Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 849 (2007) 32–42
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Review

Leveraging protein purification strategies in proteomics☆

Ipsita Roy[a], Kalyani Mondal[b], Munishwar N. Gupta[b,*]

[a] *Department of Biotechnology, National Institute of Pharmaceutical Education and Research (NIPER), Sector 67, SAS Nagar, Punjab 160062, India*
[b] *Department of Chemistry, Indian Institute of Technology, Delhi, Hauz Khas, New Delhi 110016, India*

Received 15 June 2006; accepted 9 November 2006
Available online 1 December 2006

Abstract

The proteomic studies, although, tend to be analytical in nature, yet many strategies of preparative protein purification can be usefully employed in such studies. This review points out the importance of purification techniques which are capable of dealing with samples which are suspensions rather than clear solution, e.g. aqueous two phase partitioning, three phase partitioning, expanded bed chromatography, etc. The review also outlines the potential of non-chromatographic techniques in dealing with fractionation of proteomes. Separation protocols which can deal with post-translationally modified (PTM) proteins are also considered.

Keywords: Post-translational modification; Protein arrays; Affinity precipitation; Three phase partitioning; Aqueous two phase system

Contents

1. Introduction

It is not uncommon that an emerging area attracts research workers from different disciplines. In the process, the area develops faster but the boundaries of the area quite often get blurred (Table 1). The area of proteomics is a good example of this. Irrespective of the perception (about the connotation of the term proteomics) or the intended application, there are an equally frequent references to "protein purification" [1,2] and "protein analysis" [3,4]. This should not cause any confusion since any analysis of a complex mixture requires a pre-fractionation and pre-sorting stage. The proteome is undoubtedly complex. This complexity has many dimensions:

☆ This paper is part of a special volume entitled "Analytical Tools for Proteomics", guest edited by Erich Heftmann.

* Corresponding author. Tel.: +91 11 2659 1503; fax: +91 11 2658 1073.
E-mail address: munishwar48@yahoo.co.uk (M.N. Gupta).

1570-0232/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2006.11.016

Table 1
Development of the field of proteomics over the years

1975	Discovery of two-dimensional gel electrophoresis [167] Anderson et al. employed selective denaturation in a temperature gradient two-dimensional gel electrophoresis as an 'initial resolving method' [168]
1981	First reports of development of a high field magnet coupled to fast atom bombardment mass spectrometric studies of peptides [169] Electron capture negative chemical ionization for tandem mass spectrometry of neuropeptides [170]
1983	Improved reproducibility of two-dimensional gel electrophoresis using immobilized pH gradients [171]
1988	Second generation immobilized pH gradient leading to development of horizontal micro two-dimensional gel electrophoresis [172]
1995	"Total protein complement of a genome" [173] The term 'proteomics' was coined by the Australian Proteome Analysis Facility Ltd. to emphasize similarity with genomics and was viewed as "the next step". Although Macquarie University PhD candidate Marc Wilkins is credited with the christening of this term in 1995, the origin of proteomics goes back to 1970–1980s, as evidenced above
1996	Establishment of mass spectrometry as a validated tool for proteomics [174]
2000	Importance of proteomics in the area of biomarker discovery for disease diagnosis and therapeutic monitoring recognized [175]
2001	"The analysis of complete complements of proteins. Proteomics includes not only the identification and quantification of proteins, but also the determination of their localization, modifications, interactions, activities, and, ultimately, their function." [176]
2004	Invitrogen Corporation introduces first commercially available high density protein microarray containing 1800 unique human proteins, including those involved in cell signaling
2006	"Proteomic and genomic analyses, while frequently not hypothesis-driven, offer the opportunity to accelerate the pace of discovery, particularly clinically relevant discovery" [177] Fully automated computational program (BlastPro) developed for rapid comparison of large proteomic databases leading to identification of biomarkers for metastatic cancers [178]

Table 2
An overview of the techniques used in 'conventional' and 'proteomic' separation

Conventional separation	Proteomic separation
Pre-separation steps	Pre-separation steps
Solid–liquid separation	Protein depletion or protein fractionation by any of the separation techniques
(a) Filtration [180]	
(b) Centrifugation [181]	
Precipitation	
(a) Salt [182]	
(b) Organic solvent [183]	
(c) Polymer [184]	
(d) Detergent [185]	
Chromatographic steps	Identification steps
Ion exchange chromatography	Two-dimensional gel electrophoresis
Hydrophobic interaction chromatography [186]	Mass spectrometry (coupled to liquid chromatography)
Affinity chromatography [187]	Orthogonal chromatography
Gel filtration chromatography [188]	Two-dimensional chromatography
Radial flow chromatography [189]	Direct analysis of large protein complexes (DALPC)
Perfusion chromatography [190]	Isoelectric focusing using nonporous reverse phase HPLC (IEF-NP RP HPLC)
Expanded bed chromatography [42,43]	
Displacement chromatography [33,34]	Bioimaging
Monoliths [191]	Microarray profiling
Non-chromatographic steps	Microfluidics
Aqueous two phase extraction [67–69]	
Three phase partitioning [63,64]	
Reverse micellar extraction [192]	
Crossflow ultrafiltration [193]	
Preparative electrophoresis [194]	

The techniques listed here are illustrative and not all-inclusive. In the 'proteomics' scenario, separation is often synonymous with identification. The emphasis is on 'automation, miniaturization and integration' [179].

a large number of components, a wide range of molar concentrations and the dynamic nature. The last feature itself has many facets. Proteins in a living cell continue to be "born" and "die". What is more, these molecules change their structure and hence shape/properties by aggregation or reversible covalent changes. These are the challenges of proteomics. Hence, the skills acquired and the tools developed in the area of protein purification have to be stretched to meet these challenges. It is worthwhile to keep the differences in the objectives of a protein purification exercise per se and in the context of a proteomic study in mind. It is also instructive to list the wide range of available protein purification strategies as people working in the area of proteomics may not be familiar with all of these (Table 2). Right early, there seems to have been a consensus that it is prudent to analyze proteins by looking at peptides that are generated (upon proteolysis) rather than analyzing the protein directly [5]. The early studies on proteomics were based upon electrophoretic separation of peptides which were analyzed by MALDI [6]. Alternatively, liquid chromatography was used for separation and analysis was carried out by ESI-MS [7]. A good account of how MS has evolved for proteomic applications is available [8]. With time and the widening of the horizons, it was soon realized that there is a need to have pre-fractionation stages before analysis [1,9]. These pre-fractionation stages may initially deal with intact proteins and later on with peptides. This is where protein/polypeptide purification strategies have become extremely relevant. The advent of protein/peptide arrays has brought the area of affinity-based separations into sharp focus.

This review looks at some of the concepts and strategies in the area of protein purification which have become relevant to proteomics. As the concept of affinity interactions dominate even the purification and, of course, the detection/analysis by protein arrays, it is wise to start with that.

2. The concept of affinity and affinity-based separations/analysis

Of all the separation methods used by biochemists, affinity chromatography is considered most selective. This method originated in the observation that a coenzyme or a competitive inhibitor would selectively bind to its corresponding enzyme even if the latter is present in a complex protein mixture [10]. With time, it was realized that for this selective molecular recog-

nition to occur, the in vivo relationship between the "affinity ligand" and the cognate enzyme/protein is not necessary (for a fuller discussion on the evolution of the affinity concept, see [11]). Simple textile dyes and metal ions were the next generation of affinity ligands. There was a natural concern that affinity chromatography with textile dyes was not as highly selective as with a coenzyme/competitive inhibitor/antibodies. This led to a substantial body of work on the design of biomimetic ligands [12–14]. The structures of biomimetic ligands are designed by molecular modelling techniques which determine the best fit between the affinity ligand and the protein molecule in terms of complementary shape and minimum energy of the complex [15]. While the bulk of the biomimetics ligands are designer dyes, it is possible to think of any kind of structure and use this approach. Phage display technology ushered in the next paradigm shift in obtaining affinity ligands [16]. This technology creates large libraries of peptides on the phage surface which are screened for selective binding to a chosen protein [15]. It became necessary to adopt and adept high throughput screening methods and automation. Ribosomal display libraries and oligonucleotide libraries (aptamers) by SELEX are other recent approaches for obtaining affinity ligands [17,18]. The use of fusion tags or affinity tags for exploiting affinity interactions is already adapted in proteomics and has been reviewed quite frequently [19,20].

So what is an affinity ligand? It is a molecule which can recognize the target protein with reasonable selectivity. Its structure is immaterial. It is also immaterial whether this molecular recognition has any relevance in the in vivo context. The degree of selectivity is governed by the dissociation constant of the affinity ligand-protein complex. For purification/analytical purposes, it is the relative selectivity which is important. The dissociation constant of the complex formed by the affinity ligand and the chosen protein should be much different (lower) than the corresponding dissociation constants of the complexes which this affinity ligand forms with other proteins present in the mixture. In an affinity chromatography, it is necessary that the bound protein also be dissociated from the complex. The typical steps in an affinity chromatography are:

(i) creating an affinity media by linking suitable affinity ligand to a matrix.
(ii) affinity capture.
(iii) separating the affinity complex from rest of the solution.
(iv) elution of the desired protein by dissociation of the affinity complex.

In fact, it is often possible to bring in an additional selectivity step at the elution stage. However, more relevant in the current context is the fact that very low dissociation constants (observed in the case of highly selective ligands) may not be ideal as it may not be possible to dissociate the affinity complex and recover the desired protein. Dissociation constants in the range of 10^{-8}–10^{-10} M observed with highly specific antigen–antibody interactions illustrate this [21]. Elution of the protein in affinity chromatography based upon antigen–antibody interactions is a tedious process and is dealt with very nicely in an earlier excellent review [22]. In the case of protein/peptide arrays (see later discussion), this restriction does not apply as only the formation of the affinity complex is monitored. This paves the way for using highly selective ligands in the design of such arrays. However, wherever an affinity-based separation method is used as a pre-fractionation device, the facile dissociation is a critical requirement and must be kept in mind while choosing/designing affinity ligands.

Finally, affinity chromatography is not the only affinity-based separation process. Other affinity-based separation processes, both chromatographic and non-chromatographic have been described [23].

3. Chromatographic methods in proteomics

Kellner mentioned that developing a multi-dimensional chromatography (on the line of 2D gels) may be difficult [24]. The concern expressed was that chromatographic steps are run in a serial mode and this would hamper a high throughput format. Contemporary developments show that this view is rather pessimistic. Chromatography is already adapted and available in high throughput platforms [25]. The multi-dimensional LC is now a well established tool in proteomics. Shi et al. have reviewed the role of liquid chromatography in proteomics [26]. The role of reverse phase liquid chromatography coupled with mass spectrometry has been extensively documented in proteomics literature. Shi et al. mention the promise of "ultra" high-pressure capillary RPLC-MS [26]. An exciting approach called LC-Fourier transform ion cyclotron resonance MS based upon the concept of "accurate mass tag" (AMT) has also been described [26]. This uses identification of the protein via a peptide whose mass is measured with extremely high accuracy (at 1 ppm level) and obviates the need for tandem MS (MS–MS) approach. The AMT approach was successfully used for global analysis of *Deinococcus radiodurans* proteome [27] and is credited with extension of the sensitivity, dynamic range, and throughput of conventional LC-based proteomic tools [28,29].

There has been great concern about analysis of low abundance components which are obscured by the presence of protein with high abundance [30]. Identifying a biomarker in blood, for example, is complicated by the high abundance of serum albumin. Depletion techniques are generally used before looking at low abundance proteins [31,32]. A recent work describes how both high and low abundance proteins present in goat serum can be separated and even recovered by judicious application of protein separation methods [32]. Displacement chromatography (which utilizes the displacement of the proteins by displacers rather than more frequently used elution mode) has a long history [33,34]. It has shown a good promise in enrichment of low abundance peptides in recombinant human growth hormone [35].

Among the orthogonal (multi-dimensional) chromatographic techniques in proteomics, RPLC is preceded by ion exchange or affinity or size exclusion chromatography [26]. RPLC remains the second dimension because of its compatibility with MS techniques. Shi et al. mentions an interesting 2D chromatography called MudPIT (combination of cation exchange and reverse phase chromatographies) coupled to MS which could be used to

profile the entire proteome of *S. cervisiae* and turned out to be more powerful than 2D-PAGE [26].

The isotope-coded affinity tag (ICAT) technique is fairly well known in quantitative proteomics [36,37]. At present, it is limited to the utilization of avidin–biotin-based affinity and ion-exchange chromatography [38]. It should be possible to extend this to other formats in coming years.

The review by Mirzaei and Regnier describes an innovative concept for using chromatography in proteomics [39]. The outline of this concept is that proteolysis should be followed by the picking of the peptide containing a specific amino acid like histidine, cysteine or methionine. As sufficient experience in the form of vast literature on peptide separation and modification of these amino acids exist, the combined knowledge, as discussed by these authors, can lead to some interesting resolutions of peptides obtained after proteolysis. The authors have summarized the successful results in selecting N- or C-terminal peptides or peptides containing post-translationally modified (PTM) amino acids.

4. Non-chromatographic protein purification processes

It is universally agreed that wherever high resolution is desired, chromatography scores over any other separation method; it has also been convenient to adapt it to automated and high throughput platforms. Nevertheless, a brief discussion on some emerging non-chromatographic options may be worthwhile. At least for some of these, it is easy to visualize their adaptation to high throughput format via micro titer plates and ELISA readers.

4.1. Affinity precipitation

The design of this process is based upon smart polymers [40]. Such polymers are also called stimuli-sensitive or reversibly soluble-insoluble polymers [41]. Essentially, such polymers can dissolve in aqueous solution but precipitate out if an appropriate condition is changed. This condition can be a change in pH, temperature or ionic strength of the solution. Addition of a chemical specie such as Ca^{2+} or other less common stimuli such as ultrasonication or microwave irradiation are also applicable. This insolubility can be reversed by removing/reversing the stimulus [40]. Such polymers occur in nature (e.g. alginate, chitosan) but can be synthesized as well [e.g. poly(*N*-isopropylacrylamide), methyl methacrylate polymer]. All such polymers have enough functionalities for coupling chemistries to be used for linking a suitable affinity ligand. The resultant smart affinity macroligand can form affinity complex with the cognate protein in a selective fashion in a free solution. More importantly, this affinity complex can be removed from contaminating proteins by applying suitable stimulus. The precipitated affinity complex then can be dissolved as usual for the recovery of the protein [42]. In many cases, it has been found that such smart polymers as such show inherent affinity towards various proteins and no affinity ligand is needed to be linked for creating smart affinity macroligands [42,43]. Some early results suggest that these smart polymers can act as pseudochaperonins [44] and affinity precipitation can be used for simultaneous purification/refolding of proteins [45,46].

Is there a relevance of refolding in proteomics? Many proteins are rather labile in nature and undergo inactivation due to conformational changes. In fact, for protein purification workers, such labile proteins pose a special challenge [47]. In the context of proteomics, it is possible that unfolding/refolding strategies, especially if these have inbuilt concentration/purification features may turn out to be valuable in some cases.

Some recent work combines the concept of fusion tags with affinity precipitation [48,49]. In this approach, the fusion tags play the role which is played by smart polymers in affinity precipitation.

4.2. Three phase partitioning:

Three phase partitioning (TPP), in fact, was originally described for interfacial precipitation of proteins. TPP has proved valuable as such for purification of several proteins [50–52] (Fig. 1) as well as for protein refolding [53,54]. As such, it lacks selectivity yet has proved adequate for obtaining protein preparations of reasonable purity [50,52].

4.3. Macro-affinity ligand facilitated three phase partitioning (MLFTPP)

Three phase partitioning has been around for quite sometime but it is only in recent years that its full potential seems to have been appreciated [50,55,56]. Its recent version, MLFTPP, can work with feed containing suspended matter [57] (Fig. 2). Its elegance lies in its simplicity. It essentially consists of the following steps: (i) mix an organic solvent (highly miscible with water) with crude mixture of proteins (and other suspended impurities) and a smart affinity macroligand in the presence of salt; (ii) the organic solvent separates out as an upper phase and aqueous

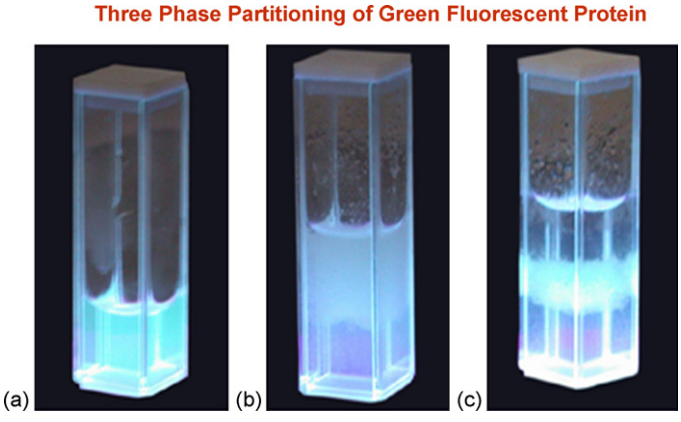

Fig. 1. Three phase partitioning of Green Fluorescent Protein (GFP). (a) GFP solution (1 mL) was placed in a quartz cuvette under UV light. (b) 60% wt. v^{-1} $(NH_4)_2$ SO_4 and *tert*-butanol (1:1 vol. v^{-1}) were added. The solution was once more placed under UV-light. (c) After 3 min phase separation has begun. After 30 min, three phases viz. lower aqueous phase, the interfacial precipitate and the upper layer of *tert*-butanol are clearly seen. The aqueous phase once fluorescent is now a clear solution, while the interfacial precipitate containing GFP shows green fluorescence.

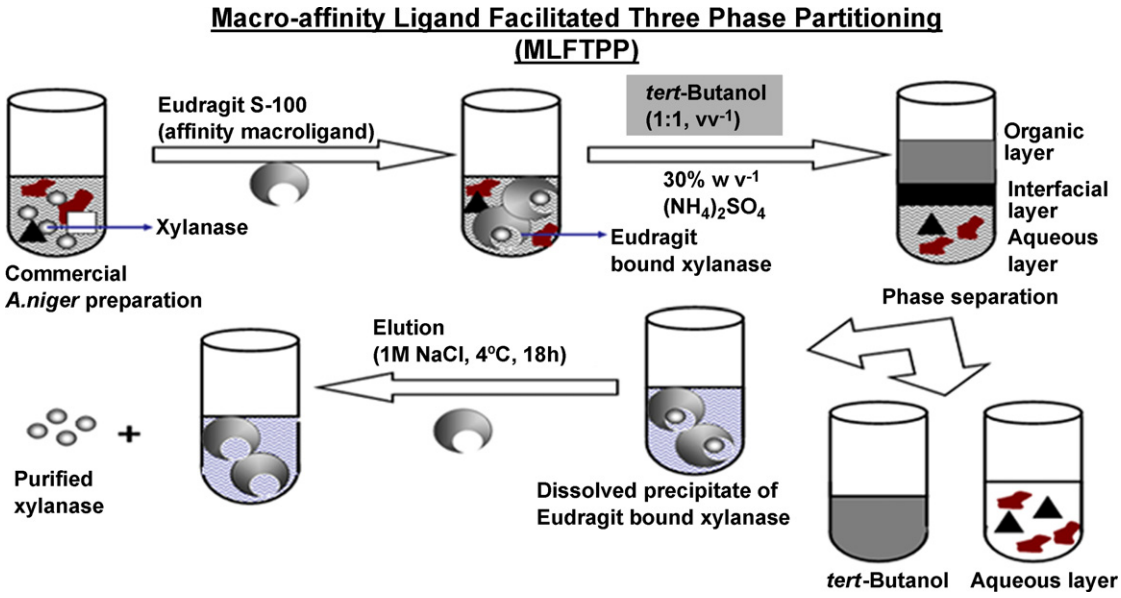

Fig. 2. MLFTPP of xylanase using eudragit S-100 as the affinity macroligand. Xylanase enzyme was purified from the commercial preparation of *Aspergillus niger* using eudragit S-100 as the smart affinity macroligand [51].

phase containing suspended material forms the lower phase; (iii) the smart affinity macroligand with the bound cognate protein forms interfacial precipitate. The affinity complex can be dissociated just like in any affinity-based separation process and the cognate protein recovered. MLFTPP can simultaneously concentrate and purify proteins [57–59]. It has been shown that MLFTPP can also refold proteins starting with their inactive forms [45].

4.4. Aqueous two phase systems (ATPS)

Aqueous solutions of two polymers (e.g. PEG and dextran) or a polymer and a salt at high ionic strength (PEG and phosphate solutions) when mixed, form two phases. A mixture of proteins when introduced into such ATPS, would partition between two phases. The suspended matter settles down at the bottom constituting a separate solid phase. The separation of proteins can be achieved by manipulating the partition coefficient of the proteins by varying the average molecular weights of the polymers, the ionic strength of the salts or by introducing an affinity ligand (of the cognate protein if the biological function of the protein is known) [60]. Such a step maybe especially valuable at a presorting stage in a proteomic study. It was, for example, found that a proteome of *E. coli* when partitioned in PEG-dye/salt ATPS resulted in 30% of the protein partitioning in PEG phase containing PEG-dye and 70% remained in the lower salt phase [61].

It should be added that ATPS, which has already been described is yet another non-chromatographic technique. In fact, both ATPS and EBC can be made more selective by incorporating affinity interactions in the process. In ATPS, the affinity ligand can be linked to a phase forming polymer. PEG-dye, as an example, has already been mentioned. This approach has many success stories to its credit [62–64]. In another variation, a smart affinity macroligand can be incorporated into one of the phases. The application of the appropriate stimulus allows separation of the affinity complex from rest of the solution and its dissociation leads to recovery of the protein(s) [65,66].

5. Separation methods that can deal directly with suspensions

Even in traditional enzymology, all the samples with which purifications are started, are generally suspensions. That is true of proteomics as well. High speed cold centrifugation and microfiltration (using membranes which retain the particles in the size range of 0.1–10 μm) are thus the initial steps in protein purification/analysis. This is because any packed bed chromatography step is compatible with only clear feed. The suspended materials would cause choking of the column, generate back pressures and ultimately stop the flow through the column. There are three purification strategies which however can deal directly with suspensions. The two of these, namely, ATPS and MLFTPP has already been discussed. The third one called expanded bed chromatography is briefly described below.

5.1. Expanded bed chromatography (EBC)

Expanded bed chromatography utilizes all the concepts of traditional packed bed chromatography but the bed is in a fluidized form. This is achieved by (i) using tailor made chromatographic media which forms “stable” fluidized bed, i.e. there is no backmixing during the chromatographic process; (ii) this fluidization is achieved by introducing the feed in upward direction. In such a bed, the inter-particle distance is higher (than packed bed) and the suspended impurities (such as cell debrii) can pass through the resultant voids [67,68]. While expensive commercial grade chromatographic materials for expanded beds in ion-exchange, hydrophobic interaction and affinity formats are available, it is possible to use less expensive alternatives such as calcium-alginate or zinc alginate beads [68–70]. EBC combines

filtration/centrifugation, concentration and purification in a single step. While there is no work available on its adaptation to a high throughput format at present, small fluidized columns have been used for analytical purposes [71,72]. EBC, while a chromatographic approach, has also benefited by using an affinity media based upon chromatographic material which can fluidize [67]. Considering that fusion tags like polyhistidine are extensively used in the purification of recombinant proteins as well as in proteomics [73], it is necessary to mention that fluidized beds for the immobilized metal affinity chromatography have been described and can be used for recovery of polyhistidine tagged proteins [74,75].

6. Dealing with post-translational modifications

Look at any standard textbook in biochemistry and you would find that it mentions 20 amino acids which occur naturally! However, as early as 1987, the review by Uy and Wold mentioned that this list actually consists of several hundred amino acids [76]. All these extra ones arise by post-translational modifications of the polypeptide. In fact, hydroxy proline, included in the textbook list is also a result of post-translational modification. These PTM amino acids do not have a separate codon. This means that the gene sequence will tell us nothing about the presence of these PTM-amino acids in the polypeptide. It is now well known that these PTM (Fig. 3) play an important role in defining conformation, stability and function of these proteins. This is also the reason why genomics does not have all the answers and is the challenge that proteomics has to meet.

A large list of PTM (than the illustrative Fig. 3) is given by Meri and Baumann [77]. To start with, it should be appreciated that many proteomic studies (like everything else in life sciences) tend to be based upon recombinant systems. In that context, there is a serious concern of expression systems whose PTM patterns are identical to the original organism. Braun and LaBaer [78] provide a very pertinent discussion on this and point out that insect cells score over other systems like yeast in this respect as these cells carry out most eukaryotic PTMs more properly. Another aspect to be kept in mind is that PTMs can be transient. This is especially true of regulatory enzymes and proteins involved in signal transduction. PTMs can also occur as a result of infection or tissue engineering [77]. So analysis of PTM has to keep these dynamics in mind.

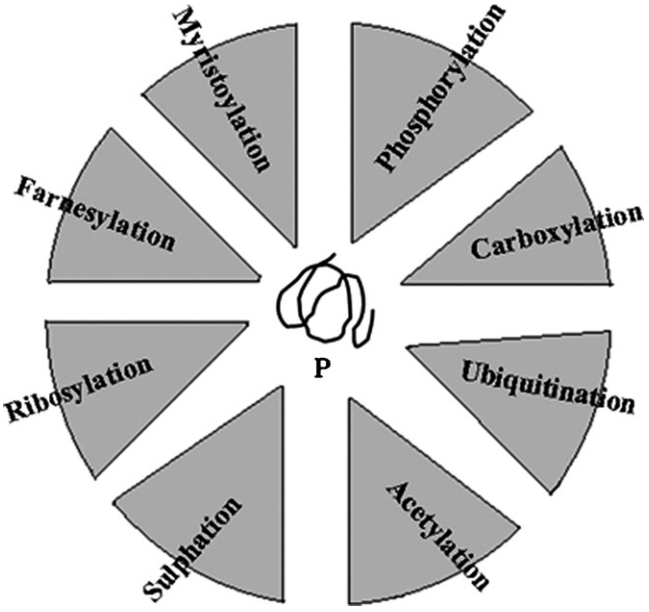

Fig. 3. Post-translational modification of proteins (P).

Some analytical approaches for looking at PTMs in the context of proteomics have been discussed at several places [30,39,79]. Understandably, affinity chromatography has played a very useful role here. Lectin affinity chromatography has been a powerful tool for looking at glycoproteins over the years [80,81]. A large number of lectins with a variety of specificity in terms of sugars are known [82]. In some cases like the lectin Concanavalin A, information about the fine details of how oligosaccharide structure influences the binding with lectin is available [83,84]. It may be pertinent to point out that efficient downstream processing strategies now allow purification of these lectins at a large scale [71,85]. Thus, lectin affinity chromatography is a viable technology and constitutes an unlimited opportunity for analyzing the important PTM of glycosylation. Similarly, IMAC with Fe^{3+} charged media had proven its usefulness in looking at phosphorylation several years back [86] and has been exploited in proteomics [87]. Antibodies specific to phosphopeptides have also been tried as affinity ligands [88]. However, both IMAC and antibody-based affinity chromatography seem to suffer from nonspecific binding. To overcome this, introducing an affinity tag at phosphorylation sites has been tried [89]. Nevertheless, immunoprecipitation or affinity binding (in solid phase) using anti-phosphotyrosine antibodies and IMAC have given impressive results [79]. What is more, these approaches are able to monitor the phosphorylation dynamics of the cell. The selectivity of IMAC could be improved by a prior methylation step (to eliminate acidic but nonphosphorylated peptides binding to IMAC in a nonspecific fashion). Lambert et al. also cites the work based upon stable isotopic amino acids in cell culture (SILAC) strategy which looked at the time course of tyrosine phosphorylation in HeLa cells as a result of epidermal growth factor stimulation [79]. SILAC is also based upon the use of antiphosphotyrosine antibodies. Size exclusion chromatography before IMAC and reverse phase chromatography after IMAC have been used to improve enrichment and resolution for phosphopeptides. Lambert et al. have also cited the evaluation of graphite powder and TiO_2 for binding of phosphorylated peptides in a selective manner with good success [79]. In all such cases, it has been necessary to work out elution conditions as well since final analysis has been invariably with MS techniques.

There has been much less work on analyzing other PTMs. Linked enzyme assay and traditional affinity chromatography have been used for quantifying *N*-myristoylation and farnesylation [79]. Mirzaei and Regnier have aptly pointed out that there are nonenzymatic PTMs as well. Glycation via Maillard reaction is a key process in diabetic complications and ageing [79]. Similarly, oxidative modifications are implicated in ageing and in various disease conditions like arthritis, muscular dystro-

phy and various neurological disorders. As these modifications invariably generates carbonyl groups, textbook chemistry like derivatization with 2,4-dinitro phenylhydrazine (2,4-DNP) or biotin hydrazide has proved valuable. The derivatized peptides could be separated on RPC coupled to MS–MS system.

Given the vast variety of PTMs, it is obvious that a lot more needs to be done in terms of selective separation strategy development before this challenge can be effectively met.

6.1. Challenges in design of protein arrays

Apart from 2D-PAGE and mass spectrometry, microarrays have emerged as a powerful tool in proteomics [90,91]. This is one of those examples where borrowing of a concept from genomics has paid dividends. DNA microarrays, based upon the concept of hybridization of the single stranded DNA sequences arranged as an ordered array on a (chip) surface with complementary test sequences enabled development of a high throughput technology for genome analysis [92,93]. Two complementary DNA strands recognizing each other is, just another example of the affinity phenomenon which extends beyond protein structures. Hence, it follows naturally that protein microarrays would be designed for proteome analysis. A good review of what has been possible is available [90].

As has been mentioned before, for an analytical application based upon the concept of affinity, the dissociation of the affinity complex is not an issue (it becomes a major issue in preparative level protein purification; otherwise protein recovered is very low). Appreciation of this leads to the understanding of two important implications. Firstly, one can work with affinity ligands which have very high binding constants. Secondly, this also enables one to operate with high selectivity (as the selectivity is based upon binding constants). Hence, it is not surprising that antibodies have been the most frequently used affinity ligands in protein arrays so far [94,95]. One can generate antibodies which are highly specific without worrying about the low dissociation constants of the resulting affinity complexes. Phage display antibodies provide a robust approach to generate sequences corresponding to variable regions of antibodies [96]. Antibody fragments derived from polyclonal sera or monoclonals have been used in other contexts for quite some time now [97–99]. Application of "naïve" antibodies to screen for antigens present in a human foetal brain library has been demonstrated [100]. So there is a good knowledge base. The information and experience with generation of antibodies in the context of proteomics was reviewed recently [2]. A subsequent review also provides an extensive discussion on screening antibodies on high throughput platforms [3].

Looking at the current scene, one gets a distinct feeling that this approach suffers from the lack of cross-fertilization of the ideas. As has been appreciated elsewhere too, the main drawback of this approach is in the intrinsic instability of protein reagents [90]. While it is true that as compared to other affinity ligands, proteins are more fragile, the issue of protein stability and protein inactivation mechanisms have been very extensively studied [101,102]. Perhaps, more relevant is the vast literature on protein stabilization which describes a variety of approaches like chemical modification [103,104], immobilization [105], site-directed mutagenesis [106] and directed evolution [107]. The same review also talks of the manipulation of protein activities by derivatization and the efforts involved in immobilization on solid chips. This is like rediscovering wheel in the context of a new car design! One may have to modify it, but the concept of the wheel is there. There is an enormous body of work spanning the last few decades which has dealt with the issues of minimizing structural and functional damage to a protein during chemical modification and immobilization. This includes the contexts in which the intended application is in water [108,109], aqueous-organic co-solvent mixtures [110,111], neat organic solvents [112,113] and ionic liquids [114,115]. Some of the books [105,116] and reviews [111,117] dealing with this are available. For protocols, the reader may profit from looking at [118]. Without intending to belabour the point, Cutler cites a reference with the year 1999 which "facilitated the immobilization of antibodies on the surface via the glycosylated region (^{297}Asp) in the F_c region of the protein." The approach was described by O'Shannesy and Hoffman in 1987 [119]. Even in 1992, hydrazide-derivatized supports for this purpose were commercially available [120]. Extensive experience with this approach have since been gained [121]. The approach is called oriented immobilization and includes the use of biotin and avidin (or streptavidin), oligosaccharide moiety (of the protein) and lectin interactions and protein A and IgG interactions [121].

Hence, it is worthwhile to provide some information originating in other areas which may prove to be helpful for workers in the area of protein array.

- For any chemical modification (including protein immobilization), it maybe worthwhile to "protect" the active site by adding a suitable substrate analog. Of course, this presumes that some idea regarding the functional aspects of the cognate protein is available.
- The key approaches for immobilization are: noncovalent immobilization [108,122], covalent immobilization [109,123], entrapment [124] and chemical aggregation [125]. Noncovalent immobilization such as ionic binding or adsorption is the gentlest method. It also generally retains the maximum activity of the protein. Unfortunately, the resultant immobilized protein is also prone to slow leaching off the surface [126]. Bioaffinity immobilization is a special class of noncovalent immobilization and exploits affinity interactions. It is a promising approach [127,128] and some results in the context of protein arrays are already available [129,130]. The huge literature on self-assembling layers [131,132] and the recent work on affinity layers are also worth mentioning [133,134]. While covalent immobilization invariably affects the structure and function of the protein, vast options in terms of coupling chemistry are now available [135]. As Scope pointed out [136], even with the same affinity ligand but with a different matrix and/or coupling procedure and/or a spacer, the selectivity of the immobilized affinity ligand may change in a critical fashion. Entrapment, because of mass transfer constraints is generally not suited for immobilizing an affinity ligand. Chemical aggregation [125] as a protein immobiliza-

tion approach has recently given rise to biocatalyst designs such as CLEA and CLEC [137–140]. Both immobilization approaches have shown usefulness in the area of biosensors [141–143] and this experience may be useful while designing protein arrays.

- In general, affinity arrays form a part of the area of biosensors [144,145]. The latter area has been largely dominated by protein immobilized by covalent methods [145]. It may be highly instructive to refer to the results reported on Surface plasmon resonance (SPR) by multiple users [146,147]. The reliability of Surface plasmon resonance assays was evaluated by different participating laboratories. While each lab used the same protocol, the coupling reagents were from separate individual labs. In both reports [146,147], this led to large variations both in the amounts of immobilized protein and the extent to which the immobilized protein molecules retained their activity. However, it did not affect the quality of kinetic data obtained both for small molecule/enzyme [146] or high affinity antibody-antigen interaction [147].
- A considerable amount of research activity has been initiated in the area of bionanofabrication [148]. The technologies in this area encompass biological micro-electromechanical systems (bioMEMs) and microfluids [148,149]. The work of Cabodi et al. is viewed as an alternative to other conventional separation methods by size but utilizes nanoscale physical phenomenon of entropic force [150]. The work of Hess and Bachand with motor proteins on the other hand deals with engineering proteins for compatibility with artificial interfaces and creating novel ways to link such proteins on nanomaterials [151].

Some of the challenges mentioned by Cutler in the area of protein arrays are worth emphasizing [90]. In general, affinity ligands do not distinguish between isoforms of a protein. So protein arrays would not be successful in distinguishing populations of various protein isoforms. The second issue is of the dynamic nature and larger range of protein expression levels. It has been suggested that antibodies of different affinities may be used. Thirdly, the nonspecific binding as well as cross-reactivities is a major complication which is amplified in proteomics in view of the highly complex systems involved. So far antibodies have been mostly used in protein arrays for many successful applications [91]. Peptide arrays wherein peptides act as affinity ligands have also been successfully used for epitope mapping, detection of pathogen infections, monitoring signal transduction, enzyme profiling and identifying ligands involved in cell adhesion [91].

A peptide/protein array using a supramolecular hydrogel has also been described a few years ago [152]. This shows how entrapment can work as an approach for anchoring the affinity ligand. Also, the presence of sufficient water in the hydrogel is believed to be conducive to maintaining the native structure of the protein.

The stage of detection of the molecular recognition is equally important. Cretich et al. classify the detection strategies into label-free and labelled probe methods [91]. The former strategy includes atomic force microscopy (AFM), MEMS and quartz crystal microbalance (QCM). The latter comprise of fluorescence, chemiluminescence, elastochemiluminescence and radioactivity measurements.

A recent review advocates the use of biomimetic ligands as affinity ligands in proteomics [153]. It describes small molecular weight with the structure based upon the triazine-scaffold which can recognize a variety of protein molecules. It is not unlikely that in future, we will see arrays based upon such and other kinds of affinity ligands.

7. Conclusion

The diverse research activities classified under the umbrella of proteomic studies, are united by a common set of tools A proteome being too complex, needs to be fractionated to get any meaningful picture. For both fractionation and final analytical stages, enzymology, protein chemistry and what is sometime called applied biocatalysis have much to offer to proteomics. To be fair, some of that knowledge and skills belonging to these older areas is already being fast adapted. Hopefully, this review would accelerate this adaptation by crossfertilization across various disciplines. This need for crossfertilization cannot be overemphasized. Firstly the workers as peer reviewers in proteomics need to be familiar with enzymology especially the bioseparation area. A recent work published in a proteomics journal rediscovered TPP and called it NATP [154]. This is in spite of the fact that TPP is known since 1987 [155] and not less than 30 papers on role of TPP in separation and fractionation are available [50,63,64]. In the area of protein separation, for example, no new unit process except expanded bed chromatography has been discovered in the last several decades. Even in that narrower area, the advancements have come by innovation. Protein purification is achieved either by electrophoretic or non-electrophoretic methods. Among the latter are chromatographic and non-chromatographic methods. This review has mentioned some precipitation and partition methods as examples of the latter kind. Membrane-based separation technique [156,157] is another powerful tool in enzymology which does not seem to have been adapted. From the viewpoint of workflow automation, membrane-based separations would fit in very well. As a large number of membranes for ultrafiltration [156], nanofiltration [158] and affinity membranes [159,160] are available, this may have a good potential in proteomics. From analytical perspective, enzyme analysis in nonaqueous media is more or less an unexplored territory [161,162]. There is a lot which is happening in the area like biosensors and protein refolding [163–166]. It is very likely that this may become quite relevant to proteomics.

Acknowledgements

The partial financial support provided by Department of Science and Technology (DST) and Council of Scientific and Industrial Research (CSIR) (Extramural Division & Technology Mission on Oil Seeds, Pulses and Maize), all Government of India Organisations, is acknowledged. The financial support provided by IIT Delhi to K.M. in the form of Senior Research Fellowship is also gratefully acknowledged.

References

[1] S.A. Lesley, Protein Expr. Purif. 22 (2001) 159.
[2] A. Bradbury, N. Velappan, V. Verzillo, M. Ovecka, L. Chasteen, D. Sblattero, R. Marzari, et al., Trends Biotechnol. 21 (2003) 275.
[3] A. Bradbury, N. Velappan, V. Verzillo, M. Ovecka, L. Chasteen, D. Sblattero, R. Marzari, et al., Trends Biotechnol. 21 (2003) 312.
[4] R.C. Panicker, S. Chattopadhaya, S.Q. Yao, Anal. Chim. Acta 556 (2006) 69.
[5] J.R. Kettman, J.R. Frey, I. Lefkovits, Biomol. Eng. 18 (2001) 207.
[6] F. Lottspeich, Angew. Chem. Int. Ed. 38 (1999) 2476.
[7] S.J. Valentine, M. Kulchania, C.A.S. Barnes, D.E. Clemmer, Int. J. Mass Spectrom. 212 (2001) 97.
[8] R.D. Smith, Trends Biotechnol. 20 (2002) S3.
[9] A.J. Link, Trends Biotechnol. 20 (2002) S8.
[10] E. Stellwagen, Acc. Chem. Res. 10 (1977) 92.
[11] K. Mondal, M.N. Gupta, Biomol. Eng. 23 (2006) 59.
[12] C.R. Lowe, S.J. Burton, J.C. Pearson, Y.D. Clonis, V. Stead, J. Chromatogr. B: Biomed. Sci. Appl. 376 (1986) 111.
[13] Y.D. Clonis, V. Stead, C.R. Lowe, Biotechnol. Bioeng. 30 (1987) 621.
[14] N.E. Labrou, Y.D. Clonis, Arch. Biochem. Biophys. 321 (1995) 61.
[15] N.E. Labrou, J. Chromatogr. B 790 (2003) 67.
[16] R. Cortese, P. Monaci, A. Luzzago, C. Santini, F. Bartoli, I. Cortese, P. Fortugno, G. Galfre, A. Ncosia, F. Felici, Curr. Opin. Biotechnol. 7 (1996) 616.
[17] M.B. Murphy, S.T. Fuller, P.M. Richardson, S.A. Doyle, Nucleic Acids Res. 31 (2003) e110.
[18] M. Moreno, E. Rincon, D. Pinero, G. Fernandez, A. Domingo, A. Jimenez-Ruiz, M. Salinas, V.M. Gonzalez, Biochem. Biophys. Res. Commun. 308 (2003) 214.
[19] J.J. Lichty, J.L. Malecki, H.D. Agnew, D.J. Michelson-Horowitz, S. Tan, Protein Expr. Purif. 41 (2005) 98.
[20] D.S. Waugh, Trends Biotechnol. 23 (2005) 316.
[21] M. Mammen, S.K. Choi, G.M. Whitesides, Angew. Chem. Int. 37 (1998) 2754.
[22] M.L. Yarmush, K.P. Antonsen, S. Sundaram, D.M. Yarmush, Biotechnol. Prog. 8 (1992) 168.
[23] M.N. Gupta (Ed.), Methods in Affinity-Based Separation of Proteins/Enzymes, Birkhauser-Verlag, Basel, 2002.
[24] R. Kellner, Fresenius J. Anal. Chem. 366 (2000) 517.
[25] C. Draveling, L. Ren, P. Haney, D. Zeisse, M.W. Qoronfleh, Protein Expr. Purif. 22 (2001) 359.
[26] Y. Shi, R. Xiang, C. Horvath, J.A. Wilkins, J. Chromatogr. A 1053 (2004) 27.
[27] M.S. Lipton, L. Pasa-Tolic, G.A. Anderson, D.J. Anderson, D.L. Auberry, K.R. Battista, M.J. Daly, J. Fredrickson, K.K. Hixson, H. Kostandarithes, C. Masselon, L.M. Markillie, R.J. Moore, M.F. Romine, Y.F. Shen, E. Stritmatter, N. Tolic, H.R. Udseth, A. Venkateswaran, L.K. Wong, R. Zhao, R.D. Smith, Proc. Natl. Acad. Sci. U.S.A. 99 (2002) 11049.
[28] T.P. Conrads, G.A. Anderson, T.D. Veenstra, L. Pasa-Tolic, R.D. Smith, Anal. Chem. 72 (2000) 3349.
[29] R.D. Smith, G.A. Anderson, M.S. Lipton, L. Pasa-Tolic, Y.F. Shen, T.P. Conrads, T.D. Veenstra, H.R. Udseth, Proteomics 2 (2002) 513.
[30] W.-C. Lee, K.H. Lee, Anal. Biochem. 324 (2004) 1.
[31] H. Muratsubaki, K. Satake, Y. Yamamoto, K. Enomoto, Anal. Biochem. 307 (2002) 337.
[32] S. Jain, M.N. Gupta, Appl. Biochem. Biotechnol. 125 (2005) 53.
[33] E.A. Peterson, A.R. Torres, Anal. Biochem. 130 (1983) 271.
[34] A.R. Torres, B.E. Dunn, S.C. Edberg, E.A. Peterson, J. Chromatogr. 316 (1984) 125.
[35] J. Frenz, J. Bourell, W.S. Hancock, J. Chromatogr. 512 (1990) 299.
[36] S.P. Gygi, B. Rist, S.A. Gerber, F. Turecek, M.H. Gelb, R. Aebersold, Nature Biotechnol. 17 (1999) 994.
[37] M.A. Moseley, Trends Biotechnol. 19 (2001) 10.
[38] J.T. Stults, D. Arnott, Methods Enzymol. 402 (2005) 245.
[39] H. Mirzaei, F. Regnier, J. Chromatogr. B 817 (2005) 23.
[40] I. Roy, M.N. Gupta, Chem. Biol. 10 (2003) 1161.
[41] A. Hoffman, Adv. Drug Deliv. Rev. 43 (2002) 1.
[42] A. Sharma, S. Sharma, M.N. Gupta, Protein Expr. Purif. 18 (2000) 111.
[43] K. Mondal, P. Mehta, M.N. Gupta, Protein Expr. Purif. 33 (2004) 104.
[44] I. Roy, M.N. Gupta, Protein Eng. 16 (2003) 153.
[45] A. Sharma, I. Roy, M.N. Gupta, Biotechnol. Prog. 20 (2004) 1255.
[46] K. Mondal, H.B. Bohidar, R.P. Roy, M.N. Gupta, Biochim. Biophys. Acta 1764 (2006) 877.
[47] M. Kaufmann, J. Chromatogr. B 699 (1997) 347.
[48] D.E. Meyer, A. Chilkoti, Nature Biotechnol. 17 (1999) 1112.
[49] D.E. Meyer, K. Trabbic-Carlson, A. Chilkoti, Biotechnol. Prog. 17 (2001) 720.
[50] S. Jain, R. Singh, M.N. Gupta, J. Chromatogr. A 1035 (2004) 83.
[51] C. Dennison, R. Lovrien, Protein Expr. Purif. 11 (1997) 149.
[52] A. Sharma, M.N. Gupta, Biotechnol. Lett. 23 (2001) 1625.
[53] I. Roy, A. Sharma, M.N. Gupta, Biochim. Biophys. Acta 1698 (2004) 107.
[54] I. Roy, A. Sharma, M.N. Gupta, Enzyme Microb. Technol. 37 (2005) 113.
[55] E. Kiss, R. Borbas, Colloids Surf. B-Biointerfaces 31 (2003) 169.
[56] B.J.A. Paule, R. Meyer, L.F. Moura-Costa, R.C. Bahia, R. Carminati, L.F. Regis, V.L.C. Vale, S.M. Freire, I. Nascimento, R. Schaer, V. Azevedo, Protein Expr. Purif. 34 (2004) 311.
[57] A. Sharma, M.N. Gupta, Biotechnol. Bioeng. 80 (2002) 228.
[58] A. Sharma, K. Mondal, M.N. Gupta, J. Chromatogr. A 995 (2003) 127.
[59] K. Mondal, A. Sharma, Lata, M.N. Gupta, Biotechnol. Prog. 19 (2003) 493.
[60] H. Walter, G. Johnsson, Methods Enzymol. 228 (1994).
[61] K. Solanki, K. Mondal, M.N. Gupta, Anal. Biochem. 360 (2007) 123–129.
[62] G. Johansson, M. Joelsson, Enzyme Microb. Technol. 7 (1985) 629.
[63] A. Cordes, M.-R. Kula, Methods Enzymol. 228 (1994) 600.
[64] M. Joelsson, F. Tjerneld, Methods Enzymol. 228 (1994) 136.
[65] S. Teotia, M.N. Gupta, J. Chromatogr. A 923 (2001) 275.
[66] S. Teotia, K. Mondal, M.N. Gupta, Food Bioprod. Process. 84 (C1) (2006) 37.
[67] F.B. Anspach, D. Curbelo, R. Hartmann, G. Garke, W.-D. Deckwer, J. Chromatogr. A 865 (1999) 129.
[68] I. Roy, M.N. Gupta, J. Chromatogr. A 950 (2002) 131.
[69] I. Roy, S. Jain, S. Teotia, M.N. Gupta, Biotechnol. Prog. 20 (2004) 1490.
[70] I. Roy, M. Sardar, M.N. Gupta, Biochem. Eng. J. 23 (2005) 193.
[71] S. Ghose, H. Chase, Bioseparation 9 (2000) 21.
[72] M.P. Nandkumar, A. Tocaj, B. Mattiasson, Bioseparation 8 (1999) 247.
[73] K. Terpe, Appl. Microbiol. Biotechnol. 60 (2003) 523.
[74] R.H. Clemmitt, H.A. Chase, Biotechnol. Bioeng. 67 (2000) 206.
[75] W. Choe, R.H. Clemmit, H.A. Chase, A.P.J. Middelberg, J. Chromatogr. A 953 (2002) 111.
[76] R. Uy, F. Wold, Science 198 (1977) 890.
[77] S. Meri, M. Baumann, Biomol. Eng. 18 (2001) 213.
[78] P. Braun, J. LaBaer, Trends Biotechnol. 21 (2003) 383.
[79] J.-P. Lambert, M. Ethier, J.C. smith, D. Figeys, Anal. Chem. 77 (2005) 3771.
[80] A. Kamra, M.N. Gupta, Anal. Biochem. 164 (1987) 405.
[81] P.R. Satish, A. Surolia, J. Biochem. Biophys. Methods 49 (2001) 625.
[82] E.J.M. Van Damme, W.J. Peumans, A. Pusztai, S. Bardocz (Eds.), Handbook of Plant Lectins: Properties and Biomedical Applications, John Wiley & Sons Ltd., England, 1998.
[83] F.P. Schwarz, S. Misquith, A. Surolia, Biochem. J. 316 (1996) 123.
[84] V.R. Srinivas, G.B. Reddy, N. Ahmad, C.P. Swaminathan, N. Mitra, A. Surolia, Biochim. Biophys. Acta 1527 (2001) 102.
[85] R. Tyagi, R. Agarwal, M.N. Gupta, J. Biotechnol. 46 (1996) 79.
[86] L.M. Nuwaysir, J.T. Stults, J. Am. Soc. Mass Spectrom. 4 (1993) 462.
[87] A. Stensballe, S. Andersen, O.N. Jensen, Proteomics 1 (2001) 207.
[88] M. Gronborg, T.Z. Kristiansen, A. Stensballe, J.S. Andersen, O. Ohara, M. Mann, O.N. Jensen, A. Pandey, Mol. Cell Proteomics 1 (2002) 517.
[89] G.C. Adam, E.J. Sorensen, B.F. Cravatt, Mol. Cell Proteomics 1 (2002) 781.
[90] P. Cutler, Proteomics 3 (2003) 3.
[91] M. Cretich, F. Damin, G. Pirri, M. Chiari, Biomol. Eng. 23 (2006) 77.

[92] R.P. Auburn, D.P. Kreil, L.A. Meadows, B. Fischer, S.S. Matilla, S. Russell, Trends Biotechnol. 23 (2005) 374.
[93] T.C. Mockler, J.R. Ecker, Genomics 85 (2005) 1.
[94] G. Elia, M. Silacci, S. Scheurer, J. Scheuermann, D. Neri, Trends Biotechnol. 20 (2002) S19.
[95] E. Kopf, D. Shnitzer, D. Zharhary, Proteomics 1 (2005) 1271.
[96] H. Persson, J. Lantto, M. Ohlin, J. Mol. Biol. 357 (2006) 607.
[97] Y. Jiang, S.-J. Zhang, S.-M. Wu, M.Y.W.T. Lee, Arch. Biochem. Biophys. 320 (1995) 297.
[98] C.A. Rowe-Taitt, J.P. Golden, M.J. Feldstein, J.J. Cras, K.E. Hoffman, F.S. Ligler, Biosens Bioelectron. 14 (2000) 785.
[99] B. Golinelli-Pimpaneau, Curr. Opin. Struct. Biol. 10 (2000) 697.
[100] L.J. Holt, K. Bussow, G. Walter, I. Tomlinson, Nucleic Acid Res. 28 (2000) 1.
[101] V.V. Mozhaev, K. Martinek, Adv. Drug Deliv. Rev. 4 (1990) 387.
[102] A. Sadana, in: M.N. Gupta (Ed.), Thermostability of Enzymes, Springer-Verlag, Berlin, 1993, 84.
[103] M. Friedman (Ed.), Protein Crosslinking: Biochemical and Molecular Aspects, Plenum Press, New York, 1976.
[104] S.S. Wong (Ed.), Chemistry of Protein Conjugation and Crosslinking, CRC Press, Boca Raton, Florida, 1992.
[105] R.F. Taylor (Ed.), Protein Immobilization. Fundamentals and Applications, Marcel Dekker, NY, 1991.
[106] K.L. Morley, R.J. Kazlauskas, Trends Biotechnol. 23 (2005) 231.
[107] V.G.H. Eijsink, S. Gaseidnes, T.V. Borchert, B. van den Burg, Biomol. Eng. 22 (2005) 21.
[108] M. Sardar, I. Roy, M.N. Gupta, Biotechnol. Prog. 19 (2003) 1654.
[109] S. Sharma, P. Kaur, A. Jain, M.R. Rajeshwari, M.N. Gupta, Biomacromolecules 4 (2003) 330.
[110] R. Tyagi, M.N. Gupta, Biochemistry (Moscow) 63 (1998) 334.
[111] R. Tyagi, R. Batra, M.N. Gupta, Enzyme Microb. Technol. 24 (1999) 348.
[112] Y. Jiang, H. Dalton, Biochim. Biophys. Acta 1201 (1994) 76.
[113] M. Persson, E. Wehtje, P. Adlercreutz, Chembiochem. 3 (2002) 566.
[114] F. van Rantwijk, R.M. Lau, R.A. Sheldon, Trend Biotechnol. 21 (2003) 131.
[115] T. Maruyama, H. Yamamura, T. Kotani, N. Kamiya, M. Goto, Org. Biomol. Chem. 2 (2004) 1239.
[116] M.N. Gupta (Ed.), Thermostability of Enzymes, Springer-Verlag, Berlin, 1993.
[117] P.J. Halling, in: K. Drauz, H. Waldmann (Eds.), Enzyme Catalysis in Organic Synthesis, vol. I, Wiley-VCH, Weinheim, 2002, p. 259.
[118] I. Roy, M.N. Gupta, in: J.M. Guisan (Ed.), Immobilization of Enzymes & Cells, Humana Press Inc., USA, 2006.
[119] D.J. O'Shannesy, W.L. Hoffman, Biol. Chem. Hoppe-Seyler 368 (1987) 767.
[120] M.N. Gupta, B. Mattiasson, in: C.H. Suelter, L. Kricka (Eds.), Bioanalytical Applications of Enzymes, vol. 36, John Wiley & Sons Inc., New York, 1992, p. 1.
[121] J. Turkova, in: M.A. Vijayalakshmi (Ed.), Biochromatography: Theory and Practice, Taylor and Francis, NY, 2002, p. 142.
[122] M. Sardar, I. Roy, M.N. Gupta, Enzyme Microb. Technol. 27 (2000) 672.
[123] Z.L. Ding, G.H. Chen, A.S. Hoffman, J. Biomed. Mat. Res. 39 (1998) 498.
[124] N.W. Fadnavis, G. Sheelu, B.M. Kumar, M.U. Bhalerao, A.A. Deshpande, Biotechnol. Prog. 19 (2003) 557.
[125] G.B. Broun, Methods Enzymol. 44 (1976) 263.
[126] L. Cong, R. Kaul, U. dissing, B. Mattiasson, J. Biotechnol. 42 (1995) 75.
[127] M. Farooqi, M. Saleemuddin, R. Ulber, P. Sosnitza, T. Scheper, J. Biotechnol. 55 (1997) 171.
[128] M. Sardar, M.N. Gupta, Enzyme Microb. Technol. 37 (2005) 355.
[129] P. Legrain, J.L. Jestin, V. Schacter, Curr. Opin. Biotechnol. 11 (2000) 402.
[130] B. Scwikowski, P. Uetz, S. Fields, Nat. Biotechnol. 18 (2000) 1257.
[131] E.W.L. Chan, D.-C. Lee, M.-K. Ng, G. Wu, K.Y.C. Lee, L. Yu, J. Am. Chem. Soc. 124 (2002) 12238.
[132] B.A. Snopok, Yu.G. Goltsov, E.V. Kostyukevich, L.A. Matkovskaja, Yu.M. Shirshov, E.F. Venger, Sens. Actuators B 95 (2003) 336.
[133] J. Kim, R. Yamasaki, J. Park, H. Jung, H. Lee, T. Kawai, J. Biosci. Bioeng. 97 (2004) 138.
[134] Z. Liang, C. Wang, Z. Tong, W. Ye, S. Ye, React. Func. Polym. 63 (2005) 85.
[135] G.T. Hermansson, A.K. Mallia, P.K. Smith (Eds.), Immobilized Affinity Ligand Techniques, Academic Press, NY, 1992.
[136] R.K. Scopes (Ed.), Protein purification. Principles and practice, third ed., Narosa Publishing House, N. Delhi, 1994, p. 187.
[137] L. Cao, L.M. van Langen, R.A. Sheldon, Curr. Opin. Biotechnol. 14 (2003) 387.
[138] S. Shah, A. Sharma, M.N. Gupta, Anal. Biochem. 351 (2006) 207.
[139] J.L. Lalonde, M.A. Navia, A.L. Margolin, Methods Enzymol. 286 (1997) 443.
[140] C.P. Govardhan, Curr. Opin. Biotechnol. 10 (1999) 331.
[141] I.L. deMattos, L.V. Lukachova, L. Gorton, T. Laurell, A.A. Karyakin, Talanta 54 (2001) 963.
[142] J.J. Roy, T.E. Abraham, K.S. Abhijith, P.V.S. Kumar, M.S. Thakur, Biosens. Bioelectron. 21 (2005) 206.
[143] B.C. Kim, S. Nair, J. Kim, J.H. Kwak, J.W. Grate, S.H. Kim, M.B. Gu, Nanotechnology 16 (2005) S382.
[144] P.T. Charles, E.R. Goldman, J.G. Rangasammy, C.L. Schauer, M.-S. Chen, C.R. Taitt, Biosens. Bioelectron. 20 (2004) 753.
[145] J.D. Wade, K. Hojo, K. Kawasaki, T.G. Johns, B. Catimel, J. Rothacker, E.C. Nice, Anal. Biochem. 348 (2006) 315.
[146] M.J. Cannon, G.A. Papalia, I. Navratilova, R.J. Fisher, L.R. Roberts, K.M. Worthy, A.G. Stephen, et al., Anal. Biochem. 330 (2004) 98.
[147] P.S. Katsamba, I. Navratilova, M. Calderon-Cacia, L. Fan, K. Thorton, M. Zhu, T.V. Bos, et al., Anal. Biochem. 352 (2006) 208.
[148] P.L. Gourley, Biotechnol. Prog. 21 (2005) 2.
[149] L.-Q. Wu, G.F. Payne, Trends Biotechnol. 11 (2004) 593.
[150] M. Cabodi, S.W.P. Turner, H.G. Craighead, Anal. Chem. 74 (2002) 5169.
[151] H. Hess, G. Bachand, Chemistry 10 (2004) 2110.
[152] S. Kiyonaka, K. Sada, I. Yoshimura, S. Shinkai, N. Kato, I. Hamachi, Nature Mater. 3 (2004) 58.
[153] A. Cecilia, A. Roque, C.R. Lowe, Biotechnol. Adv. 24 (2006) 17.
[154] H. Bal, F. Yang, X. Yang, J. Proteome Res. 5 (2006) 840.
[155] R. Lovrien, C. Goldensoph, P. Anderson, B. Odegaard, in: R. Burgess (Ed.), Protein Purification: Micro to Macro, A. Liss Inc., NY, 1987, p. 131.
[156] R. Van Reis, A. Zydney, Curr. Opin. Biotechnol. 12 (2001) 208.
[157] M. Feins, K.K. Sirkar, J. Membr. Sci. 248 (2005) 137.
[158] Y. Pouliot, M.C. Wijers, S.F. Gauthier, L. Nadeau, J. Membr. Sci. 158 (1999) 105.
[159] F. Cattoli, G.C. Sarti, Biotechnol. Prog. 18 (2002) 94.
[160] L.R. Castilho, F.B. Anspach, W.D. Deckwer, J. Membr. Sci. 207 (2002) 253.
[161] K. Ramanathan, B.R. Jonsson, B. Danielsson, in: M.N. Gupta (Ed.), Methods in Non-Aqueous Enzymology, Birkhauser-Verlag, Basel, 2002, p. 174.
[162] M.S.-P. López, E. López-Cabarcos, B. López-Ruiz, Biomol. Eng. 23 (2006) 135.
[163] K.M. Hansen, T. Thundat, Methods 37 (2005) 57.
[164] A. Rasooly, J. Jacobson, Biosens. Bioelectron. 21 (2006) 1851.
[165] J. Carlsson, F. Winquist, B. Danielsson, I. Lundström, Anal. Chim. Acta 547 (2005) 229.
[166] H. Roder, K. Maki, H. Cheng, Chem. Rev. 106 (2006) 1836.
[167] P.H. O'Farrell, J. Biol. Chem. 250 (1975) 4007.
[168] N.L. Anderson, W.J. Eisler, N.G. Anderson, Anal. Biochem. 91 (1978) 441.
[169] H.R. Morris, A. Dell, R.A. McDowell, Biomed. Mass Spectrom. 8 (1981) 463.
[170] D.F. Hunt, A.M. Buko, J.M. Ballard, J. Shabanowitz, A.B. Giordani, Biomed. Mass Spectrom. 8 (1981) 397.
[171] R. Westermeier, W. Postel, J. Weser, A. Gorg, J. Biochem. Biophys. Methods 8 (1983) 321.
[172] A. Gorg, W. Postel, S. Gunther, C. Friedrich, Electrophoresis 9 (1988) 57.

[173] V.C. Wasinger, S.J. Cordwell, A. Cerpa-Poljak, J.X. Yan, A.A. Gooley, M.R. Wilkins, M.W. Duncan, R. Harris, K.L. Williams, I. Humphery-Smith, Electrophoresis 16 (1995) 1090.
[174] J.R. Yates III, A.L. McCormack, Anal. Chem. 68 (1996) 534A.
[175] G. Chambers, L. Lawrie, P. Cash, G.I. Murray, J. Pathol. 192 (2000) 280.
[176] S. Fields, Science 291 (2001) 1221.
[177] W.G. Yarbrough, R.J.C. Slebos, D. Liebler, Head Neck 28 (2006) 549.
[178] Y. Wang, R. Hanley, R.L. Klemke, J. Proteome Res. 5 (2006) 907.
[179] A.M. Thayer, Chem. Eng. News 81 (2003) 17.
[180] R.M. Mandaro, S. Roy, K.C. Hou, Bio/Technology 5 (1987) 928.
[181] M. Roman, P.R. Brown, J. Chromatogr. 592 (1992) 3.
[182] L.T. Wen, M. Henneberger, M. Nguyen, R.A. McPherson, J. Clin. Lab. Anal. 8 (1994) 44.
[183] S. Doonan, Methods Mol. Biol. 244 (2004) 117.
[184] K.C. Ingham, Methods Enzymol. 182 (1990) 301.
[185] D.M. Morre, M. Sweeting, D.J. Morre, J. Chromatogr. B Biomed. Sci. Appl. 711 (1998) 173.
[186] H.P. Jennissen, Methods Mol. Biol. 305 (2005) 81.
[187] C. Tozzi, L. Anfossi, G. Giraudi, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 797 (2003) 289.
[188] Y. Kato, Y. Yamasaki, H. Moriyama, K. Tokunaga, T. Hashimoto, J. Chromatogr. 404 (1987) 333.
[189] T. Sun, G. Chen, Y. Liu, F. Bu, M. Wen, J. Chromatogr. B Biomed. Sci. Appl. 742 (2000) 109.
[190] J.N. Losso, R.R. Bansode, H.A. Bawadi, J. Agric. Food Chem. 51 (2003) 7122.
[191] A. Jungbauer, R. Hahn, Curr. Opin. Biotechnol. 15 (2004) 487.
[192] E.P. Melo, M.R. Aires-Barros, J.M. Cabral, Biotechnol. Annu. Rev. 7 (2001) 87.
[193] I. Hernandez-Pinzon, F. Millan, J. Bautista, Biosci. Biotechnol. Biochem. 61 (1997) 1240.
[194] Y. Kashino, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 797 (2003) 191.