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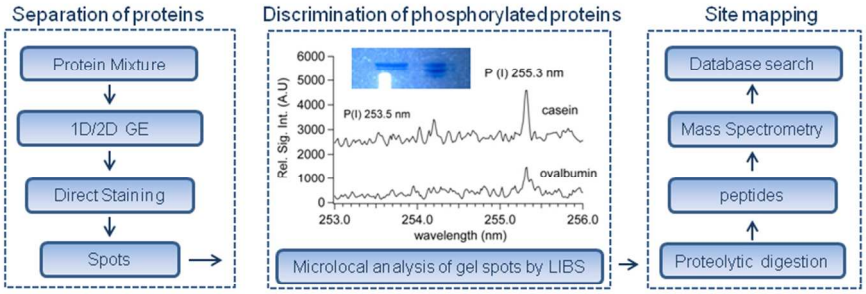

Graphical Abstract
254x190mm (96 x 96 DPI)

We discribe a methodology for rapid identification of phosphorous containing proteins in polyacrylamide gel matrix by Laser-Induced Breakdown Spectroscopy, LIBS.

Rapid Identification of Phosphorous Containing Proteins in Electrophoresis Gel Spots by Laser-Induced Breakdown Spectroscopy, LIBS

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Abstract

A novel method for rapid in-gel identification of phosphorous containing proteins, specifically casein and ovalbumin prior to mass spectrometric analysis for the elucidation of phosphorylation sites was developed. After polyacrylamide gel-electrophoretic separation, staining and drying, protein bands were subjected to focused laser pulses at the center or the vicinity of the protein band. Phosphorous containing proteins were recognized from their prominent phosphorous lines in the luminous plasma formed by energetic laser pulses. LIBS emission intensity of phosphorous lines at 253.5 nm and 255.3 nm has been optimized with respect to laser energy and detector timing parameters by using pure casein in pellet form. The method was applied to casein, ovalbumin, two commercially available standard protein mixtures and proteins extracted from the canola plant. It was shown that LIBS was capable of identifying phosphorous containing proteins directly in the gel matrix in nanogram amounts. Mass spectrometric analysis of the ovalbumin spot after in-gel digestion procedure has proved the accuracy of the technique. With the speed and spatial resolution that LIBS offers, this technique shows a promise in micro-local spotting of phosphorous containing proteins in polyacrylamide gel matrix prior to MS analysis for the determination of the phosphorylation sites.

Keywords: Laser-induced breakdown spectroscopy; micro-local analysis; phosphorous containing proteins; SDS-PAGE.

Introduction

Protein phosphorylation, the reversible addition of the phosphate group to one or more amino acids of a protein, is one of the main cellular processes that regulates and controls the protein activity and cellular functions in all cells.[1] It is one of the post-translational modifications of proteins that contain serine, threonine and tyrosine residues and has a vital role in cell signalling.[2]

Due to the presence of small quantities of total protein in real samples, the ratio of phosphorylated proteins to non-phosphorylated ones can be relatively small and the techniques used for phosphoprotein analysis sometimes suffer from a limited dynamic range and low sensitivity.[3] Phosphoprotein analysis includes separation, identification and quantification steps in which laborious and time consuming biochemical procedures are complementarily used. 1-D or 2-D-Polyacrylamide Gel Electrophoresis, PAGE, is a technique used for the separation of a large number of proteins based on their electrophoretic mobility as they migrate through polyacrylamide gel matrix. After separation, the labeling and identification of phosphoproteins is achieved by using: $^{32}P/^{33}P$ radioactive isotopes[4], phosphoamino acid specific antibodies[5] or direct staining techniques. In direct staining technique, proteins are visualized by staining dyes[6], *Coomassie Blue* or *Silver Stain*, in which staining intensities of patterns are proportional to protein abundance. Both staining techniques are specific to total proteins present in the sample and no information about the phosphorylation status of the protein is obtained during this stage. Mass spectrometry, MS, is a highly sensitive and selective analytical tool for comprehensive analysis of protein phosphorylation[7-10] and is widely used for sequencing of the peptides with femtomole level sensitivity.[11] In order to perform phosphoproteome analysis by MS, stained gel spots are excised and in-gel digested to produce peptides before being subjected to peptide-mass-fingerprinting for the elucidation of phosphorylation sites.[12,13] Hundreds of stained spots may

need to be analyzed after 2-D separation of real samples. Besides, during these excision and bringing into solution steps, protein samples are vulnerable to contamination and sample losses are inevitable. Therefore, pre-enrichment method development has been an active area of research in the last decade.[14] Any technique towards direct identification of phosphorylated proteins from the non-phosphorylated ones in the gel will substantially reduce the analysis time by MS for peptide-mass fingerprinting and would, therefore, be of considerable interest in phosphoproteomics research. In the last decade, laser ablation sample introduction coupled to atomic mass spectrometric detection, (LA-ICP-MS), has been used for the quantification of phosphorous and metal-binding proteins present in the gel.[15-22] In one of those studies, a detection limit of 1.5 pmol β-casein was reported. LA-ICP-MS has the advantages of high sensitivity and multi-element capability along with the micrometer to nanometer scale resolution of a laser probe. On the other hand, Laser-Induced Breakdown Spectroscopy, LIBS[23-24], has been in use for decades for rapid and real-time analysis of a variety of sample types for numerous applications. LIBS technique provides simple, fast and multi element analysis with several microns of spacial resolution and is suitable for in-situ analysis of samples present in inaccessible or harsh environments. Recently, there is a growing interest in biomedical applications of LIBS. A recent progress, future potential and prospects of LIBS for biomedical applications have been reviewed in articles by Rehse et.al.[25] and Singh et.al.[26] Some of these applications include: analysis of calcified tissue materials like teeth[27,28], bones[29], nails[30], soft tissue materials like human skin[31], hair[32], plant leaves[33,34], wood[35], identification and discrimination of bacteria[36], some types of bio-fluids like blood[37] and diagnosis of certain types of malignant tissues[38,39].

The main objective of this study was to evaluate the potential of the LIBS technique in rapid differentiation of phosphorylated proteins from the non-phosphorylated ones prior to mass spectrometric analysis for the identification of the protein and elucidation of the phosphorylation sites. The purpose is not to make quantitative phosphoprotein analysis however, the minimum amount of phosphorous that can be detected in phosphoproteins in gel matrix was determined.

Materials and Methods

Reagents and Materials

Most of the reagents used throughout the experiments were in the most pure form commercially available. Powder casein and ovalbumin were obtained from Sigma-Aldrich and two commercially available unstained protein molecular weight markers PhosDecor^{TM}Control and Fermentas were from Sigma-Aldrich and Thermo Fisher Scientific, respectively. Fermentas unstained protein molecular weight marker is a mixture of seven native proteins (14.4 kDa to 116 kDa) for use as size standards in protein electrophoresis (SDS-PAGE). Total phosphorous content in casein and ovalbumin standards were determined as 0.80±0.05 % and 0.24±0.06 % from ICP measurements, respectively. Casein and ovalbumin standards were prepared in dilute NaOH and de-ionized water, dH_20, respectively and diluted with the sample buffer where necessary. Sample buffer (62.5 mM Tris-HCl (pH: 6.8), 20% glycerol, 2% SDS, 5% beta-mercaptoethanol), running buffer (25mM Tris, 192mM glycine, 0,1% SDS, pH: 8,3) and de-staining solution (5% methanol, 10% acetic acid, 85% dH_20) were prepared from no phosphate containing high purity solvents. 0.1% silver nitrate and 0.05% commassie brilliant blue R250 (in 50% methanol, 10% acetic acid and 40% dH_20) staining solutions were prepared once and used throughout. Monomer concentrations for separating and stacking gels were selected as 12% and 4%, respectively.

Fresh Canola plant (*Brassica Napus*) leaves were frozen in liquid nitrogen after harvest and ground to powder prior to the extraction of the plant proteins by the phenol extraction technique[40]. Total protein concentration in 1 g of plant extract was determined by the Bradford assay[41].

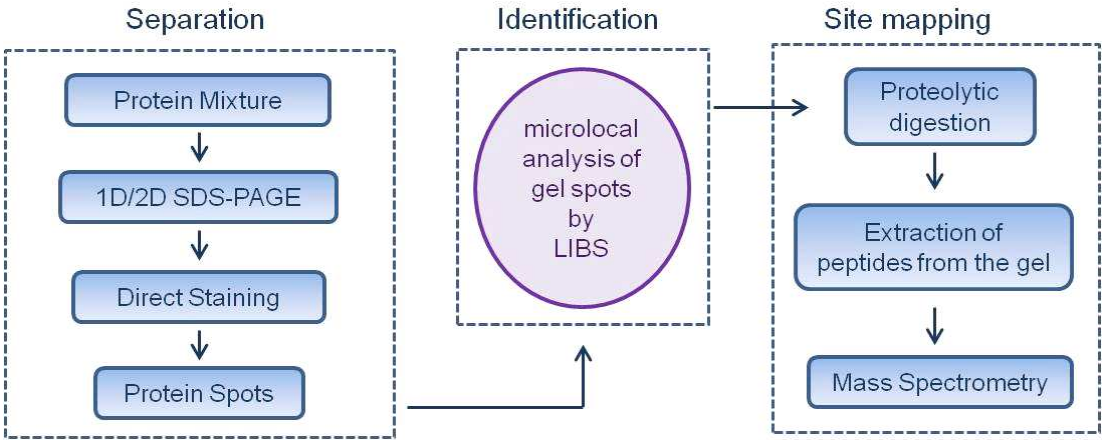
Optimizations of instrumental LIBS parameters like laser energy, delay time and gate width were initially studied on powder casein and inorganic Na_2HPO_4 in the form of pellets and , experimental conditions did not change much when blank gels were used as a matrix. Pellets were prepared in stoichiometric amounts by mixing with powder KBr (Sigma-Aldrich). To avoid the accumulation of moisture on pelletized samples, pellets were prepared fresh just before the analysis and kept in a desiccator when not in use for a short period of time.

Methodology

A general scheme for the proposed phosphoprotein analysis based on laser-induced breakdown spectroscopic identification of phosphorylated proteins followed by MS analysis is given in Scheme 1. Here, the protein mixture is separated by 1D/2D gel electrophoresis and visualized by either Coomassie Blue or Silver Staining techniques. Protein spots on the gel are subjected to laser ablation by highly energetic laser pulses. Spectrochemical analysis of the luminous plasma produced by the focused laser pulses on stained gel spots reveals information about the elemental content and the presence of phosphorous in specific protein spot. Gel spots identified by LIBS for their phosphorous content are then further analyzed by MS for structural identification after in-gel digestion and extraction procedures were applied.

In this study, the method was applied to casein, ovalbumin, commercially available standard protein mixtures and canola plant protein extract for identification of phosphorous containing proteins.

Scheme 1. A general scheme based on SDS-PAGE separation, LIBS identification and MS detection of phosphorylated proteins.



1D-SDS-PAGE separation and staining of the gels

1D-SDS-PAGE separation was carried out according to well-known Laemmli SDS-PAGE procedure[42] by using SDS-PAGE apparatus (BIO-RAD). Each sample was boiled at 100 ºC for 30 minutes, before loading into the gel in order to denature the protein. After separating and stacking gel solutions were prepared, standard protein mixtures were loaded into the wells of SDS-PAGE apparatus in different amounts and electric field was applied at 100 V for 2 hours. The volume of samples loaded into the wells was between 10-50 microliters. Separated proteins were then visualized by Coomasie Blue or Silver Staining techniques. After several hours of de-staining in de-staining solution, gel samples were removed, laid flat and left to dry overnight for LIBS analysis. Cracking in different parts of the gel is inevitable during drying process and if cracking occurs on the protein spots, necessary information would then be get lost. In order to increase the drying rate and prevent cracking of the gel, the gel samples were cut apart for the stained spots of interest and let to dry separately before being placed on XYZ-translational stage for LIBS analysis. Alternatively, one could use GelAir Drying System, (Bio-Rad, Cat.No. 1651772) , for drying any type of polyacrylamide or agarose gels without cracking. In this

system, wet gels are placed between the two cellophane sheets which are then clamped over the edges of top and bottom plastic frame to lock in its place and placed in the dryer for operation with or without heat. Gel drying times are primarily dependent upon gel thickness and an approximate drying time for 1.0 mm thick gel is about 45-60 minutes. Dry gel between cellophane sheets is then separated from the frames (the gel will not peel or separate from the cellophane) and is placed on XYZ-translational stage for LIBS analysis.

Instrumental LIBS setup

An experimental LIBS set-up used to form plasma and detect plasma emission from protein samples, either in the gel or in the form of a pellet, is schematically shown in Figure 1.

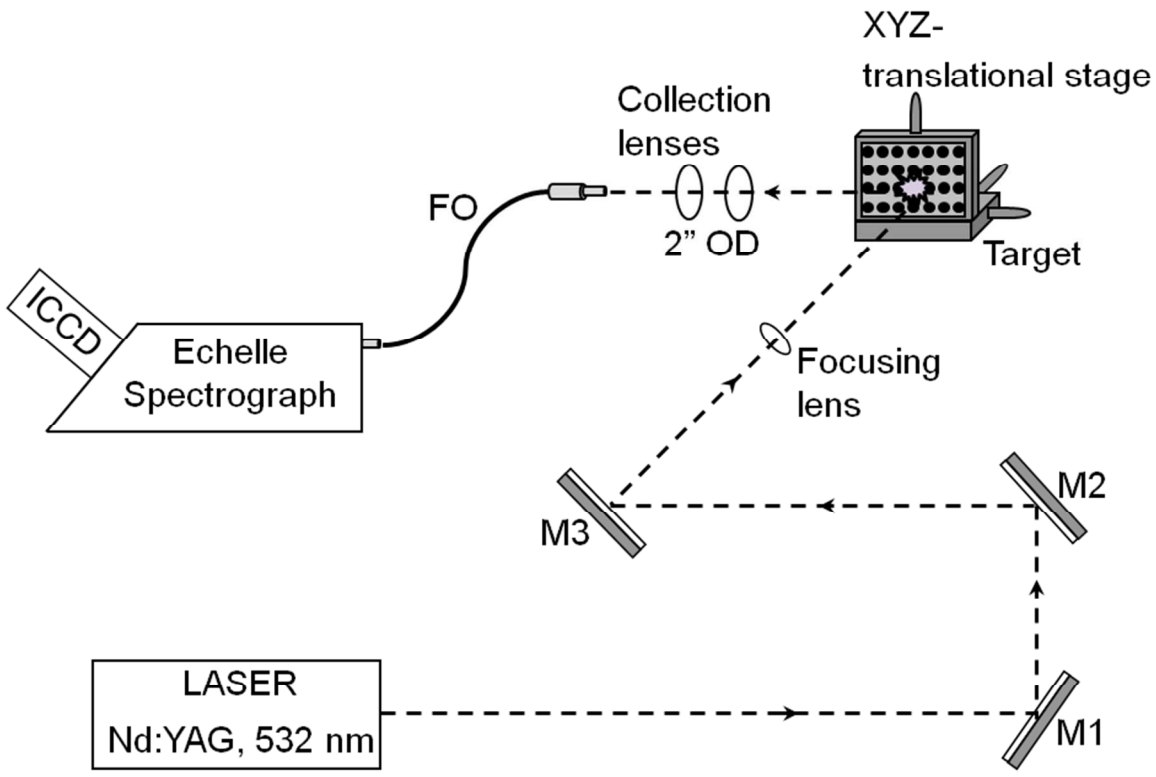


Figure 1. Experimental LIBS set-up. M1, M2 and M3: reflecting laser mirrors, FO: fiber optic cable, ICCD: Intensified Charge Coupled Detector.

A Q-switched Nd:YAG laser source (Quanta-Ray Lab-170, Spectra Physics) with 10 ns pulse duration operating at its second harmonic, 532 nm, was used for plasma formation. The laser beam was directed by using reflective mirrors and was focused on the sample via 20 cm focal length plano-convex lens down to a spot size of 100 micrometers, which corresponds to $1.27*10^{11}$ Wcm^{-2} irradiance when 100 mJ laser pulse energy is used. Samples were mounted on an XYZ-translational stage to provide fresh spots during sampling. Luminous plasma emission was collimated and imaged onto the core of a fiber optic cable (600 μm diameter) by using two 10 cm focal length, 2 inches diameter plano-convex lenses. The end of the optical fiber was connected to the entrance slit of an echelle spectrograph (Mechelle 5000, Andor Inc., f/7) equipped with an ICCD detector (iStar DH734, Andor Inc.). The spectral range of the spectrograph was between 200-850 nm with 0.08 nm resolution at 400 nm and wavelength calibration of the spectrograph was performed by using a Hg-Ar spectral calibration lamp.

Results and Discussion

Optimization of instrumental LIBS parameters

When nanosecond laser pulses focused onto a target surface, the ablated amount from the surface and hence the signal intensity is largely dependent on the incident laser pulse energy. In order to achieve the best analytical performance of a LIBS system, signal intensity should be maximized by careful selection of the experimental parameters. For this purpose, optimization of instrumental LIBS parameters such as detector delay time, gate width and laser energy were performed by using a phosphoprotein sample, -casein, in pellet form. Gel analyses were then performed under these optimum conditions.

The variation of relative signal intensity of neutral phosphorous emission line, P(I), at 255.3 nm with respect to detector delay time, T_d, detector gating time, T_g, and laser pulse energy is presented in Figure 2(a), (b) and (c), respectively.

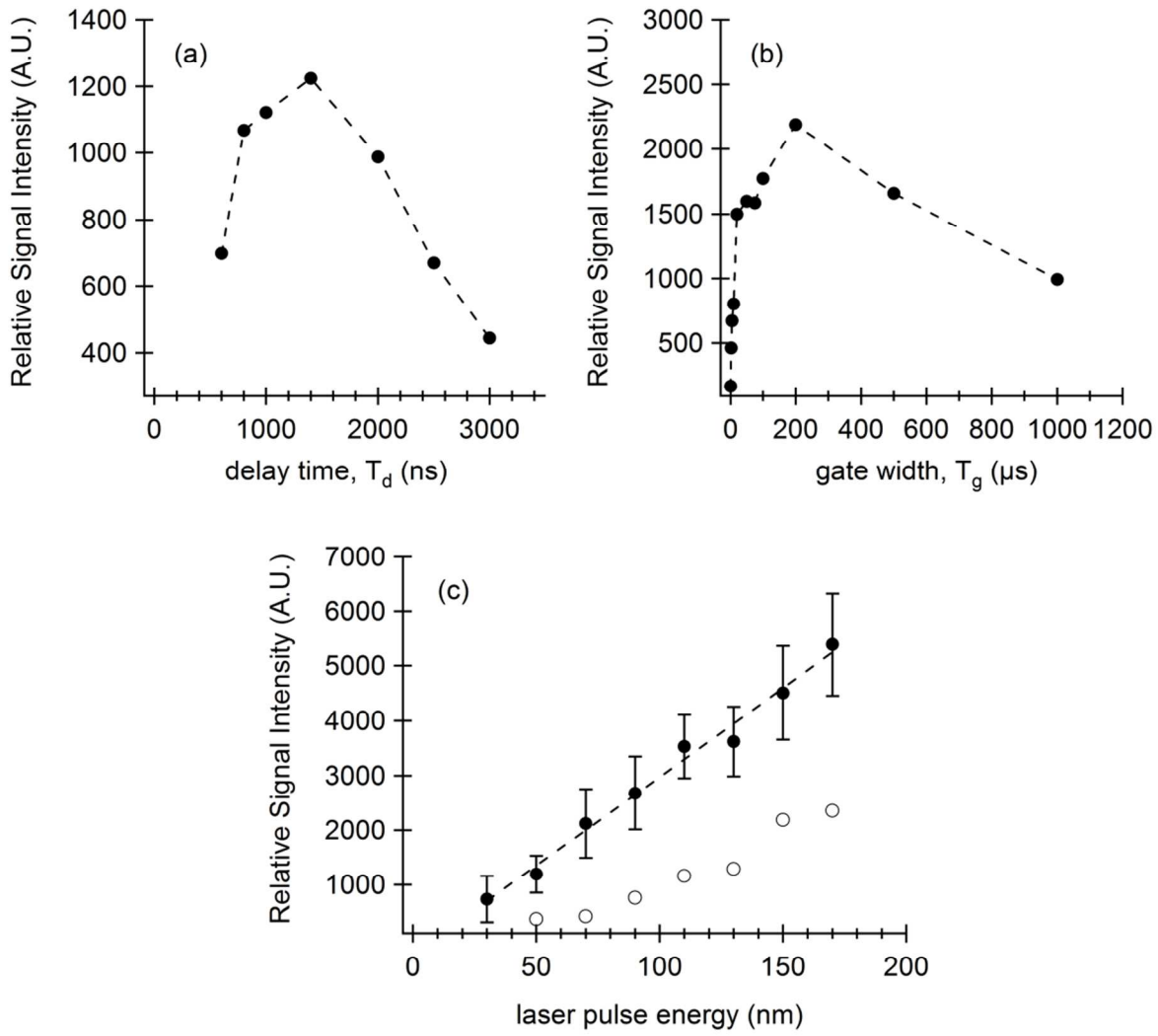


Figure 2. Variation of relative signal intensity with respect to (a) detector delay time, T_d, (b) detector gating time, T_g and (c) laser pulse energy. P(I) emission at 255.3 nm from Casein were used.

As can be seen from Figure 2(a) and (b), relative signal intensity from the neutral P(I) line at 255.3 nm increases as the gate delay and gate width increases. After reaching maxima, a sharp

decrease in signal intensity is observed. Optimum delay time and gating time at which maximum phosphorous signal observed, were found as 1.2 microseconds and 200 microseconds, respectively. Each data point was obtained from the accumulation of ten sequential laser pulses with 150 mJ energy. Detector gain setting of 150 was used for all measurements.

A linear increase in phosphorus signal intensity with respect to increase in laser pulse energy is given in Fig. 2(c). The minimum laser pulse energy to observe LIBS signal for phosphorous emission line was found as 30 mJ. Although signal intensity increases linearly as laser pulse energy increased, with a regression constant of 0.987, LIBS measurements usually suffer from the high noise signals at high laser pulse energies. Signal increases with the expense of an increase in noise levels. The variation of background noise with respect to laser energy has been shown in Fig. 2(c) as empty circles. Therefore, in order to avoid high noise signals in LIBS spectra, laser pulse energy was kept at 120 mJ for most of the measurements.

LIBS analysis of pure protein standards in pellet form

Laser-induced breakdown spectroscopic measurement of phosphorous signal in phosphoprotein samples was carried out under optimized instrumental conditions. Samples from commercially available standards were mixed with powdered KBr in differing stoichiometric proportions and were pressed into 1 cm diameter pellets. Representative LIBS spectra, between 200-850 nm spectral range, obtained from phosphor containing proteins (ovalbumin, α-casein, casein hydrolysate) and inorganic Na_2HPO_4 (as a reference) are presented in Figure 3. Each spectrum contains atomic emission lines representative of the sample. In addition to neutral C emission line at 247.85 nm, strong emission lines of Na, Ca, Mg, H and O were observed in all three phosphoprotein samples: ovalbumin, casein and casein hydrolysate.

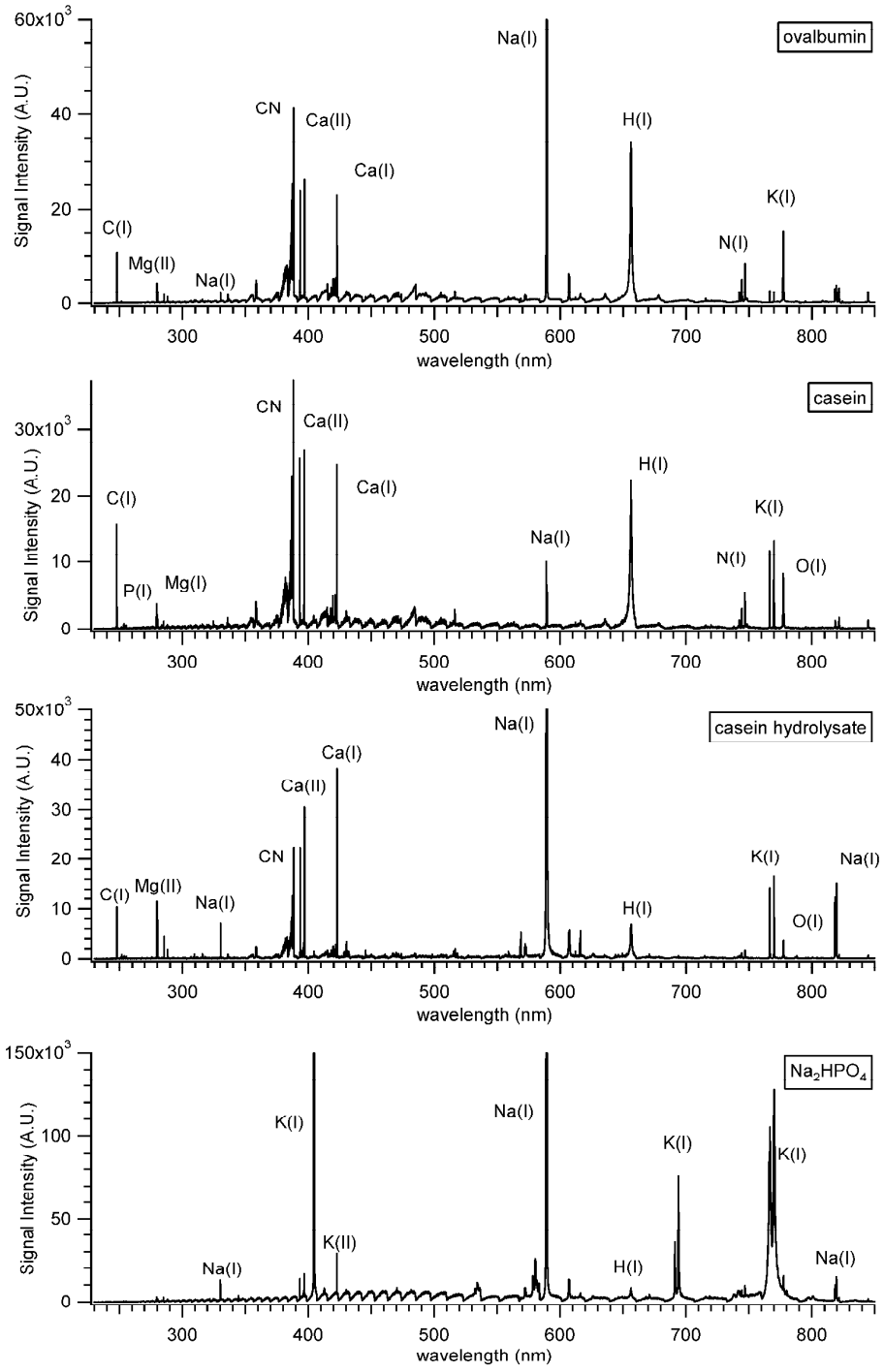


Figure 3. Representative LIBS spectra from ovalbumin, α-casein, casein hydrolysate and Na_2HPO_4, from their pellets. Each spectrum was obtained from the accumulation of 10 laser pulses under optimum instrumental conditions of T_d: 1.2 microsecond, T_g: 200 microseconds and 120 mJ/pulse laser energy.

Strong K(I) emission lines at 766.0 nm and 766.8 nm and Br(I) lines at 386 nm were dominant in each spectrum due to the KBr added during the preparation of pellets. P(I) signal at 253.5 nm and 255.3 nm cannot be visualized in the same scale along with other dominant emission lines of Na, K, Ca and H, due to the presence of low amounts of phosphorus in phosphorylated proteins. Therefore, phosphorous signal from their respective samples were given in Fig. 4, in enlarged scale. In accordance with the phosphorus content listed for casein in the literature [43], P(I) signal obtained from casein and casein hydrolysate is relatively higher compared to the one obtained from the ovalbumin sample.

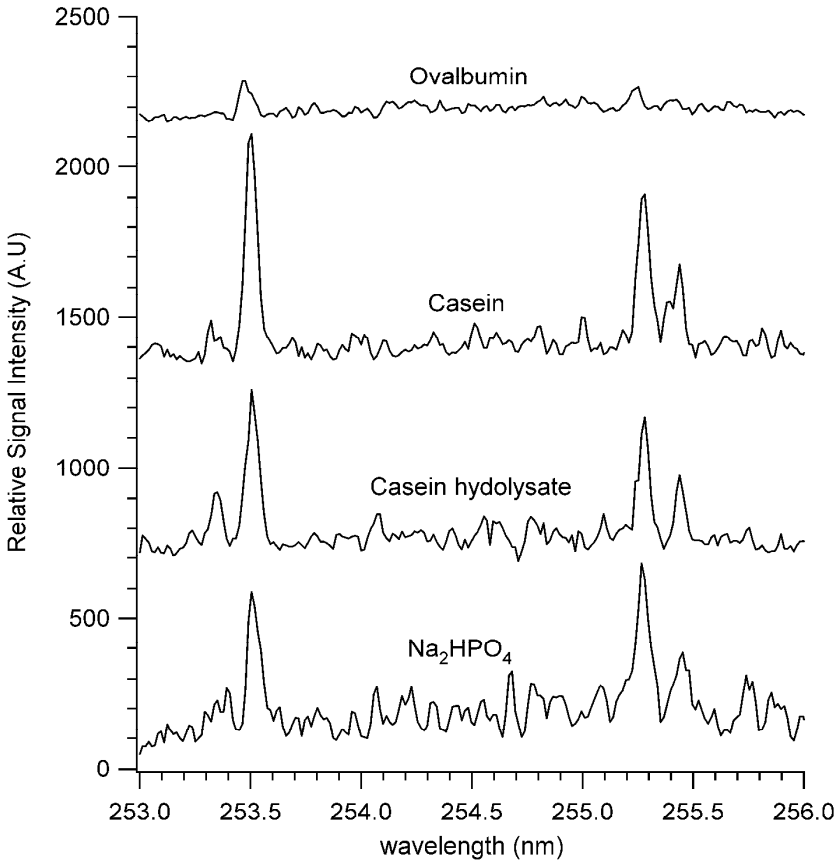


Figure 4. Enlarged spectra showing P(I) emission lines from the plasma formed on phosphoprotein and Na_2HPO_4 samples in pellet form.

Identification of phosphoproteins in SDS-PA gel matrix by LIBS

In order to test the applicability of the LIBS technique for the detection of phosphorous signal in SDS-PA gel matrix, electrophoretically separated, stained and dried protein spots were subjected to micro local analysis by LIBS. Technique was applied to; protein molecular weight markers (*Phosdecor^{TM} Control and Fermentas),* casein and ovalbumin standards prepared from their powders in the laboratory, and the canola plant extract.

Phosphorous signal from the protein molecular weight markers in gel

Two different commercially available protein molecular weight markers, *Fermentas and Phosdecor^{TM} Control,* that contain ovalbumin and casein, were used for LIBS analysis of the protein bands separated in SDS-PA gel matrix. A picture of the gel including electrophoretically separated and Coomassie Blue stained seven proteins of Fermentas molecular weight marker is given in Figure 5 on the right, and LIBS spectra between 253-258 nm region, each corresponding to different protein bands on the gel, are given on the left side of the Figure 5.

Each LIBS spectrum was obtained from the accumulation of the signal from 20 single laser pulses scanned around the center of the protein band. Among seven proteins of different molecular weights, only one of them (ovalbumin) is known to be phosphorylated. As can be seen in the spectra given in Fig. 5, LIBS technique was able to identify a single phosphorylated protein (ovalbumin) from the non-phosphorylated ones with neutral emission lines of phosphorous observed at 253.5 nm and 255.3 nm. LIBS analysis of the other protein bands separated on the gel, namely β-galactosidase, bovine serum albumin, lactate dehydrogenase, Bsp98I, β-lactoglobulin and lysozyme did not result with any phosphorus signal, as expected, within the spectral range of interest.

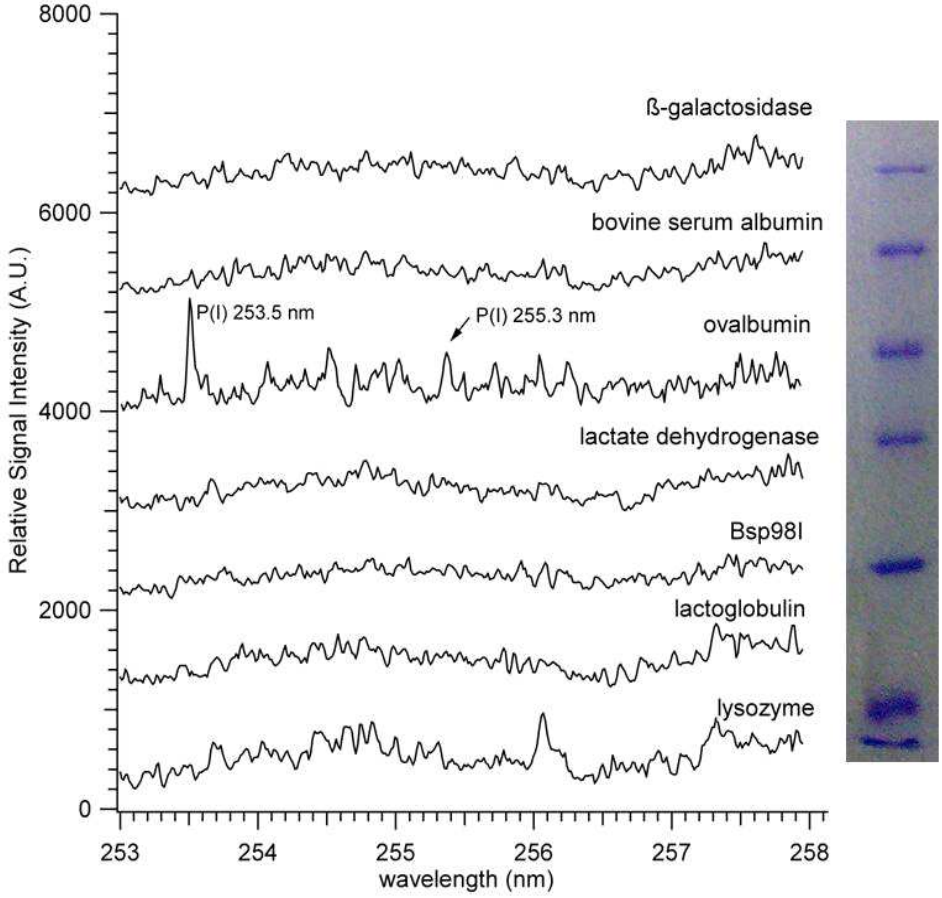


Figure 5. LIBS spectra obtained from the accumulation of 20 single laser pulses scanned around the center of each protein bands of Coomassie Blue stained gel from Fermentas protein molecular weight marker. Phosphorus (I) signal at 253.5 nm and 255.3 nm was only observed in the ovalbumin band. A picture of electrophoretically separated seven proteins of Fermentas molecular weight marker is given on the right.

The other protein molecular weight marker, *Phosdecor* ^{TM} *Control,* was also used to test the LIBS technique's discriminating power by observing phosphorous signal in specific protein spots of the gel matrix. *Phosdecor* ^{TM} *Control* is a mixture of six different proteins, in which two of

them are phosphorylated, casein and ovalbumin. Silver staining procedure was applied on SDS-PA gel separated proteins for better visualization. As in the case of Fermentas protein marker, no phosphorous signal was detected from the protein bands of *Phosdecor* ^{TM} *Control*, except from the casein and ovalbumin bands. The spectra between 253.0 nm - 256 nm range obtained from the protein spots of interest is given in Figure 6. However, in contrast to strongly observed phosphorous line at 253.5 nm from the ovalbumin band of Fermentas biomarker, 255.3 nm line of phosphorous was more strongly detected from the casein and ovalbumin bands of *Phosdecor*^{TM} *Control*. This could be associated with the matrix effect due to different staining protocols applied.

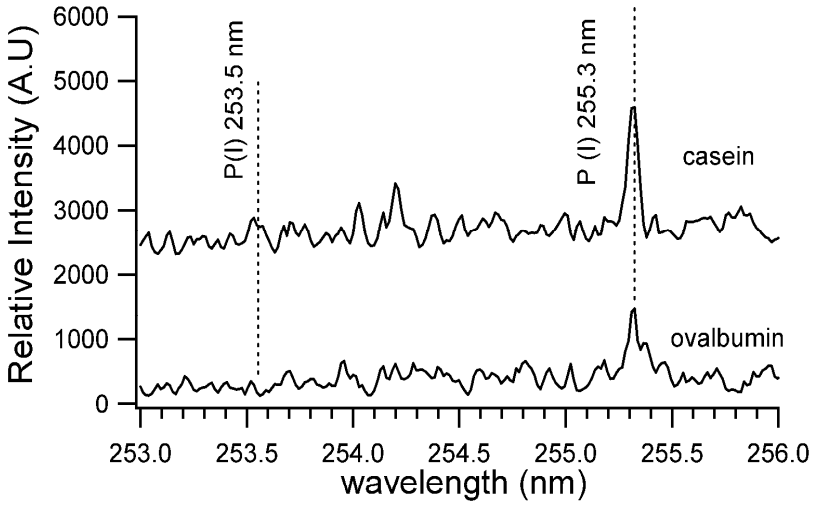


Figure 6. LIBS spectra showing phosphorus signals in *Phosdecor*^{TM} *Control* molecular weight marker, corresponding to casein and ovalbumin spots, in silver stained gel. Spectra were obtained from the accumulation of 10 single laser pulses focused around the center of the protein spots.

Total protein concentrations in molecular weight biomarkers, *Fermentas* and *Phosdecor*^{TM} *Control* are listed as 200 mg L^{-1} and 250 mg L^{-1}, respectively. Injection of 10 microliters of biomarker samples to run through the polyacrylamide gel corresponds to 2.0 and 2.5 micrograms of total protein loadings in which each protein forms 2-3 mm wide protein bands after separation. In order to constitute an observable signal, the entire region of the protein bands required to be scanned with up to 20 to 30 consecutive laser pulses of 100 micrometers diameter. Considering the fact that casein contains 0.7-0.9 % phosphorous covalently bound to protein[42], the amount of phosphorous detected in gel spots by LIBS can be estimated as a few nanograms. LIBS spectra presented in Fig. 5 and Fig. 6 were obtained from the accumulation of 20 and 10 single laser pulses around the center of the protein spots, respectively. Laser pulse energy of 120 mJ, delay time, t_d, 200 ns, gate time, t_g, 0.2 ms and detector gain of 150 were used throughout the measurements.

Phosphorous signal from the pure protein standard in gel

Phosphorous signal from the pure protein standard in gel and also the effect of staining dye on phosphorous signal strength has also been studied. For this purpose, two aliquots of protein standard, α-casein, prepared in the laboratory from its powder were loaded into different channels of electrophoresis apparatus. After separation, visualization of the protein spots was performed by two different staining dyes; Comassie Blue and Silver stain. Figure 7(a) and (b) presents P(I) signal identified in SDS-PA gel matrix stained with Comassie Blue and Silver stain, respectively.

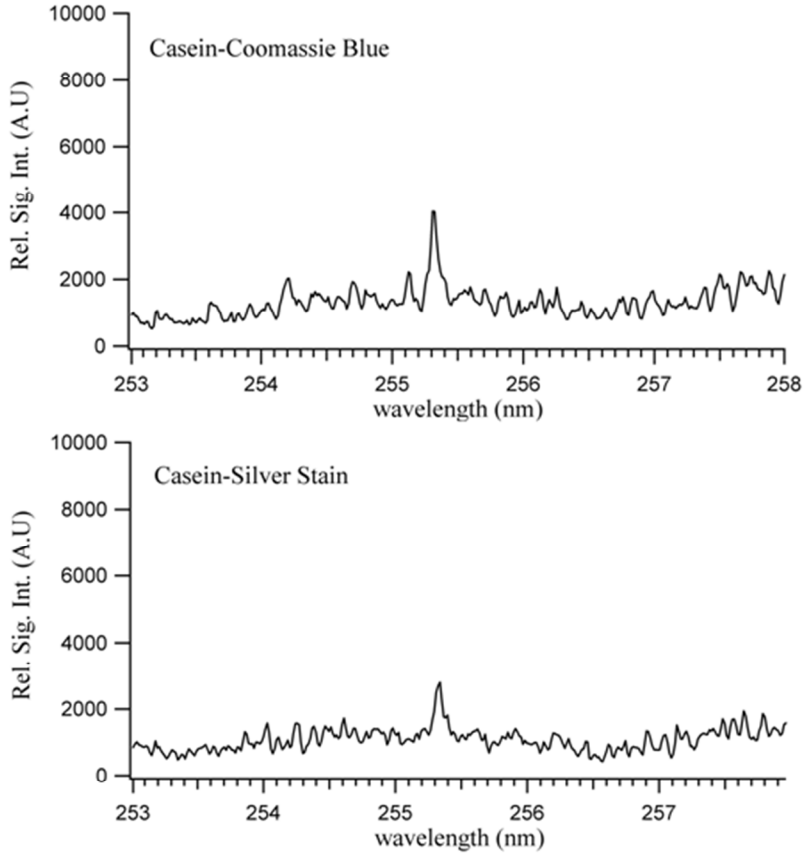
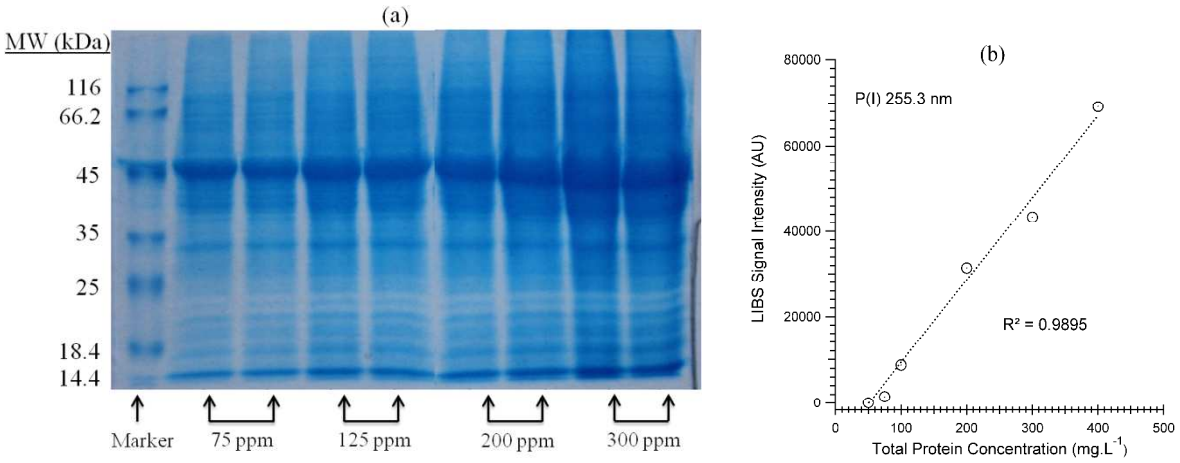


Figure 7. Phosphorus signals from α-casein loaded gels after a) Coomassie Blue and b) Silver staining. Laser pulse energy: 120mJ, delay time, t_d: 200 ns, gate time, t_g: 0.2 ms and detector gain: 150 were used. Signal was observed from the accumulation of 16 laser pulses for Coomassie Blue and 10 laser pulses for silver stained gels

Application of the method to the real samples, canola plant extract

The applicability of the LIBS method to identification and detection of phosphorous containing proteins in real samples was tested on canola plant *(Brassica Napus)* extract. For this purpose, 50 microliters of diluted protein extracts at different total protein concentrations (75 ppm, 125 ppm, 200 ppm and 300 ppm) were loaded in duplicates into the wells of polyacrylamide gels along with Fermentas molecular weight marker and run for about an hour for separation. Then, the gel

306 was stained with Coomassie Blue staining dye. Figure 8(a) shows the picture of the
307 electrophoretic patterns after 1-D separation of the plant protein fractions. Highly intense bands
308 from the protein loadings were observed at the region where 45 kDa ovalbumin band is
309 observed. Micro-local analysis of these bands by LIBS detection revealed the presence of
310 phosphorous in these protein bands. Moreover, in accordance with the concentrations of the
311 proteins loaded, increase in the size and the LIBS signal strength of these protein bands in the gel
312 matrix were also observed. A calibration graph drawn for the variation of signal intensity of the
313 P(I) line at 255.3 nm with respect to total protein concentration (R^2=0.9895) is given in Fig. 8(b).



314

315 **Figure 8.** (a) The picture of the electrophoretic patterns after 1-D separation of plant protein
316 fractions, (b) Calibration graph drawn for variation of 255.3 nm P(I) signal with respect to total
317 protein concentration.

318 A steady increase observed in phosphorous signal intensity with respect to increase in protein
319 concentration is a promising result for potential use of LIBS in semi-quantitative analysis of
320 phosphorous containing proteins in SDS-PA gel matrix.
321 To this end, it has been demonstrated, for the first time, that the LIBS technique in combination
322 with gel electrophoresis is able to identify the presence of phosphorous in protein spots,

however, does not provide information on the sequence of the proteins. Protein sequencing is a technique to determine the order of the amino acid sequence of a protein in which mass spectrometry is widely used as a direct method of analysis. Mass spectrometric analysis of the protein spots after excision and digestion procedures provides further information. In order to justify the applicability of the LIBS technique prior to MS analysis for phosphoproteome research, some experiments were performed. For this purpose, protein bands of the canola plant extract at 45 kDa ovalbumin band region were excised, in-gel digested and subjected to MALDI-MS analysis. Results were compared against a database of previously sequenced proteins. Database searching of the MS-MS data revealed the presence of an enzyme ***Ribulose-1.5-bisphosphate carboxylase/oxygenase*****,** commonly known as ***Rubisco***. Rubisco is the most abundant protein present in plant leaves and consists of eight large (~56 kDa each) and eight small (~14 kDa each) subunits[44]. Therefore, the next highly intense protein bands observed around 14.4 kDa in Fig. 8(a) may be attributed to the small subunits of this enzyme. Further studies based on 2D-gel separation of these protein bands may provide more detailed information for phosphoproteome research; however, low detection power of the LIBS technique is an issue to deal with.

Conclusion

In this study, the applicability of the LIBS technique for fast identification and detection of phosphorous containing proteins directly in SDS-PA gel matrix prior to MS analysis have been demonstrated, for the first time. Initially, the experimental LIBS parameters such as detector delay time, gate width and laser energy were optimized in order to maximize phosphorous signal. Under optimum experimental conditions, prominent P(I) lines at 253.5 nm and 255.3 nm were

used to identify phosphoproteins in commercially available molecular weight markers (Phosdecor^{TM} Control and Fermentas), in laboratory prepared protein standard (Casein) and in canola plant extracts. Proteins were run according to 1D-SDS-PAGE separation technique and were stained by two different staining procedures. Nanogram levels of phosphorus present in proteins were detected by LIBS. It has been demonstrated that SDS-PAGE separation of proteins followed by LIBS detection can be used as a versatile tool for micro-local spotting of not only phosphorous, but all elements present in protein spots. This methodology can be extended for the identification of metal binding proteins or presence of metals in protein samples. Mass spectral measurements and database searching of the mass spectral data has proved that LIBS can accurately be used as a fast and easy identification technique, prior to MS analysis, for phosphoproteome research; however, more detailed study is required for low levels of quantitative analysis.

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FIGURE CAPTIONS

Figure 1. Experimental LIBS set-up. M1, M2 and M3: reflecting laser mirrors, FO: fiber optic cable, ICCD: Intensified Charge Coupled Detector.

Figure 2. Variation of relative signal intensity with respect to (a) detector delay time, T_d, (b) detector gating time, T_g and (c) laser pulse energy. P(I) emission at 255.3 nm from Casein were used.

Figure 3. Representative LIBS spectra from ovalbumin, α-casein, casein hydrolysate and Na_2HPO_4, from their pellets. Each spectrum was obtained from the accumulation of 10 laser pulses under optimum instrumental conditions of T_d: 1.2 microsecond, T_g: 200 microseconds and 120 mJ/pulse laser energy.

Figure 4. Enlarged spectra showing P(I) emission lines from the plasma formed on phosphoprotein and Na_2HPO_4 samples in pellet form.

Figure 5. LIBS spectra obtained from the accumulation of 20 single laser pulses scanned around the center of each protein bands of Coomassie Blue stained gel from Fermentas protein molecular weight marker. Phosphorus (I) signal at 253.5 nm and 255.3 nm was only observed in the ovalbumin band. A picture of electrophoretically separated seven proteins of Fermentas molecular weight marker is given on the right.

Figure 6. LIBS spectra showing phosphorus signals in *Phosdecor*^{TM} *Control* molecular weight marker, corresponding to casein and ovalbumin spots, in silver stained gel. Spectra were obtained from the accumulation of 10 single laser pulses focused around the center of the protein spots.

Figure 7. Phosphorus signals from α-casein loaded gels after a) Coomassie Blue and b) Silver staining. Laser pulse energy: 120mJ, delay time, t_d: 200 ns, gate time, t_g: 0.2 ms and detector gain: 150 were used. Signal was observed from the accumulation of 16 laser pulses for Coomassie Blue and 10 laser pulses for silver stained gels

Figure 8. (a)The picture of the electrophoretic patterns after 1-D separation of plant protein fractions, (b)Calibration graph drawn for variation of 255.3 nm P(I) signal with respect to total protein concentration.

SCHEME TITLES

Scheme 1. A general scheme based on SDS-PAGE separation, LIBS identification and MS detection of phosphorylated proteins.

For Graphical Abstract