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Superoxide dismutase isozyme detection using two-dimensional gel electrophoresis zymograms

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ABSTRACT

Superoxide dismutases (SODs) are ubiquitous antioxidant enzymes involved in cell protection from reactive oxygen species. Their antioxidant activities make them of interest to applied biotechnology industries and are usually sourced from plants. SODs are also involved in stress signaling responses in plants, and can be used as indicators of these responses. In this article, a suitable method for the separation of different SOD isoforms using two-dimensional-gel electrophoresis (2D-GE) zymograms is reported. The method was developed with a SOD standard from bovine erythrocytes and later applied to extracts from *Stemona tuberosa*. The first (non-denaturing isoelectric focusing) and second (denaturing sodium dodecylsulphate-polyacrylamide gel electrophoresis) dimensions of duplicate 2D-GE gels were stained with either Coomassie brilliant blue G-250 for total protein visualization, or SOD activity (zymogram) using riboflavin/nitroblue tetrazolium. For confirmation, putative SOD activity positive spots were subject to trypsin digestion and nano-liquid chromatography tandem mass spectrometry, followed by searching the MASCOT database for potential identification. The method could separate different SOD isoforms from a plant extract and at least partially maintain or allow renaturation to the native forms of the enzyme. Peptide sequencing of the 2D-GE suggested that the SODs were resolved correctly, identifying the control CuZn-SOD from bovine erythrocytes. The two SODs from *S. tuberosa* tubers were found to be likely homologous of a CuZn-SOD. SOD detection and isoform separation by 2D-GE zymograms was efficient and reliable. The method is likely applicable to SOD detection from plants or other organisms. Moreover, a similar approach could be developed for detection of other important enzymes in the future.

1. Introduction

Superoxide dismutase (SOD, EC 1.15.1.1) is an antioxidant metalloenzyme [1]. The different isozymes are currently classified according to the metal cofactor in their active sites into the four main and broad types of CuZn-SOD, Mn-SOD, Fe-SOD and Ni-SOD [2–5]. SODs have an important role in catalyzing the destruction of superoxide radicals, which are cytotoxic agents to cell membranes, DNA and other biomolecules, to hydrogen peroxide and oxygen [6–8]. In general, SODs have been found in microorganisms, animals and plants. The SODs used as antioxidant agents for applications in medicine, and in the cosmetic, chemical and food industries are currently sourced and extracted (or cloned and recombinant produced) from plants [9]. Furthermore, at least some SOD isoforms in plants are involved in the defense against pathogens and in signaling responses for various stresses. Since the different SOD isoforms in plants show different responses to infection and stress [10–12], the level of expression of specific SOD isozymes in key tissues can be used as an indicator to determine the growth stage or stress/infection circumstances of that plant [13].

Currently several methods have been reported for SOD detection with one of the popular methods being detection by zymograms. This method detects active enzymes across the spectrum of the four broad types of SODs, rather than, for example, immunological detection which often cannot discriminate between enzymically active and inactive forms, and can only detect those isoforms with conserved epitopes. The original zymogram detection method for SODs was created by Beauchamp and co-workers and used native polyacrylamide gel electrophoresis (Native-PAGE) followed by enzymatic staining for SOD activity [14]. There are two combined reactions in the SOD activity assay. The first is the autooxidation from riboflavin (oxidizing agent) and the second is the

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riboflavin/nitroblue tetrazolium (NBT) reduction, which uses NBT as a chromogenic substrate. The SOD enzyme can be determined as an achromatic zone on Native-PAGE because the enzyme inhibits NBT reduction [15], but Native-PAGE does not allow determination of valuable information, such as the subunit molecular weight (M_W), nor discriminate between isozymes with a different M_W or isoelectric point (pI). However, the simultaneous detection of SOD activity and evaluation of their M_W on one dimensional denaturing sodium dodecyl sulphate-PAGE (1D-SDS-PAGE) zymograms cannot separate the different SOD isoforms that share a similar M_W but differ in their pI.

Two-dimensional gel electrophoresis (2D-GE) is one of the important techniques for protein detection and is often coupled with tryptic digestion and mass spectrometry (MS) to identify the resolved protein(s) [16]. Proteins are separated by 2D-GE according to their pI in the first dimension and their apparent M_W (three dimensional size) in the second dimension, using isoelectric focusing (IEF) and denaturing SDS-PAGE, respectively [17]. Consequently, proteins with a different pI but similar M_W, as well as those that differ in apparent M_W, can be resolved even in complex samples, such as different SOD isozymes from a whole organism or tissue extracts [18]. According to 2D-GE theory, the capacity of resolution for the method is up to 10,000 different proteins, and it can detect less than 2 ng per spot [17].

However, due to the denaturing running conditions of 2D-GE (SDS, dithiothreitol and urea plus sample heating) this method is typically unsuitable for SOD (and many other enzymes) zymograms due to the inactivation of the enzyme [16].

Accordingly, in this research, a 2D-GE approach was developed that appears to maintain SOD activity and so it is suitable for zymogram based detection of enzymically active SOD isoforms from tissue extracts. The method combined a non-denaturing IEF first dimension and SDS-PAGE second dimension, before staining duplicate gels with either Coomassie blue or SOD activity. Finally, in-gel trypsin digestion and peptide extraction was coupled with liquid chromatography tandem MS (LC–MSMS) of a known standard (CuZn-SOD from bovine erythrocytes) and an experimental tissue extract (crude total protein extract from the root of the medicinal plant *Stemona tuberosa* Lour.) to evaluate the reliability and usability of the method.

2. Methods and materials

2.1. Isolation and extraction of SOD from S. tuberosa

Fresh tubers of *S. tuberosa* (~3 kg, fresh weight) were purchased from Chatujak market, Bangkok, Thailand, in June 2009. A voucher specimen (BK 244965) is deposited at the Sirindhorn Bangkok Herbarium, Bangkok, Thailand.

The fresh roots of *S. tuberosa* were peeled, cut into small cubes and homogenized in 5 L of extraction buffer (0.1 M NaCl, 20 mM phosphate buffer pH 7.2) at 4 °C. The suspension-solution mixture was stirred at 4 °C overnight before being clarified by centrifugation (10,178 × *g*, 30 min, 4 °C) and harvesting the supernatant. Ammonium sulfate was then added to the supernatant to 90% saturation and left overnight at 4 °C, before harvesting the precipitated material by centrifugation (15,904 × *g*, 30 min, 4 °C), dissolving the precipitate in 400 mL de-ionized water, and dialyzing against 4–5 changes of 1000 mL each of 20 mM phosphate buffer pH 7.2 over 18 h at 4 °C. The dialyzed extract was then freeze-dried to yield the dark brown crude protein extract, from which the IC_{50} value of the SOD preparation was determined as reported [19]. In addition, a commercial preparation of the CuZn-SOD isozyme from bovine erythrocytes (Merck, USA), a homodimer enzyme of 32.50 kDa M_W and a pI of 4.95, was used as a positive control.

2.2. Resolution and detection of SOD isozymes

2.2.1. One dimensional reducing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (1D-SDS-PAGE)

Reducing 1D-SDS-PAGE was run according to the modified method as previously reported [19,20]. Either 12.5 μg (per track of the gel) of the reference pure CuZn-SOD homodimer enzyme from bovine erythrocytes, as a positive control, or 25 μg (per track of the gel) crude protein extract from *S. tuberosa* tubers was mixed with reducing sample buffer (62.5 mM Tris–HCl pH 6.8, 14.4 mM 2-mercaptoethanol, 10%, v/v glycerol, 2%, w/v SDS) and 1% (w/v) bromophenol blue at room temperature and then subjected to duplicate 1D-SDS-PAGE resolution using a 10% or 12.5% (w/v) acrylamide resolving gel for the bovine erythrocyte CuZn-SOD or the crude protein extract from *S. tuberosa*, respectively. After electrophoresis, one of each pair of duplicate gels was stained with Coomassie brilliant blue G-250 to visualize the protein bands, whilst the other, as a SOD zymogram, was washed and stained for SOD enzyme activity (see Section 2.3) to determine the presence of enzymically active SOD.

Crude protein extracts were also mixed with reducing sample buffer or non-reducing sample buffer (62.5 mM Tris–HCl pH 6.8, 10%, v/v glycerol and 1%, w/v bromophenol blue) and applied (25 μg per track of the gel) to a 1D-SDS-PAGE and 1D-Native-PAGE (both 12.5%, w/w gel resolving gel), respectively, for comparison of the SOD activity level.

2.2.2. Non-denaturing two-dimensional polyacrylamide gel electrophoresis (2D-GE)

The reference CuZn-SOD isozyme from bovine erythrocytes (40 μg per gel), and the crude protein extract from *S. tuberosa* (150 μg per gel) were dissolved in lysis solution (40 mM Tris, 4%, w/v 3-(3-cholamidopropyl) dimethylammonio-1-propanesulfonate (CHAPS), 1 mM ethylenediaminetetraaceticacid (EDTA), 2%, v/v immobilized pH gradient (IPG) buffer, 10%, v/v glycerol and 1%, w/v bromophenol blue), vortexed every 30 min for 1–2 h and left on ice as previously reported [21]. Each protein sample was loaded onto duplicate 7-cm, pH 3–6 IPG gel strips (Bio-RAD Laboratories, CA, USA) and left overnight at room temperature (RT). The first-dimensional IEF electrophoresis was performed at 4 °C on a Pharmacia LKB Multiphore II system at 300 V for 1200 Vh and then increasing the voltage step wise to 1000 V for 300 Vh, 5000 V for 4500 Vh and 5000 V for 1000 Vh. After IEF, the IPG strips were equilibrated in equilibration buffer (50 mM Tris–HCl buffer pH 6.8, 6 M urea, 1%, w/v SDS, 30%, v/v glycerol, 1%, w/v dithiothreitol (DTT)) and were alkylated with equilibration buffer containing 2.5% (w/v) iodoacetamide (IAA). Finally, the equilibration IPG strips were separated in the second dimension using the reducing SDS-PAGE and then the duplicate gels stained for protein and SOD activity, respectively, as described in Sections 2.2.1 and 2.3, respectively.

2.3. Gel washing procedures and SOD staining activity assay

After electrophoresis, the 1D-SDS-PAGE or 2D-GE gel was washed as reported previously [22,23]. In brief, the gel was first soaked twice in 100 mL of 25% (v/v) isopropanol in 0.01 M Tris pH 7 for 10 min each at RT to remove the SDS. The gel was then washed twice with 100 mL of 2 μM $ZnCl_2$/0.01 M Tris pH 7 for 10 min each at RT to remove the isopropanol, then twice with 100 mL of 0.1 M Tris pH 7 for 20 min each, and once with 100 mL of 0.01 M Tris pH 7 for 10 min, all at RT.

2.4. SOD isozyme identification by in-gel trypsin digestion and tryptic peptide mass spectrometry analysis

Protein spots on the Coomasie stained 2D-GE that matched the spots on the SOD activity zymogram in the paired duplicate gel