

POSTER R8-W / ABRF-PRG03: Phosphorylation Site Determination

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MISSION

The mission of the Proteomics Research Group (PRG) is to assist ABRF members in evaluating their capabilities to identify "unknown" proteins in order to establish realistic expectations for this technology.

ABSTRACT

A fundamental aspect of proteomics is the analysis of post-translational modifications, of which phosphorylation is an important class. Site-specific phosphorylation and dephosphorylation of proteins in signal transduction pathways, for example, is key to the regulation of many cellular processes. The mapping of phosphorylation sites in proteins traditionally involved radioactive methods; modern mass spectrometric techniques now make possible phosphorylation site determination at high sensitivity, avoiding the use of radioactivity. ABRF laboratories are currently engaged in phosphorylation mapping projects using these techniques. It is not known, however, how many laboratories routinely take on such projects, which methods are most often applied, and how successful the laboratories and particular methods are. The Proteomics Research Group has undertaken a study to help answer these questions.

A sample described as one or more proteins and two synthetic phosphopeptides was distributed in the form of a tryptic digest, with participating laboratories challenged to identify the component protein(s), identify the phosphorylated peptides, and determine the site of phosphorylation on each peptide. The phosphopeptides were included at low stoichiometries relative to the unphosphorylated protein since this is a common challenge in practice. It was anticipated that several strategies would be employed for the detection of phosphopeptides, including subtractive analysis, enrichment by affinity chromatography, diagnostic mass differences, and characteristic fragmentation patterns in post-source decay or collision-induced dissociation. Determination of sites of phosphorylation could involve MS/MS or Edman degradation. This study has the following goals: 1) Provide a mechanism for participating laboratories to evaluate their capabilities in locating sites of phosphorylation in non-radioactive samples; 2) Provide an introduction to phosphorylation site analysis for laboratories that do not currently do such analyses; 3) Compare different strategies for phosphorylation site mapping; and 4) Help establish realistic expectations for the analysis of phosphorylation sites.

INTRODUCTION

Post-translational modifications play important roles in determining protein function. In particular, phosphorylation controls a wide range of metabolic processes including signal transduction, differentiation and development, and metabolism. In order to understand the function of a protein of interest, it therefore is necessary to locate the sites of modification. Classically, locating sites of phosphorylation has been done by radiolabeling the phosphopeptides with ³²P-labeled inorganic phosphate, subjecting the labeled protein to an enzymatic or chemical digestion, followed by isolation of the radio-tagged phosphopeptides using HPLC. The labeled peptides then are sequenced using either Edman chemical degradation or MS/MS analysis. However, it is not always feasible to radiolabel the phosphopeptides, and hence, locating sites must, in these cases, be done on the cold sample. Approaches used to identify phosphopeptides in non-radiolabeled samples include phosphopeptide enrichment^{1,2}, tagging of the phosphopeptides using an isotope affinity tag³, neutral loss experiments⁴, precursor ion scanning⁵, and mass mapping⁶.

The ABRF-PRG03 sample contained a protein mixture of ~ 5 pmols bovine protein disulfide isomerase (PDI) and ~ 200 fmols bovine serum albumin (BSA). In addition, 2 synthetic tryptic peptides, each containing 1 phosphorylated residue and corresponding to peptides found in PDI, were added at the 1 pmol level (peptide amounts determined by amino acid analysis).

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METHODS

- Excised individual protein bands of PDI and BSA from preparative SDS-polyacrylamide gel
- Reduced with 20 mM TCEP/ 25 mM ammonium bicarbonate (pH 8.0), 15 min at 37°C
- Alkylated with 40 mM iodoacetamide / 25 mM ammonium bicarbonate (pH 8.0), 15 min at 37°C
- Digested with 0.62 µg/µl Trypsin (Promega modified), 18 hr at 37°C
- Mixed PDI & BSA digests with the two synthetic phosphopeptides in a 5 : 0.2 : 1 : 1 molar ratio, respectively
- Aliquoted into tubes to give final composition of:
 - 5 pmol PDI
 - 200 fmol BSA
 - 1 pmol SVPSDYEGK (P1; S268)⁷
 - 1 pmol THILLFLPKpSVSDYEGK (P2; S266)⁸
- Dried
- Tested by PRG member laboratories
- Mailed out to 106 requesting laboratories

⁷ Both peptides are derived from the PDI sequence, and they normally are present in tryptic digests in their phosphorylated forms. Peptide P1 contains S268 of the PDI sequence, and Peptide P2 contains S266 of the PDI sequence. Peptide P2 was designed to mimic an incompletely digested tryptic peptide, where the phosphorylated S266 might interfere with trypsin digestion at K265.

RESULTS: PROTEIN IDENTIFICATION

Table 1. Protein Identification

| Lab Number | Method | Protein | Phosphopeptide | Site | Phosphorylation | Correct Site | Correct Site |
|------------|----------|----------------------|----------------|------|-----------------|--------------|--------------|
| 1000 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1001 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1002 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1003 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1004 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1005 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1006 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1007 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1008 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1009 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1010 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1011 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1012 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1013 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1014 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1015 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1016 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1017 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1018 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1019 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1020 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1021 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1022 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1023 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1024 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1025 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1026 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1027 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1028 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1029 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1030 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1031 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1032 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1033 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1034 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1035 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1036 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1037 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1038 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1039 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1040 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1041 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1042 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1043 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1044 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1045 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1046 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1047 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1048 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1049 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1050 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1051 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1052 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1053 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1054 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1055 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1056 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1057 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1058 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1059 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1060 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1061 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1062 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1063 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1064 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1065 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1066 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1067 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1068 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1069 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1070 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1071 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1072 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1073 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1074 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1075 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1076 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1077 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1078 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1079 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1080 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1081 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1082 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1083 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1084 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1085 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1086 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1087 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1088 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1089 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1090 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1091 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1092 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1093 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1094 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1095 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1096 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1097 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1098 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1099 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1100 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |

Protein Identification Summary:

- A total of 54 different laboratories (51%) returned data.
- 12 laboratories ran multiple analyses, for a total of 67 analyses. These responses are marked with * in the above table.
- 96% of the responses correctly identified PDI at the 5 pmol level. In the ABRF-PRG02 study, 96% of the participants correctly identified PDI at the 2 pmol level.
- For the minor component BSA, at the 200 fmol level, only 10% of the responses correctly identified this protein.
- There were 5 positive wrong and 17 tentative wrong identifications.

Table 2. Protein Identification Summary

| Lab Number | Total Analyses | Correct | Wrong | Partial | Not Done | Total Wrong |
|------------|----------------|---------|-------|---------|----------|-------------|
| ALL | 67 | 34 | 1 | 1 | 3 | 5 |
| BSA | 34 | 3 | 0 | 0 | 3 | 3 |
| PDI | 34 | 31 | 1 | 0 | 2 | 2 |

RESULTS: IDENTIFICATION OF PHOSPHOPEPTIDES & THE SITES OF PHOSPHORYLATION

Table 3. Phosphopeptide Analysis

| Lab Number | Method | Protein | Phosphopeptide | Site | Phosphorylation | Correct Site | Correct Site |
|------------|----------|----------------------|----------------|------|-----------------|--------------|--------------|
| 1000 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1001 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1002 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1003 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1004 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1005 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1006 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1007 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1008 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1009 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1010 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1011 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1012 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1013 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1014 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1015 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1016 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1017 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1018 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1019 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1020 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1021 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1022 | LC-MS/MS | Bovine Serum Albumin | ...K | ... | ... | ... | ... |
| 1023 | | | | | | | |