Nano-scale Proteomics Approach Using Two-dimensional Fibrin Zymography Combined with Fluorescent SYPRO Ruby Dye

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In general, a SYPRO Ruby dye is well known as a sensitive fluorescence-based method for detecting proteins by one- or two-dimensional SDS-PAGE (1-DE or 2-DE). Based on the SYPRO Ruby dye system, the combined two-dimensional fibrin zymography (2-D FZ) with SYPRO Ruby staining was newly developed to identify the Bacillus sp. proteases. Namely, complex protein mixtures from Bacillus sp. DJ-4, which were screened from Doen-Jang (Korean traditional fermented food), showed activity on the zymogram gel. The gel spots on the SYPRO Ruby gel, which corresponded to the active spots showing on the 2-D FZ gel, were analyzed by a matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometric analysis. Five intracellular fibrinolytic enzymes of Bacillus sp. DJ-4 were detected through 2-D FZ. The gel spots on the SYPRO Ruby dye stained 2-D gel corresponding to the active spots showing on the 2-D FZ gel were analyzed by MALDI-TOF MS. Three of the five gel spots proved to be quite similar to the ATP-dependent protease, extracellular neutral metalloprotease, and protease of Bacillus subtilis. Also, the extracellular proteases of Bacillus sp. DJ-4 employing this combined system were identified on three gels (e.g., casein, fibrin, and gelatin) and the proteolytic maps were established.

Introduction

Bacillus species are well known for producing a variety of extra- and intracellular proteases, such as alkaline proteases and neutral metalloproteases, into the culture medium (Kim et al., 1996; Kim and Choi, 2000); whereas, at least two intracellular proteases are produced within Bacillus species (Hirushi and Kadota, 1976; Strongin et al., 1978). With regard to many fibrinolytic enzymes that were isolated from Bacillus strains, Sumi et al. reported that a potent nattokinase was purified from natto, a traditional fermented food in Japan (Sumi et al., 1987; Sumi et al., 1990). Kim et al. (1996) isolated subtilisin Carlsberg from the Bacillus sp. strain CK 11-4 (Chungkook-Jang). Recently, we purified and characterized the subtilisin DJ-4 from the Bacillus sp. strain DJ-4 (Doen-Jang, traditional fermented foods in Korea) (Kim and Choi, 2000). It also showed that the activity of subtilisin DJ-4 was 2.2 and 4.3 times higher than those of subtilisin BPN' and subtilisin Carlsberg, respectively.

Zymographic techniques are used to detect proteolytic enzymes following electrophoretic separation in gels (Kim et al., 1998; Choi and Kim, 1999; Kim and Choi, 1999; Choi and Kim, 2000 and 2001). These methods are based on a SDS-polyacrylamide gel, which co-polymerized with the protein substrate that is degraded by the proteases that are restored during the incubation period in the enzyme reaction buffer after the electrophoretic separation. Activities in zymogram gels are visualized as clear bands, where active bands have proteolytically degraded the substrate in the gel. Zymography is one of the most important tools for protein identification at nanogram quantities.

A SYPRO Ruby dye was developed as the new fluorescent staining system for proteomic applications, and provided high sensitivity that matched the silver staining (Lopez et al., 2000; Steinberg et al., 2000; Yan et al., 2000; Lauber et al., 2001). It was also a permanent stain that is composed of ruthenium-based metal chelate that noncovalently interacts with proteins (Berggren et al., 1999; Steinberg et al., 2000) and is less

Keywords: Bacillus, Proteomics, SYPRO Ruby, Two-dimension, Zymography
affected by the presence of nonprotein components that are present in the gel (Malone et al., 2001). This SYPRO Ruby dye also allows one-step, low background staining of proteins in gels without resorting to lengthy destaining steps. Also, it can be visualized by ultraviolet light of about 302 nm (UV-B transilluminator) or visible light of about 473 nm (Steinberg et al., 2000; Berggren et al., 1999 and 2000). Furthermore, it does not modify proteins covalently and does not interfere with mass spectrometric analysis (Yan et al., 2000; Malone et al., 2001). This SYPRO Ruby dye system is more compatible with in-gel digestion than other dyes (Valdes et al., 2000). Also, it could be useful to directly identify the proteins by mass spectrometric analysis (Valdes et al., 2000; Malone et al., 2001).

In this paper, we describe the determination of the activities of intracellular fibrinolytic enzymes from Bacillus sp. DJ-4 using the proteomic technique that was combined with two-dimensional fibrin zymography (2-D FZ) (Choi et al., 2001a) and with the SYPRO Ruby staining system. Also, our goal was to separate and characterize various proteases with high resolution and reproducibility using a new system, the combination of 2-D FZ and SYPRO Ruby dye.

Materials and Methods

Materials Bovine fibrinogen, thrombin, and ultra-pure urea were purchased from Sigma (St. Louis, USA). Ammonium persulfate, TEMED, Triton X-100, trypsin, Coomassie Brilliant Blue (CBB), and bromophenol blue were available from Sigma. Ampholytes (pH ranges of 3 to 10 and 5 to 7), pre-stained protein molecular weight standards, and the SYPRO Ruby Protein Gel Stain were purchased from Bio-Rad (Hercules, USA). All of the other chemicals were of analytical grade.

Bacterial strain and protease preparation The bacterial strain that was used in this study was isolated on the fibrin plate from Doen-Jang (a Korean traditional fermented food) and identified as Bacillus sp. DJ-4 from the Korean Collection for Type Cultures (KCTC). The cells were grown at 37°C in a tryptic soy broth (TSB, Difco, Detroit, USA) for 2 d. The cells were then precipitated by centrifugation at 10,000 × g for 10 min. The supernatant that was used for the assay was an extracellular protease. The cells were washed twice with 50 mM of an ice-cold Tris-Cl (pH 7.4) and sonicated five times on ice at the 20 amplitude microns power setting (Soniprep 150, Gallenkamp) for 2 min. The sonicated extract was centrifuged at 12,000 × g for 15 min at 4°C. The supernatant solution was used as an intracellular protease. The protein concentration was determined according to Bradford’s method (Bradford, 1976) using bovine serum albumin (BSA) as the standard.

First dimension An IEF gel containing 4 M urea was prepared, based on the technique described by the groups of Robertson (Robertson et al., 1987) and Kim (Choi et al., 2001a), with minor modifications. The IEF gel was made as described in Table 1. The samples (100 µg) were diluted with the IEF sample buffer (2 times), which consisted of 4 M urea, 2% 5-7 ampholyte, 0.4% 3-10 ampholyte, 30% glycerol, and 0.01% bromophenol blue. The cathode and anode solutions used were 25 mM NaOH and 10 mM H3PO4, respectively. Electrophoresis was carried out in a cold room (at 4°C) for 1 h at 100 V, 1 h at 150, and 1.5 h at 200 V constant voltage, then increased to 250 V constant voltage for an additional 6 h.

Zymography The casein (Raser et al., 1995), fibrin (Kim et al., 1998), and gelatin (Kleiner and Stetler-Stevenson, 1994) zymograms were carried out as described previously (Choi et al., 2001b). The separating gel solution (12%, w/v) was prepared in the presence of casein, fibrinogen, and gelatin (0.12%, w/v). In the case of the fibrin gel, 100 µl of thrombin (10 NIH units/ml) was added in the fibrinogen solution to make a soft fibrin. The samples (1.0 µg) were diluted with a zymogram sample buffer (5 times), which consisted of 0.5 M Tris, pH 6.8, 10% SDS, 20% glycerol, and 0.03% bromophenol blue. After electrophoresis in a cold room (at 12 mA constantly), the gel was incubated for 30 min at room temperature on a rotary shaker in 50 mM Tris-Cl (pH 7.4), which contained 2.5% Triton X-100. The gel was washed with distilled water for 30 min to remove Triton X-100, then incubated in a zymogram reaction buffer (30 mM Tris-Cl, pH 7.4, and NaNO3) at 37°C for 12 h. The gel was stained with Coomassie blue for 1 h, then destained. The digested bands were visualized as the non-stained regions of the zymogram gel.

Schematic two-dimension (SDS-PAGE and fibrin zymography) For further study, 2-D SDS-PAGE and 2-D SDS-FZ were performed (Robertson et al., 1987; Choi et al., 2001a). The IEF gel, which carried out the first gel running, was applied to both SDS-PAGE and SDS-FZ gels. After performing IEF electrophoresis, the gel slice was equilibrated with the buffer solution (62.5 mM Tris, pH 6.8, 2.3% SDS, and 10% glycerol) for 30 min. The equilibrated gel slices were placed on the stacking gel of the SDS-PAGE and fibrin gels to perform the electrophoresis. The SDS-FZ was carried out as previously described.

SYPRO Ruby staining After the 2-D electrophoresis, the 2-D FZ and 2-D gels were stained with Coomassie Brilliant Blue and SYPRO Ruby, respectively. The 2-D gel was rinsed with three changes of distilled water for 5 min, then fixed in 40% methanol-10% acetic acid for 30 min (Lopez et al., 2000; Steinberg et al., 2000; Yan et al., 2000). The gel was placed in the SYPRO Ruby staining solution. It was necessary to cover the container with

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (ml)</th>
</tr>
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<tbody>
<tr>
<td>Acrylamide-bisacrylamide (30%-0.8%)</td>
<td>2.5</td>
</tr>
<tr>
<td>Urea</td>
<td>2.4 g</td>
</tr>
<tr>
<td>Glycerol (50%)</td>
<td>2.4</td>
</tr>
<tr>
<td>3-10 Ampholyte (40%)</td>
<td>0.6</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1.9</td>
</tr>
<tr>
<td>10% (w/v) ammonium persulfate</td>
<td>0.05</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.02</td>
</tr>
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Table 1. Preparation of IEF gel containing 4 M urea

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</tr>
</tbody>
</table>

Total volume 10
Enzymatic digestion of proteins in-gel

Enzymatic digestion was basically performed as previously described (Valdes et al., 2000). The spots on the gel were excised with a razor, placed in an Eppendorf tube, and washed three times with water to remove the methanol and acetic acid. Next, the gel was mashed with a glass spatula, then dried in a speed-vac vacuum centrifuge apparatus for 30 min at room temperature. The dried samples were reconstituted in 20 µl of 25 mM ammonium bicarbonate (pH 8.5) containing 0.0125 µg/µl of trypsin and incubated overnight at 37°C. After enzymatic digestion, the supernatant was removed and the resultant peptides were extracted by adding 20 µl of 0.1% trifluoroacetic acid (TFA)-50% acetonitrile (ACN) (Merck, Darmstadt, Germany). The extraction was repeated twice with the same solution. In the final extraction, 30 µl of 100% ACN were added to dehydrate the gel completely (which turned white). Subsequently, the extract solutions were pooled and dried in a speed vac vacuum. Finally, the dried samples were dissolved in 20 µl of a solution of 0.1% TFA-ACN (2:1, v/v).

MALDI-TOF mass spectrometric analysis and database search

All of the analyses were performed using a PE Biosystems MALDI-TOF Voyager-DE STR Mass Spectrometer. The peptide mixtures were analyzed using a saturated solution of α-cyano-4-hydroxycinnamic acid. For the database searches, the peptides were selected in the mass range of 900-3000 Da and evaluated using the ExPasy server on the Worldwide Web at http://prospector.ucsf.edu/.

Extracellular protease map of Bacillus sp. DJ-4 using 2-D zymography

To study the enzymatic specificity of Bacillus sp. DJ-4, the lane that carried out the first IEF gel running was divided into four parts of gel slices with the same width. The gel slices were then applied on the SDS-PAGE gel and three different substrate gels (casein, fibrin, and gelatin), as previously described.

Results

Identification of the intracellular fibrinolytic enzymes (IFEs) from Bacillus sp. DJ-4 on the fibrin zymography

We established the fibrin zymographic technique (Kim et al., 1998) that detects plasmin-like fibrinolytic enzymes within nanogram quantity. This FZ gel was applied to Bacillus sp. DJ-4 that was isolated from Doen-Jang (a Korean traditional fermented food). As shown in Fig. 1, four intracellular fibrinolytic enzymes (IFEs) were detected by the FZ gel.

Visualization of spots in 2-D gels

For further study, 2-D SDS-PAGE and 2-D SDS-FZ were performed. The IEF gel, which was carried out the first gel running, was applied to both SDS-FZ (Fig. 2A) and SDS-PAGE (Fig. 2B), as described in Materials and Methods. Five spots were detected on the 2-D SDS-FZ gel (Fig. 2A). Also, as shown in Fig. 2B, the 2-D SDS-PAGE gel was visualized using the SYPRO Ruby dye. Five spots (No. 1-5) on the SYPRO Ruby dye stained gel that correspond to the 2-D SDS-FZ gel (A) were selected for the MALDI-TOF mass spectrometric analysis.
MALDI-TOF mass spectrometric analysis As shown in Fig. 2B, five making spots that showed fibrinolytic activity on the 2-D SDS-FZ were excised and digested with trypsin. The resulting peptides were extracted and analyzed by a MALDI-TOF mass spectrometric analysis. The trypsin-digested peptide fingerprinting data were evaluated using the ExPasy server at http://prospector.ucsf.edu/. As shown in Table 2, spots 1, 2, and 5 were quite similar to the ATP-dependent protease, extracellular neutral metalloprotease, and protease of Bacillus subtilis, respectively. On the other hand, spots 3 and 4 showed little similarity with the known proteins in the protein database.

Identification of the extracellular proteases (EPs) from Bacillus sp. DJ-4 on the three zymogram gels The EPs in the culture supernatant from Bacillus sp. DJ-4 were analyzed using the three different substrate (e.g., casein, fibrin, and gelatin) gels (Fig. 3). As shown in Fig. 3B and C, the same pattern of molecular weight of seven EPs was detected on the fibrin and gelatin zymogram gels. On the other hand, two EPs (EP6 and EP7) were not detected on the casein gel (Fig. 3A).

Enzymatic maps using 2-D zymography The enzymatic specificity of the extracellular proteases (EPs) of Bacillus sp. DJ-4 was studied using three different substrate (e.g., casein, fibrin, and gelatin) gels. These results are summarized in Fig. 4. Namely, the 2-D SDS-PAGE gel was visualized using the SYPRO Ruby staining system (Fig. 4A). When the three differentzymographic maps of EPs from Bacillus sp. DJ-4 are weed, the same enzymatic maps on the fibrin and gelatin gels then show the activity (Fig. 4B and C). On the other hand, three spots with low molecular weight were not visualized on the casein gel (Fig. 4D).

Discussion

A major focus of proteomics is the development of methods and technologies that permit the integrated analysis of numerous biomolecules in total proteome from various sources. With the rapid development of proteomics technologies, as well as a great deal of current interest in the application of proteomics to study the various proteolytic enzymes, our knowledge of how proteolytic maps vary in Bacillus sp. DJ-4 is accruing at an impressive pace. Recently, the total genome sequence of Bacillus subtilis enabled us to establish a theoretical two-dimensional (2-D) zymographic map.

Zymography is a versatile two-stage technique that involves protein separation by electrophoresis, followed by the detection of proteolytic activity (Kim et al., 1998; Kim and Choi, 1999; Choi and Kim, 1999, 2000, and 2001; Choi et al., 2002). This technique is routinely used to identify the protease activity in polyacrylamide gels under nonreducing conditions.

To visualize the proteins on gels, the SYPRO Ruby dye system was used (Lopez et al., 2000; Steinberg et al., 2000; Yan et al., 2000; Lauber et al., 2001). This dye readily visualizes a wide range of proteins in 2-D gels. The dye allows one-step, low background staining of proteins in gels without resorting to lengthy destaining steps (Berggren et al., 1999, 2000; Steinberg et al., 2000). The merits of zymography and the SYPRO Ruby dye led us to design an advanced proteomic technique (Figs. 2 and 4). The IFE and EFE maps in Bacillus sp. DJ-4 were established by this advanced system. Four bands (IFE1-4) were detected on the 1-D zymography gel (Fig. 1). However, the IFE-4 band was separated into two spots (Nos. 4 and 5) using the 2-D zymography (Fig. 2). Three spots (Nos. 1, 2, and 5) were then identified as ATP-dependent protease, extracellular neutral metalloprotease, and protease, respectively (Table 2).
particular, the extracellular neutral metalloprotease seems to be primarily produced as an intracellular protein, and then secreted into the culture broth after some modifications.

Also, three different zymogram maps (e.g., casein, fibrin, and gelatin), which could be used to identify the specific proteases, were also elucidated in the same bacterium (Fig. 4). Through 1- and 2-D zymogram gels, the enzymatic pattern on the casein gel showed different activity than the others did (Figs. 3 and 4).

In conclusion, the 2-D zymography is a powerful tool for the analysis of proteolytic enzymes among the total proteomes of Bacillus sp. DJ-4. It is suitable for the assay of individual enzymatic activities because active complex protein mixtures can be separated according to the differences in their isoelectric points and molecular weights. Furthermore, different substrates, such as casein (Raser et al., 1995), fibrin (Kim et al., 1998), gelatin (Kleiner and Stetler-Stevenson, 1994) (Figs. 3 and 4), and protein inhibitors, can be used to not only determine the type of proteolytic enzymes, but also to understand the functional proteins against a specific substrate.

We analyzed these three identified proteins (ATP-dependent protease, extracellular neutral metalloprotease, and protease) on the 2-D FZ and the specific enzymes that were detected on the three different substrates using the combined two-dimensional zymography (2-D Z) with the SYPRO Ruby staining system.

**Acknowledgments** This work was supported in part by a research grant from the Korean Ministry of Science and Technology (Critical Technology 21 [M1-0015000020-01A210001910]) and KGS 1130311.

**References**


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**Table 2.** Protein identity by MALDI-TOF

<table>
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<tr>
<th>Spot No.</th>
<th>Protein identity</th>
<th>Accession*</th>
<th>Molecular mass (kDa)</th>
<th>pI value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ATP-dependent protease</td>
<td>NP 390699</td>
<td>60.41</td>
<td>6.3</td>
</tr>
<tr>
<td>2</td>
<td>Extracellular neutral metalloprotease</td>
<td>NP 389353</td>
<td>56.50</td>
<td>7.2</td>
</tr>
<tr>
<td>5</td>
<td>Protease</td>
<td>NP 389414.1</td>
<td>34.84</td>
<td>8.9</td>
</tr>
</tbody>
</table>

*Accession number for the GenBank protein sequence data library


