Comparison of SYPRO Ruby and Flamingo™ Fluorescent Gel Stains With Respect to Compatibility With Mass Spectrometry

Tom Berkelman, Katrina Academia, Ning Liu, Tim Wehr
Life Science Group, Bio-Rad Laboratories, Inc., Hercules, CA 94547, USA

Introduction

Proteomics research commonly uses an analytical scheme consisting of protein separation by 2-D electrophoresis followed by proteolytic digestion and analysis of the peptide mixture by mass spectrometry (2-DGE/MS). In this scheme, the gel-separated proteins are typically detected and quantified with a nonspecific general protein stain. Fluorescent stains are well suited to this application due to the high sensitivity and dynamic range achievable using fluorescence detection. In order to be useful for 2-DGE/MS, a stain must be compatible with subsequent proteolytic digestion and mass spectrometry.

SYPRO Ruby is currently the most commonly used fluorescent protein stain, and is widely used in conjunction with proteolytic digestion of excised protein spots and MS analysis (Lopez et al. 2000). Flamingo fluorescent gel stain is a recently developed nonspecific protein stain with potential utility in proteomics research due to its high sensitivity and wide dynamic range.

This study was undertaken to assess the suitability of Flamingo gel stain for the detection of gel-separated proteins that would be identified by proteolytic digestion, followed by mass spectrometry. Proteins were excised and digested from SDS-PAGE gels loaded identically and stained with either SYPRO Ruby or Flamingo fluorescent gel stains and the peptides were analyzed by MALDI-TOF-MS. Results from each experiment were compared with respect to peptide sequence coverage. The data were also analyzed for the presence of artifactual peptide modifications.

Methods

Serial dilutions of broad range SDS-PAGE standards (Bio-Rad Laboratories, Inc.), were separated on Criterion™ Tris-HCl 4–20% gels (Bio-Rad) as indicated in Figure 1. Gels were stained either with SYPRO Ruby protein gel stain (Bio-Rad) or with Flamingo fluorescent gel stain (Bio-Rad), as indicated in the provided instructions. Gel plugs containing *E. coli* β-galactosidase, chicken ovalbumin, or chicken lysozyme were excised from the gels using an EXQuest™ spot cutter (Bio-Rad) with a 1.5 mm cutting head and UV epi-illumination to visualize the bands to be excised. Illumination times for imaging were 1 min for SYPRO Ruby protein gel stain and 3 min for Flamingo fluorescent gel stain. Following excision, the gels were re-imaged with the Molecular Imager® PharosFX™ system (Bio-Rad) using settings for SYPRO Ruby protein gel stain (Figure 1). The gel plugs were submitted for MALDI-TOF-MS tryptic peptide mass analysis (Proteome Research Services, Inc.) without identifying the stain used. The mass spectra were analyzed using m/z software (Proteometrics) and any monoisotopic mass with signal-to-noise >4 within 0.02 Da of a predicted monoisotopic mass was considered a hit.

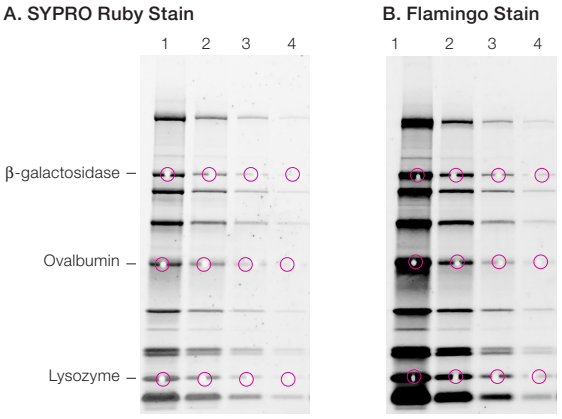

Fig. 1. SDS-PAGE, staining, and excision of stained proteins for MALDI-TOF-MS analysis of tryptic peptides. A, Gel stained with SYPRO Ruby protein gel stain according to instructions; **B**, Gel stained with Flamingo fluorescent gel stain according to instructions. Amounts of protein per band in lanes 1 thorugh 4 in **A** and **B** are as follows: 480, 120, 30, 7.5 ng. ○ indicate the position of excised plugs.

Results

Sequence Coverage by MALDI Tryptic Peptide Mass Analysis

The results of MALDI-TOF-MS peptide analysis are presented in Table 1. The data show that staining with Flamingo fluorescent gel stain resulted in more complete sequence coverage than staining with SYPRO Ruby protein gel stain in almost all instances (Table 1). Figure 2 presents the same data in graphical form with trendlines.

Table 1. Sequence coverage by MALDI mass analysis.

		Sequence Coverage, %	
Protein	Quantity*, ng	SYPRO Ruby Stain	Flamingo Stain
β-galactosidase (116352 Da)	96 (825 fmol)	27.1	33.8
	24 (206 fmol)	24.2	34.5
	6 (52 fmol)	7.5	17.4
	1.5 (13 fmol)	6.5	6.5
Ovalbumin (42750 Da)	96 (2246 fmol)	34.3	37.4
	24 (561 fmol)	42.6	33.5
	6 (140 fmol)	23.6	35.1
	1.5 (35 fmol)	11.7	27.3
Lysozyme (14313 Da)	96 (2246 fmol)	58.6	57.8
	24 (561 fmol)	31.3	57.8
	6 (140 fmol)	20.3	57.8
	1.5 (35 fmol)	20.3	28.1

* Estimated from the size of the gel plug relative to the width of the band.

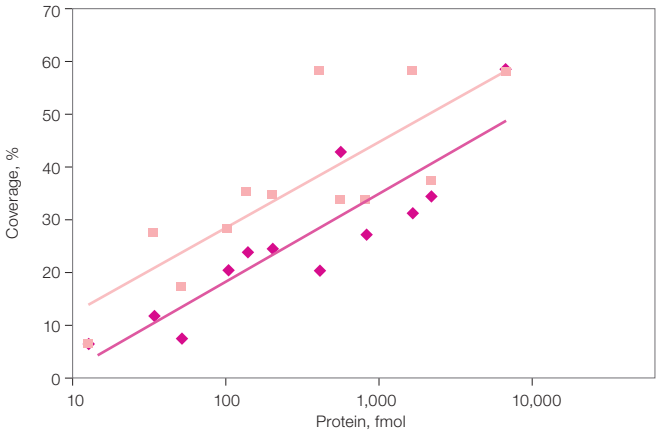

Fig. 2. Percent sequence coverage vs. protein quantity (fmol). Comparison of sequence coverage by MALDI vs. peptide quantity between SYPRO Ruby (◆) and Flamingo fluorescent gel-stained (■) peptides.

Incidence of Possible Oxidative Modification of Amino Acids

The presence of species with masses ~15.995 Da and ~31.990 Da higher than the masses of predicted peptides was observed in the mass spectra of peptides from all three proteins tested (Figure 3). The incidence of these putative modifications was higher in digests of SYPRO Ruby-stained proteins than in digests of Flamingo-stained proteins (Table 2).

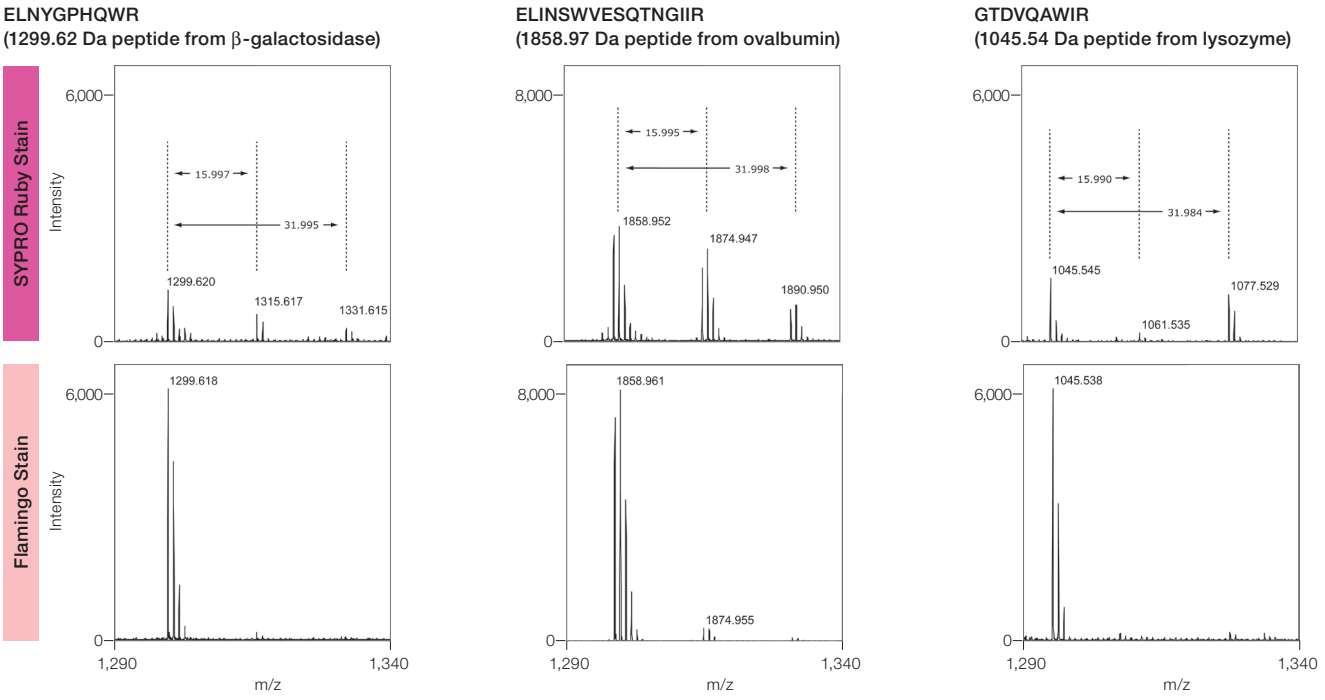

Fig 3. Examples of MALDI-TOF-MS spectra showing possible oxidative modifications. The partial MS spectra shown are of digests of gel plugs containing 24 ng of protein from gels stained either with SYPRO Ruby stains (top) or Flamingo stains (bottom).

Table 2. Sequence coverage by MALDI mass analysis.

Protein	Quantity, ng	Incidence of Potentially Oxidized Peptides* SYPRO Ruby Stain	Flamingo Stain
β-galactosidase (116352 Da)	96 (825 fmol)	18	14
	24 (206 fmol)	10	5
	6 (52 fmol)	0	0
	1.5 (13 fmol)	2	0
Ovalbumin (42750 Da)	96 (2246 fmol)	9	6
	24 (561 fmol)	8	7
	6 (140 fmol)	3	3
	1.5 (35 fmol)	0	0
Lysozyme (14313 Da)	96 (2246 fmol)	13	7
	24 (561 fmol)	4	2
	6 (140 fmol)	0	2
	1.5 (35 fmol)	0	0

* Peptides identified as monoisotopic peaks with m/z shifted +15.995 ± 0.02 or +31.990 ± 0.02 relative to predicted peptides from the source protein.

The mass shifts of these modifications correspond to the mass of one or two oxygen atoms and likely indicate oxidative amino acid modification. Several such modifications have been described in the literature (Berlett and Stadtman 1997), some of which are shown in Table 3. The exact nature of the modification cannot be determined from these data, but peptides with monoisotopic masses larger in multiples of 15.995 Da than the predicted mass are diagnostic of oxidative protein modification.

Table 3. Examples of known oxidative amino acid modifications*.

Amino Acid	Oxidized Amino Acid	Change in Mass, Da
Methionine	Methionine sulfoxide	+15.995
	Methionine sulfone	+31.990
Tryptophan	2-, 4-, 5-, 6- or 7-hydroxytryptophan	+15.995
	N-formylkynurenine	+31.990
Histidine	2-oxohistidine	+15.995
Phenylalanine	2-, 3- or 4-hydroxyphenylalanine	+15.995
	2,3-dihydroxyphenylalanine	+31.990
Tyrosine	3,4-dihydroxyphenylalanine	+15.995

* From Jori et al. 1968.

Discussion

In most cases, staining with Flamingo resulted in more complete sequence coverage than SYPRO Ruby when MALDI-TOF-MS was used to analyze the tryptic peptides from gel-separated proteins. The basis for the difference in performance has several potential explanations, as the dye component, the formulation of the solution, and the staining protocols are all different between the two products.

It was observed that mass spectra derived from SYPRO Ruby-stained samples showed a greater incidence of peaks shifted from the expected peptide masses by the mass of one or two oxygen atoms. This is indicative of oxidative amino acid modification and may provide some insight into the basis for the difference in sequence coverage between Flamingo stain and SYPRO Ruby stain.

Dye-sensitized photooxidation of certain amino acids is a well-described phenomenon (Jori et al. 1968, Nakagawa et al. 1985, Tomita et al. 1969), and ruthenium(II)–diimine complexes such as SYPRO Ruby protein gel stain (Bhalgat et al. 2001) are known to have particularly high quantum yields for generation of the reactive species (Mulazzani et al. 1994). Ruthenium(II)–diimine-sensitized oxidation of tryptophan has been described (Liu et al. 1995, Wessels et al. 1997). Flamingo fluorescent stain may be a weaker photosensitizer than SYPRO Ruby stain, resulting in higher yields of unmodified peptides. A relative lack of cysteine-containing peptides from mass spectra of SYPRO Ruby-stained gels has been noted (Lanne and Panfilov 2005), and modification of this oxidatively labile residue may be a related phenomenon.

Conclusions

Our data indicate that:

- Flamingo stain is fully compatible with polyacrylamide gel electrophoresis and MS
- Staining with Flamingo fluorescent gel stain resulted in more complete sequence coverage by MALDI-TOF-MS tryptic peptide mass analysis than did SYPRO Ruby stain
- Staining with Flamingo stain resulted in a lower incidence of oxidative amino acid modifications than did SYPRO Ruby stain

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Bulletin 5754 Rev A US/EG *08-0488 0109 Sig 0308*