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High sample throughput phosphoamino acid analysis of proteins separated by one- and two-dimensional gel electrophoresis

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Abstract

Studies on protein phosphorylation usually involve radiolabelling techniques and visualisation on gels; this limits studies to those on tissues which can be ^{32}P labelled. With the advent of reproducible micropreparative two dimensional gel electrophoresis, the proteome (protein complement) of a subcellular fraction, cell or tissue can be displayed. We have investigated the sensitivity of limited protein hydrolysis to detect in vivo phosphorylation in proteins blotted from one or two-dimensional polyacrylamide gels onto polyvinylidene difluoride (PVDF) membranes. The method uses 9-fluorenylmethyl chloroformate (FMOC) derivatisation chemistry and a modified HPLC AMINOMATE system. Conditions were established for hydrolysis of the PVDF-blotted protein (5.7 *M* HCl at 110°C for 4 h) which resulted in the recovery of phosphoserine (Ser(P)), phosphothreonine (Thr(P)) and phosphotyrosine (Tyr(P)). The chromatography was carried out on the same system routinely used for amino acid compositional analysis using a gradient elution modified from that used for separation of 16 amino acids. A chromatographic window was designed where all 3 phosphoamino acids are separated with baseline resolution in the order of Ser(P), Thr(P) and Tyr(P), and eluted before the normal protein amino acids. The total separation time is 13 min and includes the elution of excess FMOC, its derivatives and incompletely hydrolysed peptides. The FMOC fluorescence of these three standard phosphoamino acids is linear in the range 10–100 pmol. The technique is shown to be successful in finding phosphoproteins separated by two-dimensional gel electrophoresis. The method is sensitive (30 pmol of a single site phosphorylated protein, 3.8 pmol of Ser(P) detected) and allows automated sample throughput. This presents an option for rapid screening of protein phosphorylation of large numbers of proteins separated by two-dimensional polyacrylamide gel electrophoresis.

Keywords: Phosphorylation; Proteome; Proteins; Amino acids; Phosphoamino acid

1. Introduction

Protein phosphorylation is important for regulating many cell functions ranging from signal transduction to cell division [1,2]. Researchers have used a variety of approaches to identify phosphoproteins in the cell's protein complement. The most popular method remains that where growing cells are labelled in vivo and the phosphoproteins subsequently visualised on a gel by autoradiography [3]. This is a sensitive

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technique, but it is limited to organisms or cells which can be radiolabelled. An alternative method is to purify the protein, and to react it in vitro with a specific kinase and [γ-^{32}P]ATP, and again visualise by autoradiography of the gel [3–5]. However, this method is kinase specific and if the protein is already fully phosphorylated in vivo, further addition of labelled phosphate does not occur. In addition, modification of proteins can be regulated in vivo differently compared to in vitro, so that this technique may not reflect true in vivo phosphorylation. In general, neither of these methods identify the type of amino acids (serine/threonine/tyrosine) that are phosphorylated. Recently, studies on phosphopeptides by electrospray ionisation mass spectrometry (ES–MS) have been reported with pmol to fmol level sensitivity for identifying Tyr(P) [6–8]. These involved on-line MS analyses following either two-dimensional tryptic phosphopeptide mapping and two capillary HPLC separations [6,7], or dephosphorylation with a non-isotope enzyme reactor and capillary electrophoresis or capillary HPLC separation [8]. This elegant technique certainly offers high sensitivity, but it involves considerable skill and expensive instrumentation. Immunodetection with antibodies for Thr(P) and Tyr(P) presents a further option [9,10] but there is limited specificity and cross-reactivity of the antibodies. Other attempts have been focused on chemical modifications such as β-elimination of the phosphate group of serine or threonine and subsequent derivatisation by the thiol group, resulting in substitution of ethanedithiol onto the Ser/Thr residue, leaving a free thiol group which enables the identification and quantification of phosphoamino acid [11] by amino acid analysis of the eliminated adduct [12] or Edman degradation [13]. However, β-elimination eliminates the sugars from glycosylated proteins and results in the same derivatives being formed [14]. This is certainly a problem with Ser and Thr, which must be taken into account during interpretation of the data.

With the advent of micropreparative two-dimensional polyacrylamide gel electrophoresis (2-D PAGE), a proteome [15] containing a complex mixture of proteins can be separated on a single 2-D gel. 2-D PAGE allows the separation of discrete isoforms of the same molecule which typically arise from post-translational modifications such as glycosylation and phosphorylation. There is sufficient material in these preparative separations to further analyse and identify these proteins. Since mass screening of a proteome may involve hundreds or even thousands of proteins, rapid techniques are required to identify post-translational modifications. Recently we have shown that carbohydrate analysis is possible by high-performance anion-exchange chromatography–pulsed amperometric detection if sufficient protein is electroblotted onto the PVDF [16].

The few reports on the analysis of phosphoamino acids using high-performance liquid chromatography [17–19]. More recently, pmol levels of phosphoamino acids analysis of PVDF bound protein (β-casein) were reported using pre-column phenyl isothiocyanate (PITC) derivatisation of hydrolysed amino acids [20]. The PITC chemistry has shown phosphoamino acid analysis of PVDF blotted proteins can be done. Now we need a rapid approach for mass screening of protein phosphorylation in proteome studies.

We have recently described use of the GBC automated AMINOMATE HPLC system with 9-fluorenylmethyl chloroformate (FMOC) chemistry and reversed-phase separation to analyse amino acids after complete hydrolysis (5.7 *M* or 11.4 *M* HCl, 155°C for 1 h) of PVDF blotted proteins [21,22]. We report here the development of Ser(P), Thr(P) and Tyr(P) analysis of PVDF-blotted standard phosphoproteins separated on 1-D and 2-D gels using this HPLC system. Also, we demonstrate the application of this phosphoamino acid analysis on 2-D PAGE separation of a known phosphoprotein, elongation factor 1b, from human liver. This technique provides an option for the rapid mass screening of protein phosphorylation of proteins ($\geq$30 pmol) separated by 2-D gel electrophoresis.

2. Materials and methods

2.1. Sample preparation

Standard phosphoamino acids, namely O-phospho-L-serine, O-phospho-L-threonine and O-phospho-L-

tyrosine were purchased from Sigma. Stock solutions of these three phosphoamino acids were made with 0.1 *M* HCl and then diluted with 250 m*M* borate buffer (pH 8.8) to the required concentration. Standard phosphorylated proteins, β-casein (Swiss-Prot entry (SPE): casb_bovin) (90% purity by electrophoresis, Sigma, C6905) and ovalbumin (SPE: oval_chick) (99% purity, Sigma), dephosphorylated β-casein (80% purity, enzymatically prepared from C6905, Sigma, C8157) were dissolved in Milli-Q water and an aliquot either dried down for phosphoamino acid analysis, or run on 1-D or 2-D PAGE and then electro-blotted onto PVDF membrane (Bio-Rad). Elongation factor 1-beta (SPE: ef1b_human) was purified from the human liver extract by 2-D PAGE according to Sanchez et al. [23]. The protocol for sample preparation is outlined in the SWISS-2DPAGE database (http://expasy.hcuge.ch/ch2d/technical-info.html).

2.2. Gel electrophoresis

2-D PAGE was carried out using isoelectric focusing as the first dimension and sodium dodecyl sulfate (SDS)-PAGE as second dimension. Sigmoidal immobilised pH gradient (IPG) strips (pH 3–10) (Pharmacia LK Biotechnology, Sweden) were rehydrated, the sample applied and run according to the method of Bjellqvist et al. [24,25]. In the second dimension, vertical gradient slab gels (160×200×1.5 mm) were used and run according to Hochstrasser et al. [26,27]. After second dimensional electrophoresis, the gels were wet-electroblotted to PVDF with 3-cyclohexylamino-L-propanesulfonic acid (CAPS) buffer (pH 11.0) [28]. PVDF blots were stained with amido black and destained with Milli-Q water [29]. β-Casein 2 μg (80 pmol) and ovalbumin 4 μg (80 pmol) were loaded on a single gel for preparative 2-D PAGE. The loading for the human liver extract was 3 mg for each gel. 1-D PAGE was carried out using the Mini Protean II (Bio-Rad). The detailed protocols for analytical (β-casein and ovalbumin) and preparative (human liver) 2-D PAGE are summarised in SWISS-2DPAGE (http://expasy.hcuge.ch/ch2d/technical-info.html). Gel and PVDF blot images were analysed by the computer software MELANIE II (Bio-Rad).

2.3. Phosphoamino acid analysis

Gas-phase hydrolysis of PVDF blotted phosphoproteins was carried out manually in a hydrolysis vessel [22] using partial hydrolysis condition of 5.7 *M* HCl, at 110°C for 2, 4 or 6 h compared with the conditions (5.7 *M* or 11.4 *M* HCl, at 155°C for 1 h, or at 110°C for 24 h) used for complete hydrolysis of proteins. After the hydrolysis, the released phosphoamino acids were extracted from the PVDF membrane by sonication with 170 μl of 0.1% trifluoroacetic acid–Milli-Q water–acetonitrile (20:50:100, v/v/v) [22]. The PVDF membrane was removed from hydrolysis vial. The extraction solution was then dried under vacuum.

A GBC automated AMINOMATE HPLC system (GBC Scientific, Australia) was used to analyse the phosphoamino acids which were separated on a reversed-phase column (ODS-Hypersil, 5 μm packing, 150×4.6 mm I.D., Keystone Scientific) at 38°C. Flow-rate was a constant 1 ml/min. The samples were derivatised using FMOC according to Haynes et al. [30] and Ou et al. [21] and injected automatically by a LC1650 advanced autosampler. FMOC-derivatives were detected by fluorescence (LC1250 fluoro-detector system, excitation wavelength 270 nm, emission wavelength 316 nm) and peak analysis was controlled by the WINCHROM chromatography data system (GBC), version 1.2.

3. Results

3.1. Chromatography

A gradient elution program was developed which gave baseline separation of phosphoamino acids (Table 1). Amino acid standards were mixed with phosphoamino acid standards in order to monitor the separation between phosphoamino acids and other amino acids. Particular efforts were made to adjust the chromatography to separate the phosphoamino acids from aspartic acid (Asp) and glutamic acid (Glu) which are released from the protein during partial hydrolysis, have similar polarity and charge and elute close to the phosphorylated amino acids. After testing 6 m*M* ammonium phosphate buffer in the pH range 6.5–9.0, a pH value of 8.0 was found

Table 1
The gradient program for the separation of Ser(P), Thr(P) and Tyr(P)

Time (min)	A (%)[a]	B (%)[b]	C (%)[c]
0.00	20.00	68.50	11.50
1.00	20.00	68.50	11.50
6.00	19.40	66.60	14.00
6.50	20.00	68.50	11.50
7.00	0.00	0.00	100.00
12.00	0.00	0.00	100.00
12.05	20.00	68.50	11.50
13.00	20.00	68.50	11.50

[a] A=30 m*M* ammonium phosphate buffer, in buffer B, pH 8.0.
[b] B=15% (v/v) methanol.
[c] C=90% (v/v) acetonitrile.

to give the best resolution for the separation of phosphoamino acids from Asp and Glu. After 800 runs for full amino acid analyses and 500 runs for phosphoamino acid analyses (see Section 4), this column appeared quite stable at the high pH.

3.2. Chromatograms of separation of three standard phosphoamino acids

The three standard phosphoamino acids were separated with baseline resolution in the order of Ser(P), Thr(P) and Tyr(P) (Fig. 1a). The difference in retention time for these 3 phosphoamino acids was approximately 1 min with Ser(P) eluted at around 4 min. The total chromatography time was 13 min, including the elution of excess FMOC and its derivatives, such as amino acids and peptides. Fig. 1a shows the baseline separation between these phosphoamino acids and Asp and Glu, while other amino acids and incompletely hydrolysed peptides were co-eluted with FMOC. We routinely use the standard containing the mixture of phosphoamino acids and amino acids in order to confirm the retention times for the phosphoamino acids, Asp and Glu.

3.3. Multitasking of pre-column derivatisation and chromatography for high sample throughput

Ou et al. [21] increased system throughput using a multitasking method that runs a separation gradient of one sample while simultaneously derivatising the next sample to be analysed. However, the multi-

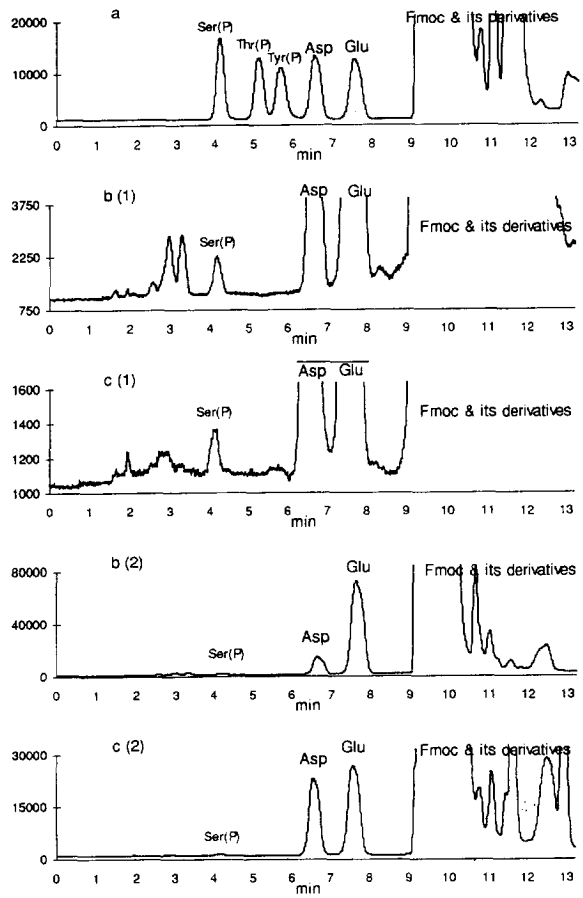

Fig. 1. Chromatogram of (a) the separation of 125 pmol of standard Ser(P), Thr(P), Tyr(P), Asp and Glu; (b) chromatograms of separation and detection of Ser(P) from casein; (c) chromatograms of separation and detection of Ser(P) from ef1b; where (1) expanded to show Ser(P) peak, (2) full scale for Asp and Glu.

tasking program (pretreatment file) was suitable only for the WINCHROM software, version 1.0 and had to be written individually for each sample to be analysed. With the newly released autosampler (LC1650) a new "pretreatment editor setting dialog box" was created in the new version (1.2) of software. This editor setting enables selection of when to start processing the next pretreatment file in the batch table during the current sample run. The advantage of this approach is to have only one pretreatment file for all the samples and the computer operates in a "smart" way to "decode" what to do with the next sample. In the case described here, pre-processing of the next pretreatment file begins 1 min after the

commencement of a sample run, with the duration of auto-derivatisation and injection being 10 min (Table 2). The autosampler waits a further 4 min to inject the next sample (2 min for finishing the run and another 2 min for pump equilibration). This multitasking program reduces total analysis time for each sample from 25 min to 15 min (pump equilibration 2 min) and reduces the consumption of solvents to 15 ml per run.

3.4. Hydrolysis time

There are several reports on hydrolysis time of proteins varying from 2–6 h for phosphoamino acid release and recovery [17–19]. It was necessary to establish an optimal recovery of the phosphoamino acids from the competing reactions of hydrolysis and dephosphorylation. The three standard phosphoamino acids (125 pmol of each) were hydrolysed for 2, 4 and 6 h to test the stability of each phosphoamino acid to vapour-phase acid hydrolysis (5.7 *M* HCl) at 110°C. We found that up to 4 h, Ser(P) and Tyr(P) were stable and 25% of Thr(P) was degraded, but after 6 h, 50% of Ser(P) and 75.5% of Thr(P) were degraded, while Tyr(P) survived. Hence, 4 h was determined as the best hydrolysis time for maximal stability of the three phosphoamino acids (Fig. 2). The acid labilities of

Table 2
Multitasking program (pretreatment file) for automated derivatisation and injection

	Command	Volume (μl)	Vial position	Parameter
1	External wash-duration			5 s
2	Vial-get	10 (needle wash)	1	
3	Needle-dump content			
4	Vial-get	10 (needle wash)	1	
5	Vial-put	10 (needle wash)	2 (waste vial)	
6	External wash-now			
7	Vial-get	2 (air)		
8	Vial-get	30 (needle wash)	1	
9	Vial-get	2 (air)		
10	Vial-get	10 (FMOC reagent)	4	
11	Vial-put	12 (FMOC+air)	[a]	
12	Vial-get	20 (air)		
13	Vial-put	20 (air)	[a]	
14	Vial-put	10 (needle washing)	2	
15	External wash-now			
16	Operation-wait			60 s
17	Vial-get	2 (air)		
18	Vial-get	10 (cleavage reagent)	5	
19	Vial-put	12 (cleavage+air)	[a]	
20	Vial-get	20 (air)		
21	Vial-put	20 (air)	[a]	
22	Vial-put	10 (needle wash)	2	
23	External wash-now			
24	Operation-wait			200 s
25	Vial-get	2 (air)		
26	Vial-get	10 (quenching reagent)	6	
27	Vial-put	12 (quenching+air)	[a]	
28	Vial-get	20 (air)		
29	Vial-put	20 (air)	[a]	
30	Vial-put	10 (needle wash)	2	
31	External wash-now			
32	Operation-park inject	[b]	[a]	

[a] Represents the vial position of the sample to be automated derivatised and injected.
[b] Represents the injection volume between 5–40 μl.

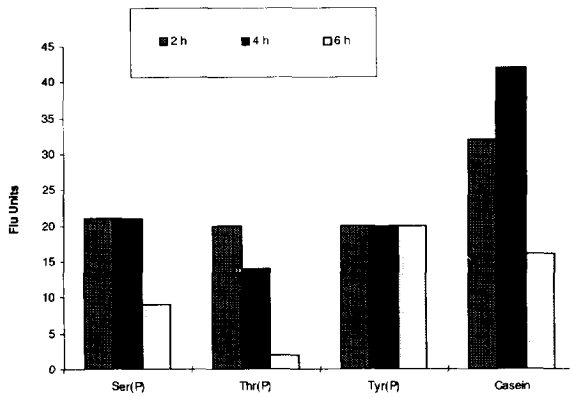

Fig. 2. Time course (2, 4 and 6 h) of hydrolyses of standard Ser(P), Thr(P), Tyr(P) (125 pmol of each) and casein (30 μg).

these three phosphoamino acids are thus Thr(P)> Ser(P)>Tyr(P). To test the maximum release of phosphoamino acids from the protein, we hydrolysed 30 μg (1.2 nmol) of β-casein for 2, 4 and 6 h. At 4 h, the maximum yield of Ser(P) was achieved, balancing the release of Ser(P) from the protein with its degradation upon prolonged hydrolysis (Fig. 2). Since casein has only serine phosphorylation, we were not able to test the optimal release of Thr(P) and Tyr(P) from proteins, but assume equal susceptibility to acid of the amide bond of these amino acids. Unfortunately to date, we have not found any standard proteins being phosphorylated on Thr or Tyr in the SWISS-PROT database.

3.5. Linearity of the detection of phosphoamino acids and recovery of Ser(P) from casein

Duplicate analyses of standard phosphoamino acids of 15.6 pmol, 31.3 pmol, 62.5 pmol and 125 pmol were conducted for investigation of linearity of detection. The regression analysis of standard Ser(P), Thr(P) and Tyr(P) detection was found to be 0.999, 0.998 and 0.996, respectively (Fig. 3a). In addition, the analysis of Ser(P) was also tested on partially hydrolysed β-casein in solution with the amounts of 6.3 pmol, 12.5 pmol, 25 pmol and 100 pmol. The linearity of release of Ser(P) from β-casein was found to be 0.982 (Fig. 3b). From the linear regression ($y=0.5707x-2.9653$) shown in Fig. 3b, its gradient (slope of the equation) is proportional to the recovery of Ser(P) from β-casein, i.e., a gradient of

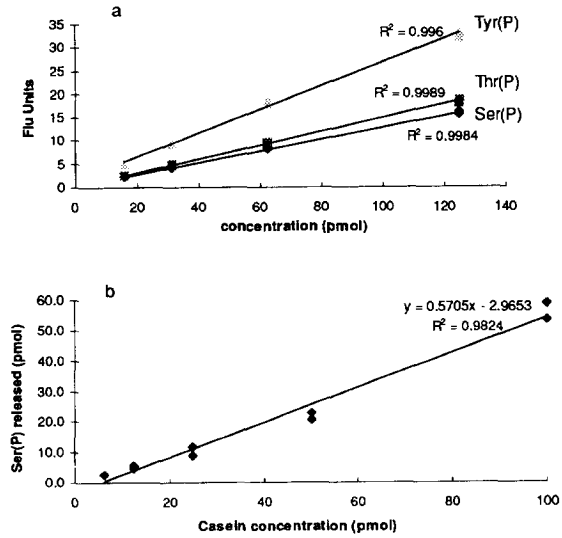

Fig. 3. Linearity of (a) the detection of Ser(P), Thr(P) and Tyr(P); (b) release of Ser(P) from casein (65–100 pmol).

0.57 indicates a 57% recovery of Ser(P) from β-casein solution. Since β-casein has 5 serine residues phosphorylated, the absolute recovery was 11.4%. Recovery of Ser(P) from PVDF-bound β-casein was found to be similar (data shown in Table 3). However, when the amount on PVDF below 30 pmol, the recovery decreased dramatically (2.6%). This has shown the limitation when the material is insufficient.

3.6. Demonstration of phosphoamino acid analysis of proteins separated by 2-D PAGE

3.6.1. Separation of the phosphoproteins using 2-D PAGE

Initially, β-casein (0.2 μg) and ovalbumin (0.4 μg) were individually subjected to 2-D PAGE and visualised by silver stain detection (data not shown) to determine their p*I* and M_r range. Subsequently, both proteins were combined and subjected to 2-D PAGE and electroblotted onto PVDF. Fig. 4 shows the separation of β-casein (2 μg) and ovalbumin (4 μg) into their respective isoforms in which each resolve into a unique pattern of proteins with the same molecular mass and different isoelectric point. These patterns may indicate alteration in p*I* due to the post-translational modification of phosphoryla-

Table 3
Absolute recovery of Ser(P) from PVDF blotted β-casein

Amount loaded (pmol)	Amount recovered (pmol)	Expected Ser(P) (pmol)	Observed Ser(P) (pmol)	Absolute recovery (%)
150	100	500	67	13.4
100	55	275	55	20
50	26	130	3.4	2.6

tion. In addition, β-casein [31] and ovalbumin [32] are glycoproteins, so glycosylation could also be a contributing factor to this heterogeneity. Four major isoforms of ovalbumin and two major spots of β-casein were detected by amido black staining of a PVDF blot (Fig. 4). The other minor spots around ovalbumin and β-casein were observed with different pI and M_r range. Elongation factor 1-beta protein (Fig. 5) was purified from the human liver extract by 2-D PAGE and identified by gel mapping, with pI/M_r of 5.43/28 227 in the SWISS-PROT database.

3.6.2. Phosphoamino acid analysis

β-Casein, ovalbumin and ef1b are reported in the SWISS-PROT database to be serine phosphorylated. Phosphoamino acid analysis showed that both isoforms of β-casein and the four isoforms of ovalbumin contained Ser(P). Fig. 1 shows the Ser(P) peak present in β-casein (b) and ef1b (c). In Fig. 1b and c, (1) are expanded chromatograms to show the Ser(P) peak, while, (2) are standardised on full scale for Asp and Glu. The ratio of Asp to Glu (Asn and Gln are converted into their acid forms during acid hydrolysis) is 1:4 and 1:1 in β-casein and ef1b, respectively. We found the release of Asp and Glu in partial acid hydrolysis was proportional compared to their compositions. Ovalbumin showed a similar pattern, with a lower yield of Ser(P) compared to β-casein (data not shown). Dephosphorylated β-casein was used as a negative control (100 pmol loaded onto 1-D gel and blotted onto PVDF mem-

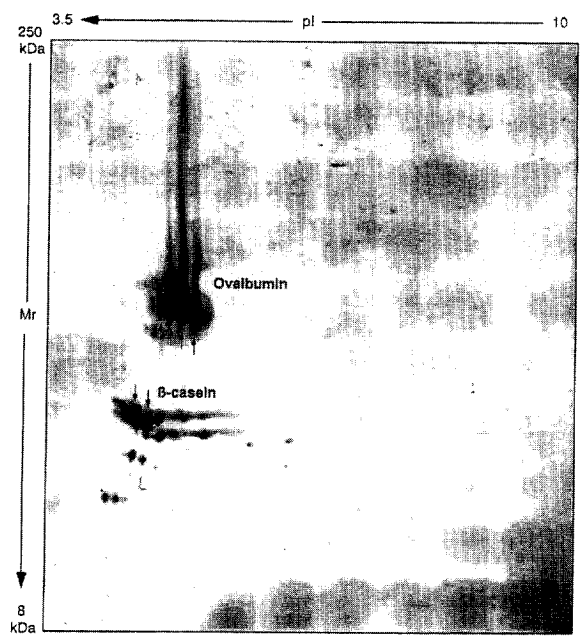

Fig. 4. 2-D PAGE blot (preparative) of casein and ovalbumin, kDa=kilodalton.

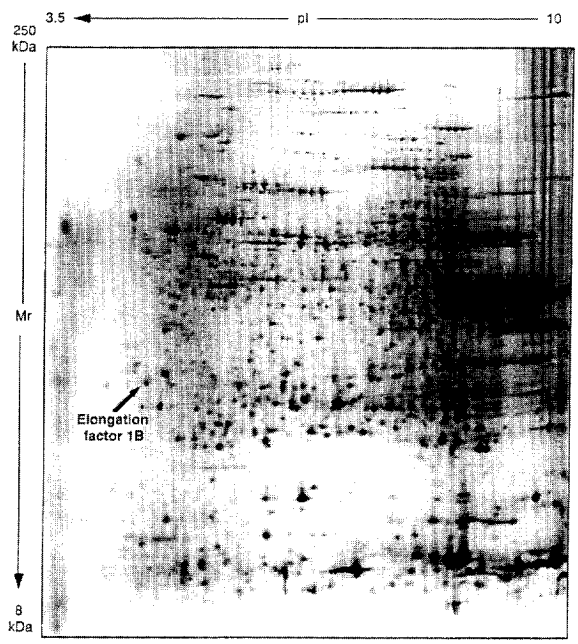

Fig. 5. 2-D PAGE of the human liver extract, indicating the position of ef1b.

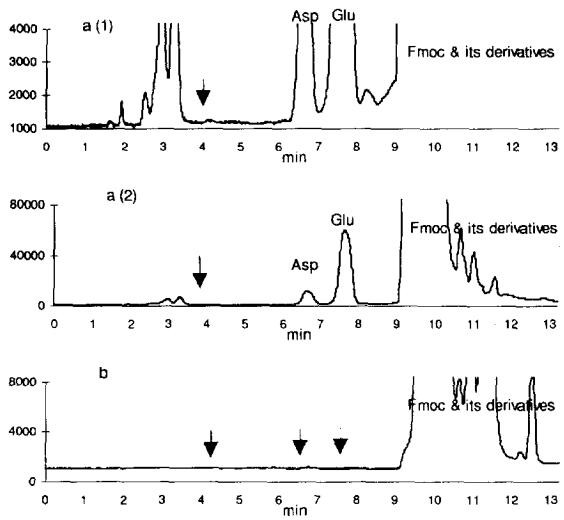

Fig. 6. Chromatograms of analysis of (a) dephosphorylated casein; (b) PVDF blank; where (1) expanded to show Ser(P) peak, (2) full scale for Asp and Glu.

brane) (Fig. 6a), which resulted in no detectable Ser(P) when compared to the same Asp and Glu pattern (Fig. 6a(2)) as phosphorylated casein (Fig. 1b(2)). A blank PVDF membrane was used for a baseline control, which showed no detectable Ser(P), Asp or Glu on the expanded scale (Fig. 6b). In the expanded chromatograms (Fig. 1b(1) and c(1) Fig. 6a(1)), it is noticeable that there are complex peaks which elute before Ser(P) in the chromatography of hydrolysed protein samples. To investigate the nature of these peaks, we partially hydrolysed bovine serum albumin (BSA; a non-modified protein), fetuin (a highly glycosylated protein) and casein (a phosphorylated glycoprotein). The chromatograms of all of these proteins showed a small cluster of peaks occurring at approximately the same retention time. The proportion of these peaks, relative to the molar quantities hydrolysed, were similar for BSA and fetuin. This demonstrates that the early eluting peaks are not amino sugars or glycoamino acids. Casein hydrolysate contained ten times the relative amount of these peaks. There was no evidence of these peaks in a blank hydrolysate (without protein) (Fig. 6b), or in any of these proteins when they were subjected to the stronger hydrolysis conditions (6 *M* HCl for 1 h at 155°C) used for total amino acid analysis (data not shown).

4. Conclusions and discussion

Micro-preparative 2-D PAGE allows tissues of any organism to be analysed. With respect to post-translational modifications this technique provides a powerful means of separation of discrete isoforms that were generated in vivo. Previously we have shown that it is possible to distinguish glycoforms using chemical analysis [16]. Here we have shown it is now possible to find phosphoproteins on 2-D gel. Importantly, this chemical method is not dependent on β-elimination and adduct formation which could lead to confusion between phosphorylated and glycosylated Ser or Thr. This is now an important consideration with a large research effort into cytoplasmic and nucleoplasmic protein glycosylation while phosphorylation can occur simultaneously on the same Ser or Thr [33,34]

Using partial acid hydrolysis, FMOC pre-column derivatisation and reversed-phase HPLC, phosphoamino acids (Ser(P), Thr(P) and Tyr(P)) have been resolved with baseline separation. The short analysis time and high sample throughput make feasible mass screening for phosphorylation of proteins separated by 2-D PAGE.

We have previously established a mass screening technique to identify proteins using amino acid composition [15,22]. The quality of the amino acid compositional data is critical and can be only achieved with a successful complete acid hydrolysis. Therefore, as this phosphoamino acid analysis is achieved using partial acid hydrolysis, we did not try to separate the phosphoamino acids and the other amino acids. There are unassigned peaks eluting before Ser(P) in the chromatography of all the partially hydrolysed protein samples. The peaks do not appear to be sugar-related as they occur to the same extent in the hydrolysate of both non-modified BSA and the highly glycosylated protein, fetuin. In addition, they are not free amino sugars, such as glucosamine and galactosamine, which are eluted much later than Asp and Glu in a normal FMOC amino acid separation (unpublished observation). The peaks do not appear in the absence of, or after strong acid hydrolysis (6 *M* HCl, 1 h, at 155°C) of, protein suggesting that they are peptide products of the incomplete hydrolysis. The extent and identity of these peaks may depend on the primary sequence of the different proteins.

Due to the high sample throughput of amino acid analysis in our facility, the life of new HPLC columns is limited (800 runs) [21]. However, for phosphoamino acid analysis, the separation requires only 5 components (Ser(P), Thr(P), Tyr(P), Asp and Glu) to be resolved. Hence, the demand for a high resolution column is reduced. The column we used in this study was indeed a "second-hand" one that had lost its high resolution for 16 aa peaks and was rejected after 800 runs. The separation of the three phosphoamino acids is remarkably good. More interestingly, the gradient buffer is used at a high pH (8.0), which is not favourable for a reversed-phase packing, yet this column has been continuously used over a year and analysed more than 500 samples for phosphoamino acid analysis and still retains efficiency. This method is thus cost-effective for mass screening in proteome studies.

Due to the partial acid hydrolysis and instability of the phosphate group, the recovery of phosphoamino acids is quite low (around 15%). Thus, for screening purposes this technique is qualitative, rather than quantitative. In proteome studies, many of the samples cannot be radiolabelled. By using this non-radiolabelling, non enzyme-specific technique, the proteins can be identified not only as to whether they are phosphorylated, but also as to which amino acids are phosphorylated. This is particularly useful to provide initial information for further detailed phosphorylation studies. For example, separated peptides of a protein after trypsin digestion can be subjected to phosphoamino acid analysis to identify the phosphopeptide. This approach is more time-effective than radiolabelling and can also be powerful for rapid screening. Affolter et al. [6] have pointed out that phosphoamino acid analysis before MS analyses would help the data interpretation, especially for unknown proteins. In their later paper [7], they have reported a highly sensitive technique of phosphopeptide identification by using two capillary HPLC systems and on-line electrospray ionisation MS with tandem MS to identify a tyrosine phosphorylated protein. With such elegant techniques, they still used phosphoamino acid analysis to confirm the Tyr(P) in those phosphopeptides. Therefore, we conclude that this rapid, simple and inexpensive method of phosphoamino acid detection and identification has an important role for day-to-day analysis and mass screening.

Demonstration of phosphoamino acid analysis of ef1b from 2-D PAGE shows the sensitivity of this technique. We used 4 ef1b spots from 4 blots (total protein amount was 30 pmol) for this successful analysis following an experiment where we failed to detect phosphoamino acids from 1 spot (around 7.5 pmol). Ef1b is reported to have only one serine residue phosphorylated. Liver tissue (3 mg) was loaded onto the gel and the intensity of this spot (amido black) indicated the low amount presented in the liver tissue (data not shown). This can also be observed on a silver stained analytical gel where ef1b is not an abundant protein in liver (Fig. 5). For this 30 pmol of ef1b, 3.8 pmol of Ser(P) was detected. Thus we believe this is a sensitive method to deal with the moderate and high abundance proteins from 2-D gels.

We propose here a strategy for mass screening of phosphorylated proteins from 2-D PAGE separation. This phosphoamino acid analysis is a sensitive method for identification of in vivo protein phosphorylation. Short analysis time, multitasking of pre-column derivatisation and chromatography and easy column maintenance provide options for high sample throughput analysis and cost-effectiveness in mass screening of protein phosphorylation in proteome studies.

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