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Enhanced Proteomic Analysis of *Streptomyces peucetius* Cytosolic Protein Using Optimized Protein Solubilization Protocol

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Abstract Improvements in the dissolution of proteins in two-dimensional gel electrophoresis have greatly advanced the ability to analyze the proteomes of microorganisms under a wide variety of physiological conditions. This study examined the effect of various combinations of chaotropic agents, a reducing agent, and a detergent on the dissolution of the *Streptomyces peucetius* cytosolic proteins. The use of urea alone in a rehydration buffer as a chaotropic agent gave the proteome a higher solubility than any of the urea and thiourea combinations, and produced the highest resolution and clearest background in two-dimensional gel electrophoresis. Two % CHAPS, as a detergent in a rehydration buffer, improved the protein solubility. After examining the effect of several concentrations of reducing agent, 50 mM DTT in a rehydration buffer was found to be an optimal condition for the proteome analysis of *Streptomyces*. Using this optimized buffer condition, more than 2,000 distinct and differentially expressed soluble proteins could be resolved using two-dimensional gel electrophoresis with a p*I* ranging from 4–7. Under this optimized condition, 15 novel small proteins with low-level expression, which could not be analyzed under the non-optimized conditions, were identified. Overall, the optimized condition helped produce a better reference gel for *Streptomyces peucetius*.

Key words: Solubilization, two-dimensional gel electrophoresis (2-DE), *Streptomyces*

Two-dimensional gel electrophoresis (2-DE) is one of the classical tools in proteomic analysis, where proteins are separated by isoelectric focusing in the first dimension and further resolved by SDS-PAGE in the second dimension according to their molecular weights. The aim of 2-DE is to separate and show as many of the gene products expressed at certain stages of cell growth as possible. The wide variety of properties of protein such as hydrophobicity, molecular weight, and p*I* value make it impossible to show all the proteins expressed in a single gel. Therefore, zoom gels have been used to overcome this problem [5, 12]. Recently, a combination of 2-DE and mass spectrometry (MS) has become one of the powerful tools for analyzing a complex mixture of proteins [8, 9, 16, 18, 30].

Among the difficulties associated with using the 2-DE technique, dissolving the protein mixtures for isoelectric focusing (IEF) is one of the most important factors, because the efficient dissolution of the protein samples is essential for high-performance 2-DE.

A protein is dissolved by breaking the intermolecular and intramolecular interactions and stabilizing the resulting polypeptide. To this end, several buffers and new compounds have been developed in 2-DE to visualize the maximum number of proteins in a specific microorganism. O'Farell [24] used a lysis buffer, a mixture of 9.5 M urea, 2% (w/v) NP-40, 2% Ampholines (1.6% with pH range of 5 to 7 and 0.4% with pH range of 3 to 10), and 5% mercaptoethanol, which contributes to protein dissolution, to resolve more than 1,000 different soluble protein spots from *E. coli*. Nandakumar *et al.* [23] dissolved the membrane and cell-wall associated proteins from *Staphylococcus aureus* using the rehydration buffer containing urea, thiourea, amidosulfobetaine-14 (ASB-14), and dithiothreitol (DTT). Even the cytosolic proteome from *Streptomyces* has been dissolved using various dissolution buffers containing 7 to 10 mM urea, 0 to 2 mM thiourea, 4% CHAPS, and 0 to 100 mM DTT

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[11, 25, 26, 33]. As reported earlier, these dissolution buffers have been used in different ways according to the strains and proteome properties [2, 11, 14, 17, 20, 25, 26, 33]. Therefore, a universal dissolution buffer for the cytosolic proteomes from *Streptomyces* is needed. Urea, thiourea, CHAPS, and DTT were selected as the key factors that influence the protein solubility and 2-DE pattern.

Urea and thiourea are chaotropic agents. Urea is used at concentrations >8 M, and converts proteins into a single conformation by loosening their secondary and tertiary structures to obtain and maintain hydrophobic proteins in solution [9]. In the case of samples containing a significant number of hydrophobic domains such as the membrane protein, a second stronger chaotrope, thiourea, needs to be added together with urea to enhance the solubility of the hydrophobic proteins [15, 28]. The optimal ratio can be different in protein samples [9, 22]. Thiourea can disturb the 2-DE patterns, resulting in a low resolution and streaking in the acidic range (pH 3–5) [26].

Detergents prevent hydrophobic interactions between the hydrophobic domains of the proteins and enhance the solubility of the cytosolic and membrane proteins. Nonidet P-40 (NP-40), Triton X-100, and dodecyl maltoside are popular; however, they are not very effective on account of their low purity as well as the lower solubility of hydrophobic proteins [9, 34]. SDS (sodium dodecylsulfate) is the most popular detergent, but cannot be used on its own in an IEF separation because of horizontal streaking in the 2-DE pattern [9, 34]. On the other hand, zwitterionic detergents such as CHAPS (3-[(3-chlolaminodopropyl)dimethylamino]-1-propane sulfonate) can be supplied in high purity and dissolve hydrophobic proteins better [6, 9, 27, 29, 34].

In addition, reducing agents are essential for reducing the disulfide bonds to allow the complete unfolding of many proteins as well as to reduce and prevent the reoxidation of protein samples [3, 12]. Originally, 2-mercaptoethanol was used to reduce proteins in 2-DE , but it is not recommended because it is not only required at high concentrations, but can also produce artifacts from its impurities [19, 31]. 2-Mercaptoethanol has been replaced by dithiothreitol (DTT) or dithioerythritol (DTE), which are used at concentrations ranging from 20 mM to 100 mM [9, 32]. Tributyl phosphine (TBP), which is an uncharged agent and can be used at low concentrations like 2 mM, unlike the sulfhydryl reductants such as DTT and mercaptoethanol, has recently been reported to enhance the dissolution of proteins [10]. However, its use is not recommended on account of its low solubility and poor stability [9].

This study reports the optimal concentrations of the compounds to dissolve the cytosolic proteins of *Streptomyces peucetius*, which have a wide range of protein properties. In order to determine the optimal composition of the IEF rehydration buffer, urea and thiourea, CHAPS, and DTT for 2-DE were combined on commercially supplied IPG gel strips. The optimized rehydration buffer could substantially improve the solubility and resolution of the protein mixtures derived from *Streptomyces peucetius* on 2-DE. It is believed that these results can be applied to the analysis of other bacterial proteomes.

MATERIALS AND METHODS

Materials

The IPG strips and buffers were purchased from Amersham Biosciences. The acrylamide was purchased from Bio-Rad (Hercules, CA, U.S.A.). The thiourea was purchased from Fluka (Buchs, Switzerland). All other chemicals were obtained from Sigma-Aldrich and were of analytical grade (St. Louis, MO, U.S.A.).

Growth Condition

The *S. peucetius* subsp. *caesius* ATCC 27952 was obtained from KCTC (Korean Culture and Tissue Collection). The strain was precultivated with the R5 medium [13] and cultivated in 250-ml Erlenmeyer flasks on an orbital shaking incubator (230 rpm) at 30°C in a defined medium [4].

Protein Extraction

The mycelial broth was harvested by centrifugation at 2,840 ×*g* for 2 min at 4°C. The cell pellet was washed twice with PBS (phosphate-buffered saline), and centrifuged at 2,840 ×*g* and 4°C for 5 min. The obtained cell pellets were stored frozen at –70°C until needed. The cells were disrupted with a cold sonication buffer, and all the disrupted cells were centrifuged at 16,000 ×*g* and 4°C for 20 min (Eppendorf, U.S.A.) to obtain the soluble protein fraction. The concentrations of proteins were determined using the Bradford method (Bio-Rad, Hercules, U.S.A.) [1].

Two-Dimensional Gel Electrophoresis

The protein samples were mixed with various rehydration buffers. For isoelectric focusing (IEF) as the first dimension, 18 cm IPG strips with a linear pH gradient from 4 to 7 (Amersham Biosciences) were rehydrated actively with 40 µl of the protein sample solution for 12 h using IPGphor (Amersham Biosciences) set at 30 V. IEF was carried out to a total of 87,000 Vh with a maximum voltage of 5,000 V. The focused strips were stored at –70°C until needed. After IEF, the IPG strips were equilibrated. The first equilibration was carried out for 15 min in an equilibration solution of 6 M urea, 2% SDS, 50 mM Tris-HCl, 30% glycerol, a trace of bromophenol blue, and 65 mM DTT (pH 8.8), and the second equilibration was carried out for 10 min in the same equilibration solution with 135 mM

iodoacetamide in place of 65 mM DTT. Separation in the second dimension was performed using 12.5% polyacrylamide gels in a Bio-Rad electrophoresis system. The running condition for each gel was 5 mA for 30 min, which was followed by 10 W until the bromophenol blue dye had migrated off the lower end of the gel. Silver staining was carried out using the method reported by Mortz *et al.* [21]. The silver-stained gel images were scanned with a POWERLook 1100 densitometer (UMAX). Image analysis was carried out using ImageMaster 2D Platinum Software (Amersham Biosciences).

Protein Identification by ESI-MS/MS

The in-gel digested peptides were analyzed by nano LC-ESI-tandem mass spectrometry on an LCQ DecaXP instrument (ThermoElectronics, U.S.A.) [7]. A fused silica capillary column (360 μm OD×100 μm ID) was pulled with a P-2000 laser puller (Shutter Instrument, Novato, CA, U.S.A.) to create a 5 mm tip. For liquid chromatography, the pulled capillary columns (360 μm OD, 100 μm ID, 12 cm long) were packed with 7–9 cm of a POROS 10 R2 5 μm hydrophobic packing material (Agilent, U.S.A.) under a nitrogen gas pressure using a homemade pressure chamber. The samples were then injected onto a C18 packed column. The bound peptides were separated using a gradient program consisting of the following: a 5 min initial isocratic elution with 5% B, followed by a linear gradient 5–70% B in 20 min, 5 min isocratic conditions with 70% B, a linear gradient of 70 to 5% B in 10 min, and 20 min isocratic 70% B to re-equilibrate (A=water-0.1% formic acid, B=100% acetonitrile-0.1% formic acid). The flow was approximately 220 nl/min, and was introduced into the mass spectrometer online.

The tandem mass spectra were obtained from data-dependent MS/MS during capillary LC analysis. The top three most intense ions were selected from the full MS scan. A 3-min dynamic exclusion was used to minimize the acquisition of the redundant MS/MS data.

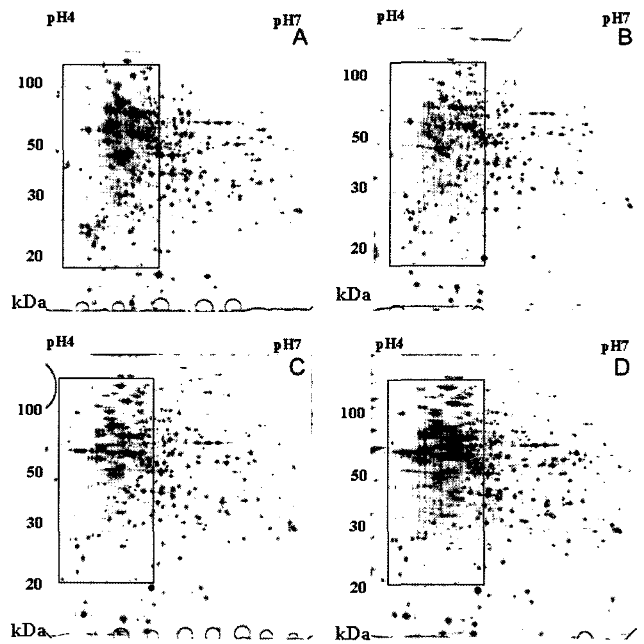

Fig. 1. 2-DE gel analysis of the cytosolic proteins of *S. peucetius* dissolved with (**A**) 9.5 M urea, (**B**) 8 M urea, (**C**) 7 M urea and 2 M thiourea, and (**D**) 5 M urea and 2 M thiourea.
The soluble protein fraction was separated on pH 4–7 linear IPG strips, followed by 12.5% SDS-PAGE gels.

RESULTS AND DISCUSSION

Chaotrope Optimization

Four different rehydration buffers with different urea and thiourea concentrations were tested. Fig. 1 shows the 2-DE images of the proteins prepared by dissolving them in different rehydration buffers. Totals of 1,628, 1,499, and 1,600 spots were detected using the rehydration buffers containing 8.0 M, 9.5 M, and 7 M urea and 2 M thiourea, respectively, whereas 1,739 spots were detected using the rehydration buffer containing 5 M urea and 2 M thiourea. The 8 M urea gave better results than the 9.5 M urea in

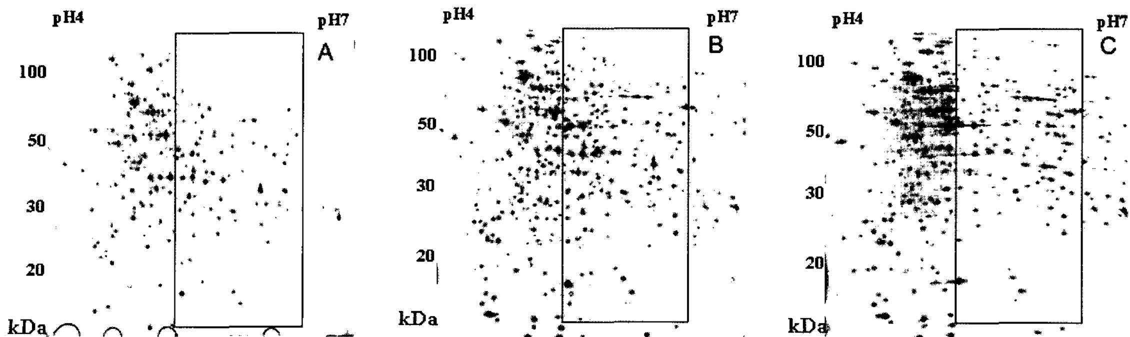

Fig. 2. Effect of CHAPS on proteome dissolution.
2-DE gel analysis of the cytosolic proteome of *S. peucetius* dissolved with (**A**) 0% CHAPS, (**B**) 2% CHAPS, and (**C**) 4% CHAPS.

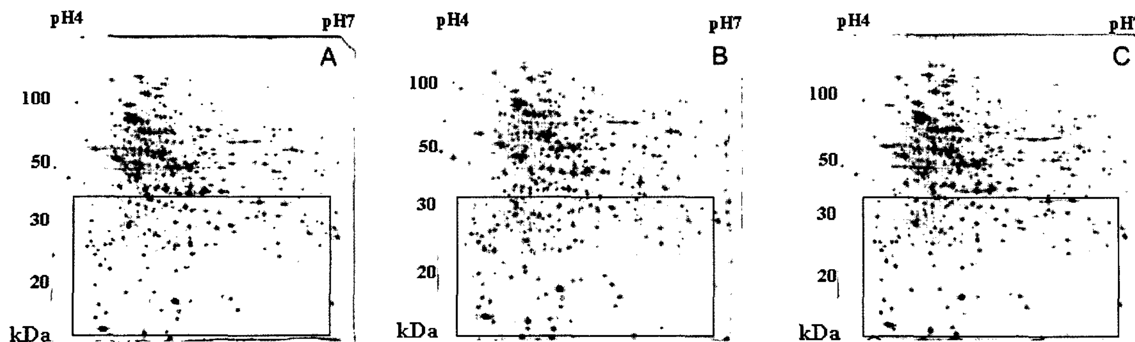

Fig. 3. The effect of DTT on proteome dissolution.
2-DE gel analysis of the cytosolic proteome isolated from *S. peucetius*. (**A**), (**B**), and (**C**), treatments with 25 mM DTT, 50 mM DTT, and 100 mM DTT, respectively.

terms of the number and distinct shape of the detectable spots. The addition of thiourea and urea to the rehydration buffer produced more spots in the p*I* range >5 than the urea alone, but with low quality. However, multiple and unresolved spots appeared in the acidic region <p*I* 5.5, even though the same protein source and amount of protein (40 μg *per* each gel) had been applied. Heuristic clustering analysis, which is a method used to blindly classify similar gels into two or more classes, showed that the 2-DE gels prepared from the rehydration buffers containing 9.5 M urea, 8 M urea, and 7 M urea and 2 M thiourea clustered on the same branch and only the 2-DE gel from the rehydration buffer containing 5 M urea and 2 M thiourea clustered on a different branch (data not shown). This was mainly attributed to the presence of thiourea, which is useful for dissolving hydrophobic and high MW proteins, but is associated with a streaking phenomenon, particularly in the acidic region. Therefore, it was concluded that the single use of more than 8 M urea was better than any combination of urea and thiourea. This concentration produced a better solubility, resolution, and clear background, even though a combination of urea and thiourea could resolve many more proteins. On the other hand, 8 M urea was found to be better than 9.5 M urea, because a higher urea concentration can cause some proteins to precipitate near the anode.

Detergent Effect

Chaotropes denature proteins by exposing the hydrophobic residues of the proteins. These denatured proteins need to be maintained in the dissolved state during IEF. Therefore, among several detergents available, zwitterionic sulfobetaine CHAPS was selected and tested from 0% to 4% to determine if it could better dissolve the proteins during IEF (Fig. 2). In the absence of CHAPS, there were approximately 1,125 detectable spots, whereas more than 2,000 spots were detected and visualized in the presence of 2% or 4% CHAPS. The presence of CHAPS gave a better resolution in the 2-DE patterns. Although 4% CHAPS resolved the highest number of detectable spots, it produced a horizontal streaking phenomenon, which decreased the number of dissolved proteins over pH 5. This demonstrates that 2% CHAPS

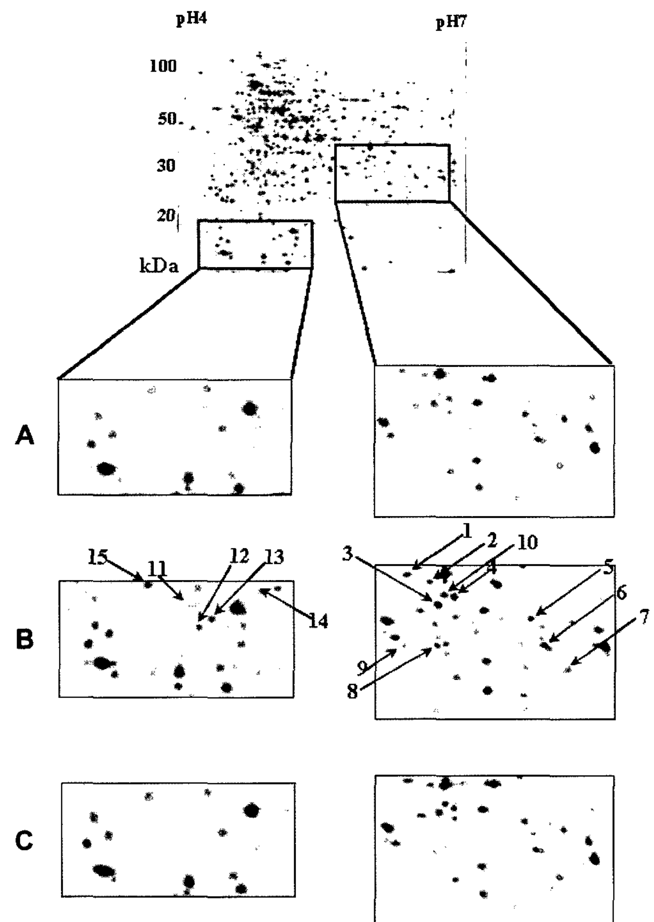

Fig. 4. Enlarged view of some sections from pH 4–7 linear gradient 2-DE gels.
The cytosolic proteome of *S. peucetius* was extracted with (**A**) 8 M urea, 2% CHAPS, and 25 mM DTT, (**B**) 8 M urea, 2% CHAPS, and 50 mM DTT, and (**C**) 8 M urea, 2% CHAPS, and 100 mM DTT, respectively.

can generate a 2-DE pattern of a large number of well-resolved proteins without the streaking phenomenon by maintaining the proteins in the dissolved state.

Effect of the Reducing Agent

The 2-DE patterns were compared using rehydration buffers containing 25 to 100 mM DTT (Fig. 3). It was found that the protein spots became clearer and better resolved with increasing DTT concentration. On the other hand, there was no significant difference in the number of spots detected. In this experiment, the addition of 50 mM DTT to the rehydration buffers resulted in the visualization of a larger quantity of resolved spots, particularly located in low molecular weight and over pH 5 regions (Fig. 4). Therefore, the rehydration buffer needs to contain more than 50 mM DTT to dissolve the *Streptomyces* cytosolic proteome.

Analysis of Low Abundant Proteins of *Streptomyces peucetius*

This study evaluated a combination of various chaotropic agents, detergents, and reducing agents to help dissolve the cytosolic protein fraction from the *Streptomyces.* A rehydration buffer containing 8 M urea, 2% CHAPS, and 50 mM DTT was best in terms of the number and shape of the resolved spots, degree of streaking, reproducibility, and resolution of the 2-DE gel images. Interestingly, the optimized rehydration buffer enhanced the quantity and quality of the protein spots detected in the 2-DE gels, particularly in the low molecular weight region (Fig. 4). Minor proteins could be detected under these optimized conditions, and identified using mass spectrometry. Xcorr is a measure of how well the theoretical spectrum cross-correlates with the observed spectrum. Most of the proteins were identified unambiguously, because identification was defined successfully when two or more peptides with Xcorr was ≥1.6 for singly-charged, 2.2 for double-charged, and 3.0 for triple-charged peptides. Fig. 5 gives an example of protein identification. The indicated spot (No. 10) shown in Fig. 4 was excised and digested with trypsin. The double-charged precursor ion *m/z*=799.7 was selected and subjected to ion trap MS/MS fragmentation. TurboSEQUEST determined the "y" ion sequence of the peptide to be STDSATFGSL(I)VI(L)D, which was used to match the protein to the putative two-component response regulator, SP4727, from the local *S. peucetius* database. This approach enabled the identification of fifteen low abundant protein spots. Table 1 lists the individual proteins identified, the gene name, theoretical molecular weight and p*I*, sequence coverage, and the number of matched sequenced peptides. The number of peptide sequences varied by at least 2 to 11 per spot. Two proteins were identified on spot No. 8, which was attributed to the spot mixture localized in the same p*I* and molecular weight region. The identified proteins were grouped into several categories such as cell division, fatty acid synthesis, protection response, cofactor biosynthesis, and protein translation and modification, *etc.*

In conclusion, the rehydration buffer, which was composed of 8 M urea, 2% CHAPS, 50 mM DTT, and 0.5% pH 4–7 Ampholine, was optimized to obtain a better 2-DE pattern that could be used to visualize more than 2,000 spots. The use of urea on its own was more effective in enhancing the number of spots and image resolution as well as decreasing the streaking phenomenon than the use of combined urea

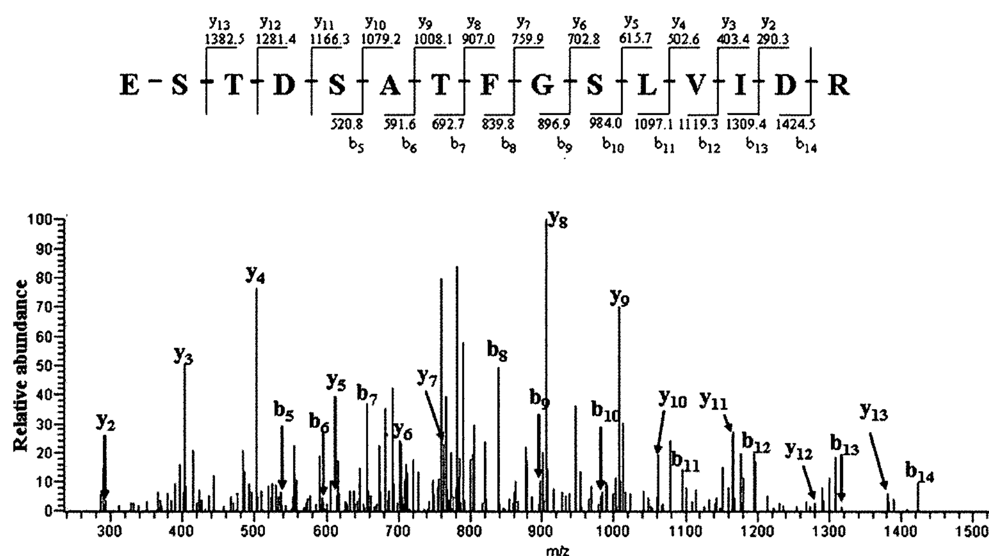

Fig. 5. Ion trap MS/MS spectrum obtained from the spot No. 10 shown in Fig. 4.
The precursor was a doubly charged ion at *m/z*=799.7. This spot was matched to the putative two-component response regulator from the local *S. peucetius* database.

Table 1. Identification of detectable proteins from 2-DE gels, following in-gel trypsin digestion and analysis of tandem electrospray ionization mass spectrometry.

Spot No.	Gene name	Identified protein	Theortical MW/p*I*	Sequence coverage (%)	No. of sequenced peptides that were matched
1	SP2452	Oxidoreductase, putative oxidoreductase	20.20/10.15	16.7	4
2	SP1964	Transcription factor	28.73/ 5.63	26.2	6
3	SP3946	Putative dihydrodipicolinate synthase	24.09/ 6.13	30.7	5
4	SP6490	Phosphate ABC transport system ATP-binding protein	29.06/ 5.72	79.4	11
5	SP3117	Glutamate uptake system ATP-binding protein	34.74/ 7.88	9.27	2
6	SP3541	Putative inhibitor of KinA	21.77/ 5.08	23.8	3
	SP0786	Unknown	23.34/ 6.62	11.0	2
7	SP4167	Putative oxidoreductase	24.82/ 6.25	16.4	3
8	SP3722	Putative 3-oxoacyl-[acyl-carrier protein] reductase	31.12/ 8.9	12.3	3
	SP2257	Putative transcriptional regulator	26.00/ 5.70	16.5	3
9	SP6641	Putative oxidoreductase	27.05/ 5.60	14.9	3
10	SP4727	Two-component response regulator	29.80/ 5.28	7.3	3
11	SP3387	Putative phage inhibition, colicin resistance and tellurite	18.68/ 5.62	8.8	2
12	SP1484	Putative ribosome-binding factor	16.54/ 4.87	17.3	2
13	SP6265	Putative riboflavin synthase beta subunit	28.21/ 4.94	27.1	5
14	SP4635	Peroxidase	19.03/ 5.30	19.2	2
15	SP1111	Phosphatidylethanolamine binding	18.93/ 4.78	29.8	3

and thiourea. In addition, 2% CHAPS and 50 mM DTT produced good quality 2-DE gel images. An enhanced protocol for dissolving the cytoplasmic proteome of *Streptomyces* was developed, which allowed 15 novel tiny spots to be analyzed by mass spectrometry. This method can be applied to an enhanced optimized protocol to construct a 2-DE reference map and analyze the differentially expressed proteome of *S. peucetius* for a study of its cell physiology and regulation.

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