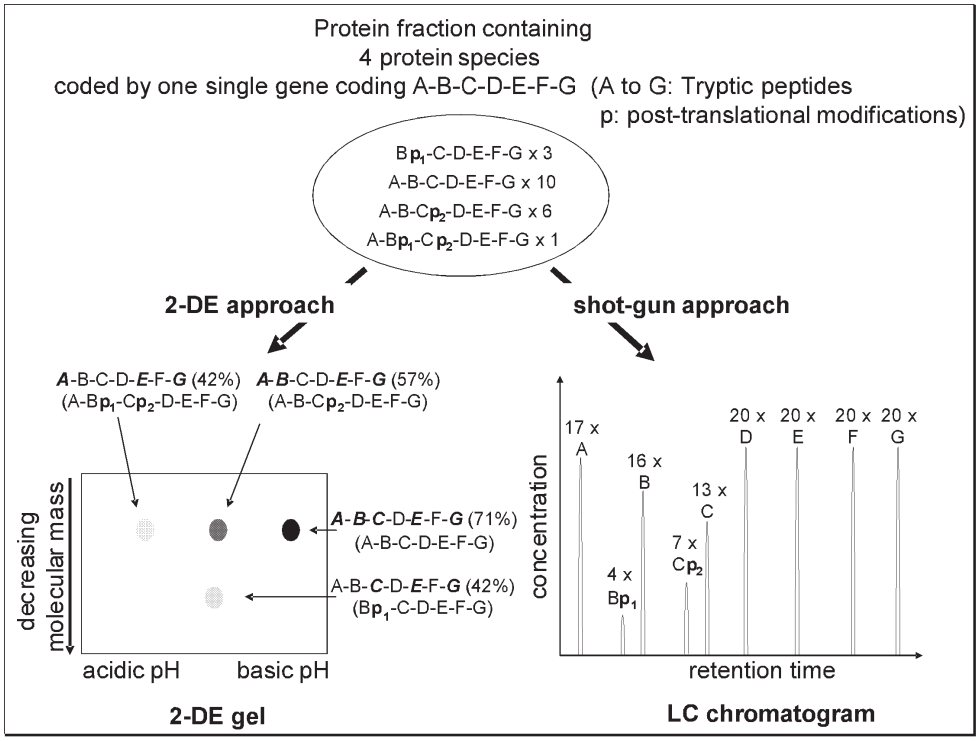

Figure 4. Simplified model of typical results of the analysis of a protein fraction containing 4 protein species present in 4 different concentrations (1x, 3x, 6x, 10x), coded by a single gene, performed either by the 2-DE approach or the shot-gun approach. The letters A to F represent peptides generated by enzymatic cleavage. Posttranslational modifications: p_1, p_2. 2-DE approach: The different quantities of the protein species are represented by the different greys. In this model only the tryptic peptides A, B, C, E, F and G (bold italic letters) yield data for the identification of the protein via data base searches resulting in 1 protein name (here named A-B-C-

D-E-F-G) for 4 different protein species (A-Bp_1-Cp_2-D-E-F-G; A-B-Cp_2-D-E-F-G; A-B-C-D-E-F-G; Bp_1-C-D-E-F-G). The peptide coverage (in percent) differs from protein species to protein species.

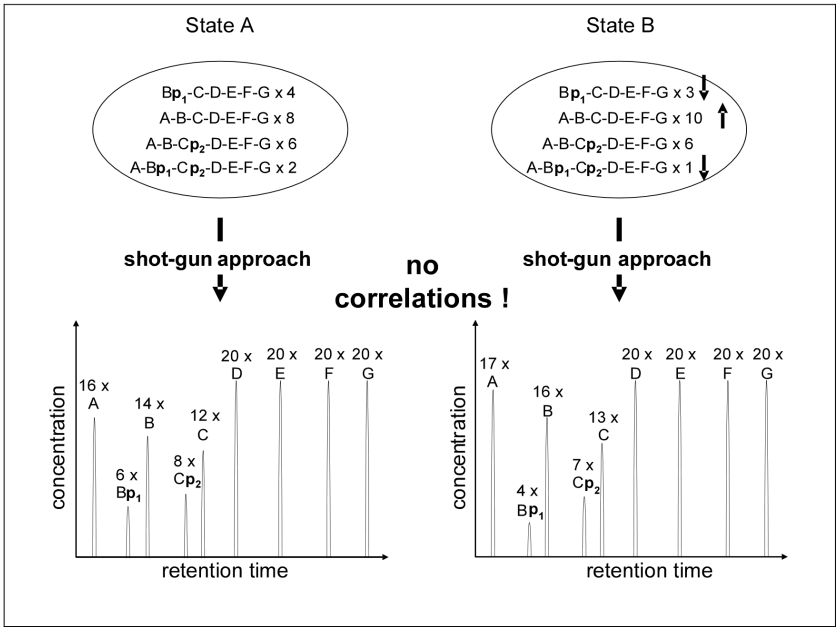

Figure 5. Simulated quantitative analysis by liquid chromatography of a protein fraction from state A and state B, differing in the concentrations of 4 protein species coded by one single gene. In the idealized chromatograms "absolute quantities" were yielded. A-B-C-D-E-F-G in state A is down-regulated 20% compared to state B. A-Bp-Cp-D-E-F-G in state A is up-regulated 100%. Bp-Cp-D-E-F-G in state A is up-regulated 25%.

Quantification of Protein Species with Labelling Reagents

Several quantitative approaches employing chemical labelling with stable isotopes are often combined with the shot-gun strategy [16], for instance ICAT (Isotope-Coded Affinity Tag) [17], ITRAQ (Isobaric Tag for Relative and Absolute Quantification) [18] and SILAC (Stable Isotope Labeling by Amino acids in Cell culture) [19]. These methods have already proved their applicability in the determination of relative protein amounts in different states of biological systems [20]. However in the case of the presence of several protein species which are all coded by one single gene, the shot-gun approach may yield false results since the enzymatic cleavage peptides have identical amino acid sequences, but originate from different protein species the concentrations of which usually change individually, can no longer be distinguished after enzymatic digestion (Fig. 4). The detection of the up- or down-

regulation of individual protein species, which may be of importance concerning their function, is not possible, as exemplified in Fig. 5. This problem can be circumvented by first labelling the intact proteins with reagents for differential quantification, followed by the separation of the intact protein species either by electrophoresis or by liquid chromatography or combinations of both. After their purification to near homogeneity the protein species will be digested enzymatically. This quantitative analytical approach can be performed with the ICAT reagent [21], the ITRAQ reagent (isobaric tags for relative and absolute quantification) [22], the ICPL reagent (Isotope-Coded Protein Labeling) [23] and the MECAT reagent [24] to mention only some of the possible labelling procedures.

In the ICAT method, proteins from the two states to be compared are labelled at cysteine residues with heavy and light tags, respectively, carrying a biotin moiety. The labelled proteins are then mixed, separated by 2-DE and digested. After a cation-exchange chromatography step, the mixed peptides are affinity purified via immobilized avidin. Peaks corresponding to the same peptide are identified as doublets in mass spectra due to the mass difference between light and heavy isotopes. The peak intensities of the peptides correlate directly with the relative abundance of the proteins in the two states.

The ITRAQ technology makes use of a four-plex and eight-plex set of amine reactive isobaric tags to derive peptides at the N-terminus and the lysine side chains, thus labelling all peptides in a digest mixture. In MS, peptides labelled with any of the isotopic tags are indistinguishable (isobaric). Upon fragmentation in MS/MS, signature ions (m/z from 114 to 117) are produced, which provide quantitative information upon integration of the peak areas [18].

The ICPL label is an isotope coded nicotinoyl group coupled to an amino reactive N-hydroxysuccinimide (NHS). NHS targets unmodified amino groups of lysine and the N-terminus. The benefit of lysine labelling is the more frequent occurrence in proteins as compared to cysteine (≈ 6% vs. 1.5%). A weakness of lysine labelling is that trypsin does not cleave ICPL-modified lysine sites. Trypsin digestion of ICPL labelled proteins results in rather long peptides, because cleavage occurs solely at arginine residues. Because of the loss of basic amino groups, proteins are shifted to the acidic side of the gel, which is an advantage for basic proteins.

The MECAT reagent contains a chelating group, which binds lanthanoid metal ions with high affinities and can be coupled covalently to proteins. With the MECAT reagent ''loaded'' with different lanthanoid cations, proteins from different states can be labelled. Since the metal elements are detected by the ICP-MS even an absolute quantification of MECAT- labelled proteins is possible. A further advantage of the ICP-MS based quantification is the large linear detection range of 7 orders of magnitude. In comparison to all other quantification techniques based on labelling, MECAT is the only quantification method which allows the detection of quantitative differences without the need for digesting the

proteins. Focusing on those proteins, which occur in different concentrations, will reduce significantly the number of proteins which have to be analysed for their identity. MECAT is also compatible with the mass spectrometric top-down approach, which introduces intact protein ions into the gas phase of the mass spectrometer by an electro-spray ionization (ESI) source, followed by determining the molecular mass of the protein and thereafter fragmenting the proteins, thus yielding more specific data for the characterization of sequence and posttranslational modifications than by peptides from the protein's digestion.

Intact protein identification by MS/MS – the top-down approach – was demonstrated first by Mortz *et al.* 1996 [25]. In the top-down approach, intact protein ions are introduced into the mass spectrometer and then fragmented, yielding the molecular masses of both the protein and the fragment ions. If a complete set of informative fragment ions are detected, this analysis can supply a complete description of the amino acid sequence of the protein and reveal all of its posttranslational modifications. The main challenge of the top-down approach is given by the problem of producing wide-ranging gas-phase fragmentation of intact protein ions. By pumping large amounts of energy into the ionized protein in the gas-phase, Han *et al.* significantly improved the applicability of the top-down approach towards the analysis of protein species [26]. The authors reported that they could obtain very informative fragmentation for proteins with molecular masses larger than 200 kDa. The essential fragmentation element of the top-down approach appears within reach since the current introduction of two other very helpful methods for fragmenting proteins – electron transfer dissociation [27] and electron capture dissociation [28]. Nevertheless top-down still is a technique for studying single purified proteins. Other challenging problems wait to be overcome before the top-down approach can be regarded as really strong for proteomics studies. The need to separate complex mixtures of proteins prior to mass spectrometric top-down analysis remains a central challenge. The extremely different physico-chemical properties of the individual proteins make them difficult to handle as mixtures without gaining awesome losses of certain proteins or leaving the proteins incompatible with mass spectrometry. Even more demanding is the need to separate the protein species which are only slightly different. Sensitivity is also a major challenge, because effective fragmentation of a high-molecular-mass protein implies that the protein will fracture in a large number of different ways. Therefore, the intensity of the resulting fragments will be weak compared to that of small peptides. The most practicable approach today is the top-down 2-DE separation of protein species combined with the bottom-up identification of the peptides by MS.

Label-Free Quantification of Protein Species

Is it possible to quantify protein species without labelling? This question can be accepted for hypothesis-driven investigations, if an enzymatic cleavage peptide of a protein species, which belongs to a family of the protein species derived from one single gene, is known, which is unique according to its chemical structure. This unique peptide can be quantified by

the selected-reaction-monitoring method (SRM, also called multiple-reaction monitoring) [29]. This type of mass spectrometric experiment is very common in quantifying drugs and their metabolites [30]. SRM experiments are designed for obtaining the maximum sensitivity for detection of target compounds, as shown by Onisko *et al*., who quantified attomole amounts of the prion protein with the SRM method in the brains of terminally ill Syrian hamsters [31]. Knowing the mass and structure of the peptide, it is possible to predict the precursor m/z and a fragment m/z (SRM transition) [32]. The advantage of measuring the fragment ions is the reduction of background interferences, especially for complex mixtures such as plasma. Fragmentation in the majority of cases offers one or more unique fragment ions. The combination of the specific parent mass and the unique fragment ion is generally an unambiguous method to monitor and quantify selectively the peptides of interest. The SRM method has already been applied for identifying and quantifying protein posttranslational modifications [29] thus demonstrating that the SRM method is suitable for quantifying protein species via their tryptic peptides which are unique within the tryptic peptides of the protein species family. Quantification of a protein by the SRM method via its tryptic peptides is reported by Berna *et al*., who analysed the time course of the protein myosin light chain 1 in rat serum following a 50 mg/kg subcutaneous dose of isoproterenol, a β-adrenergic receptor agonist known to induce cardiac injury [33].

Conclusion and Perspectives

Since the determination of the exact chemical composition in relation to its function will be time consuming, the question arises as to which data are really needed to achieve progress in life science. In the past the discovery and identification of key players of molecular processes in organisms helped in the understanding of fundamental aspects in biochemistry and molecular biology. This knowledge was also the basis for the development of new drugs. Therefore it should be beneficial to apply the new tools of proteomics for the identification of protein species linked to defined functions. This aim can be achieved by comparing quantitatively the protein species composition of two biological systems in the quiescent (control) state and in a defined activated state thereby identifying those protein species, the concentrations of which have changed. An alternative, yet classical way of identifying the function and the exact chemical composition of a protein species comprises its purification towards near homogeneity guided by a functional assay. An example following this strategy is given in Rykl *et al.,* [34] where a protease with a defined function was purified using a system (PPS [35]) for the determination of optimum chromatographic purification steps and detecting the protease via its catalytic properties with a mass spectrometric assay (MES [36]). The purified protease was identified via mass spectrometric analysis of the tryptic peptides of the purified active fraction. Although in this study the active protease was identified without determining the exact chemical structure, the workflow comprises the potential to elucidate the exact chemical structure.

Since the protein function is critically associated with the exact chemical composition of the protein species it should become obligatory in future analysing and publishing of the complete amino acid sequence and identifying every posttranslational modification of the individual enzyme under investigation. By applying targeted protein analysis to those key protein species will make it possible to follow these proteins over a longer period of time since the restriction of the analysis towards a few protein species will enable the analyses of a larger quantity of samples collected at many different time points. This reinforces the need for flexibility in our interpretation of sequence data and also illustrates the importance of more traditional approaches such as genetics and biochemistry for discovering gene functions.

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