CHAPTER III

Superoxide dismutase isozyme detection using two-dimensional gel electrophoresis zymograms

3.1 INTRODUCTION

Superoxide dismutase (SOD, EC 1.15.1.1) is an antioxidant metalloenzyme.[47] 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.[48-51] 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.[52-54] 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.[55] 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,[36, 56, 57] 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.[58]

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.[59] There are two combined reactions in the SOD activity assay. The first is the autooxidation from riboflavin (oxidizing agent) and the second is the 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,[60] 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).[61] 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.[62] 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.[63] 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.[62] 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.[61]

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*) to evaluate the reliability and usability of the method.

3.2 MATERIALS AND METHODS

3.2.1. 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 $^{\circ}$C. The suspension-solution mixture was stirred at 4 $^{\circ}$C overnight before being clarified by centrifugation (10,178 x g, 30 min, 4 $^{\circ}$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 x 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.[64] 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.

3.2.2. Resolution and detection of SOD isozymes

3.2.2.1 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.[64, 65] 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 an SOD zymogram, was washed and stained for SOD enzyme activity (see section 3.2.3) 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.

3.2.2.2 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.[66] Each protein sample was loaded onto duplicate 7-cm, pH 3–6 IPG gel strips (Bio-RAD laboratories, California, 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 reducing SDS-PAGE and then the duplicate gels stained for protein and SOD activity, respectively, as described in section 3.2.2.1 and 3.2.3, respectively.

3.2.3. Gel washing procedures and SOD staining activity assay.

After electrophoresis, the 1D-SDS-PAGE or 2D-GE gel was washed as reported previously.[67, 68] 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.

3.2.4. 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 were excised and washed three times in 100 μL distilled water before cutting into small pieces. The gel pieces were then washed with 0.1 M NH_4HCO_3 in 50% (v/v) acetonitrile (ACN) at 30 °C for 20 min to de-stain, dried in a Speed Vacuum and incubated in 50 μL of buffer solution (0.1 M NH_4HCO_3/10 mM DTT/1 mM EDTA) for 45 min at 60 °C to reduce the gels. The supernatant was discarded and the gels incubated in 50 μL of 100 mM IAA in 0.1 M NH_4HCO_3 for 30 min at RT in the dark. The supernatant was removed and the gels were washed three times with 50 μL each of 0.05 M Tris-HCl pH 8.5 in 50% (v/v) ACN and then dried in a Speed Vacuum. Tryptic digestion was performed by the addition of 30 μL of trypsin digestion buffer (0.05 M Tris-HCl pH 8.5, 0.1 μg/μL trypsin in 1% (v/v) acetic acid, 10% (v/v) ACN and 1 mM $CaCl_2$) to the excised region of the gel containing the desired spot and incubating at 37 °C overnight. The reaction was stopped by adding 20 μL of 2% (v/v) trifluoroacetic acid and incubating for 30 min at 60 °C, and then the supernatant was harvested. The gels were then extracted three

times with 50 µL 2% (v/v) trifluoroacetic acid and 0.05 M Tris-HCl pH 8.5 containing 1 mM $CaCl_2$ and 2% (v/v) formic acid, and for 10 min at 30 °C on a shaker (1500 rpm), and then sonicated for 5 min. The harvested solutions were pooled and dried in a Speed Vacuum for further characterization by nano-LC-MSMS as detailed in section 3.2.5.

3.2.5. SOD characterization by liquid chromatography tandem mass spectrometry (LC-MSMS).

Each tryptic digested zymogen spot protein (putative SOD enzyme, see section 3.2.4) was mixed with 0.1% (v/v) formic acid before being analyzed by nano liquid chromatography-electrospray ionization quadrupole-time of flight MS (nano-LC-ESI-MSMS) using an EASY-nLCII spectrometer coupled with a MicroTOF QII (Bruker, Germany). The tandem mass spectra of the tryptic peptides were searched from Mascot database as the website below. The precursor and MSMS tolerances were set to ± 1.2 Da and ± 0.6 Da, respectively.

(http://www.matrixscience.com/cgi/search_formpl?FORMVER=2&SEARCH=MIS)

3.3 RESULTS AND DISCUSSIONS

3.3.1 Evaluation of the 2D-GE method using the bovine erythrocyte CuZn-SOD isozyme as a known standard.

The CuZn-SOD from bovine erythrocytes, a known homodimer with a pI of 4.95 and M_W of 32.5 kDa, was used to determine the efficiency of the developed 2D-GE method for the detection of the SOD enzyme. Figure 3.1 shows a representative 1D-SDS-PAGE and 2D-GE resolution gel zymogram of the CuZn-SOD isozyme from bovine erythrocytes after staining for SOD activity (Figure 3.1A and B) or for protein (Figure 3.1C and D). The 1D-SDS-PAGE resolution revealed a strong protein band at

~15–16 kDa (Figure 3.1C) with a weak SOD activity (Figure 3.1A), which presumably represents the monomer (15–16 kDa). However, the intensity of this protein spot is markedly reduced in the 2D-GE (Figure 3.1D, spot 6), and showed no detectable SOD staining activity (Figure 3.1B). This is potentially due to the renaturation of the monomer to homodimeric units.

The 1D-SDS-PAGE gel also revealed three protein bands at around 55–58 and > 66 kDa (Figure 3.1C), at least two of which had a strong SOD activity in the zymogram (Figure 3.1A). These were also potentially found in the 2D-GE as two spots at 55–58 kDa with a pI of 4.95 (Figure 3.1D, spots 1 and 2) that also displayed SOD activity (Figure 3.1B). These may be the homodimer but in native forms, where the abnormal (low) levels of bound SDS reduce the charge density and so accounts for the apparently higher M_W in terms of the gel mobility.[69] If so, although these results show an advantage of the method in that it can maintain the native form of the SOD enzyme (or allow its partial renaturation) even though the second dimension was run under a denaturing condition, it also suggests that the discrimination of isoforms by M_W, and the assignment of M_W from the 2D-GE are both unreliable.

Accordingly, to ascertain if the two large Coomassie blue stained spots on the 2D-GE gel (spots 1 and 2 in Figure 3.1D) were the CuZn-SOD, the spots were cut out, digested by trypsin and the resultant peptide fragment sequences identified by nano-LC-EIS-MSMS were used to search the Mascot database to identify their likely source. Both spots 1 and 2 matched the Cu-Zn SOD from *Bos taurus* with an amino acid match of 59/59, which over the 152 amino acids gives a 38.8% sequence coverage (Figure 3.2A and Table 4.2).

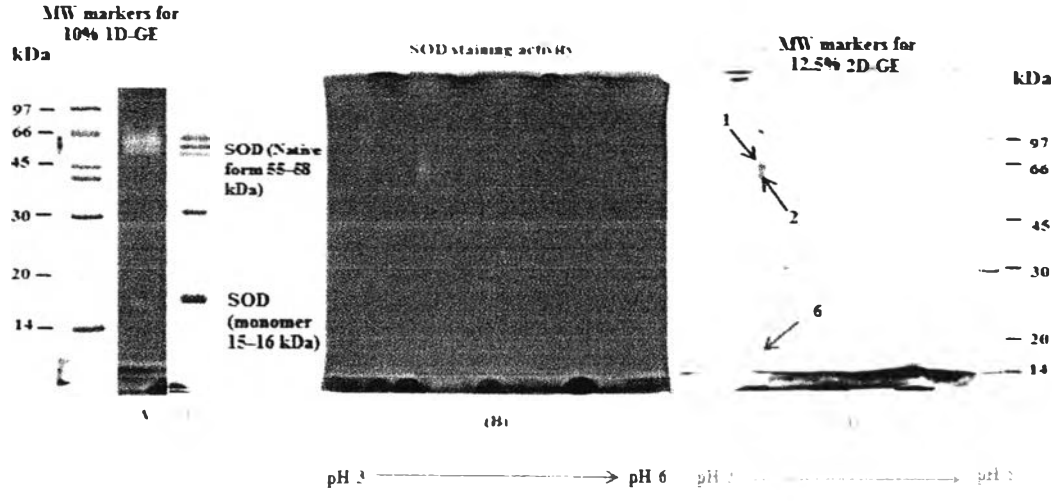

Figure 3. 1 Representative (A, C) 1D-SDS-PAGE and (B, D) 2D-GE of the CuZn-SOD *isozyme (12.5 and 40μg, respectively) from bovine erythrocytes after staining* for (A, B) SOD activity or (C, D) Coomasie blue for protein. Note that the spot appearing at ~30 kDa with pI~4.0 in C and D corresponds to the carbonic anhydrase enzyme

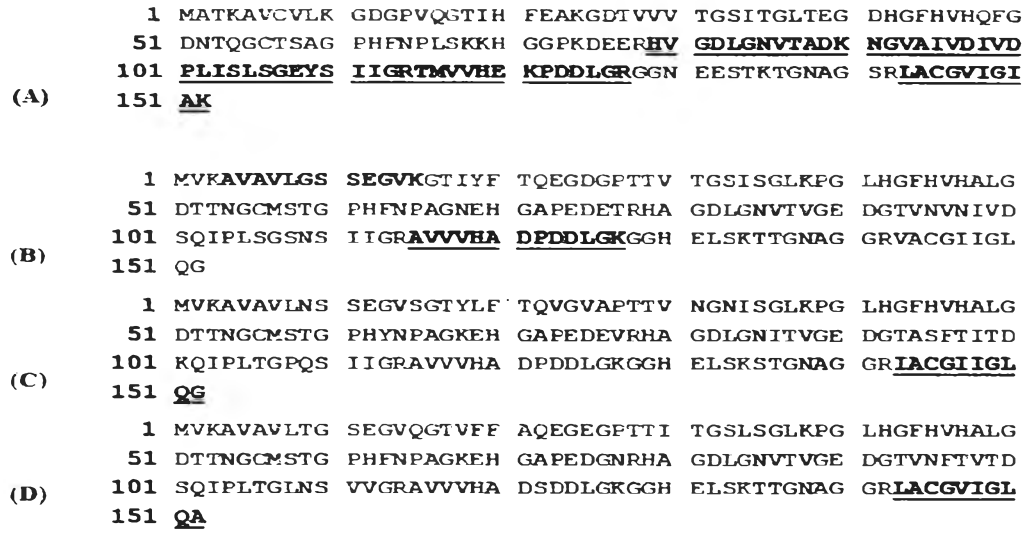

Figure 3. 2 Deduced amino acid sequences of (A) the CuZn-SOD isoforms and (B, C and D) the matches to the tryptic peptide sequences of the SOD isozymes *from a crude protein extract of S. tuberosa tubers. Matching amino acid* sequences (bold) of the tryptic peptides for (A) *B. taurus* CuZn-SOD spots 1 and 2 (see Fig. 1D) with the sequence from the bovine (*Bos taurus*) erythrocyte CuZn-SOD. For peptides from *S. tuberosa*, the putative SOD isoforms matched with the CuZn-SOD sequence from (B) Ananas comosus (Fig. 4D, spot 3), and from (C) *Solanum lycopersicum* and (D) *Zantedeschia aethiopica* (Fig. 4D, spot

3.3.2. SOD isoform separation from a crude protein extract of *S. tuberosa* tubers.

To evaluate the efficiency of this 2D-GE method in real situations, the crude protein extract from *S. tuberosa* tubers was used as a model system. The initial choice of *S. tuberosa* was based upon its SOD activity, since the derived SOD activity was higher (i.e., with lower IC_{50} value in the NBT reduction assay) than that reported in other plants (Table 4.2).

The crude protein extract (150 µg) from *S. tuberosa* was initially resolved by 2D-GE using a pH 3–10 linear IEF strip (Figure 3.3). However, most of the proteins, including those that displayed SOD activity were found to be in the acidic zone resulting in poor resolution. To solve this problem, a narrow range linear IEF strip of pH 3–6 was used in the first dimension IEF to improve the resolution of the individual SOD proteins from each other and from the other non-SOD active proteins. After comparing the spots on the gel stained with Coomassie blue with those tested for SOD activity, three spots that potentially represent different (active) SOD isoforms were revealed. Two of these had an apparently similar M_W of 53 kDa but slightly different pI values at 4.25 and 4.95 (spots 3 and 4 in Figure 3.4D, respectively), while the third was smaller at 38 kDa and more acidic with a pI of 4.0 (spot 5 in Figure 3.4D).

These three SOD-active spots (spots 3–5) were excised from the corresponding Coomassie blue stained 2D-GE, digested with trypsin and then analyzed via nanoLC-ESI-MSMS. However, only spots 3 and 5 could be identified, presumably because the limited data in the plant protein sequence databases prevented matches of the tryptic peptides from spot 4. Those from spot 3 matched

to the CuZn-SOD from *Ananas comosus* at 25/25 amino acids (a coverage of 16.4% over the total 152 amino acids), whilst spot 5 matched to the CuZn-SOD from *Solanum lycopersicum* and *Zantedeschia aethiopica* at 10/10 amino acids (a coverage of 6.6% over the total 152 amino acids) (Figure 3.2C and 3.2D, respectively). The results from the 2D-GE coupled with SOD staining activity, therefore, suggest the presence of at least three different active SODs in *S. tuberosa*. More importantly, the LC-MSMS results reveal the capability of this method in separating two different SOD isozymes from each other (Figure 3.4, spots 3 and 4 with a similar M_W but different pI).

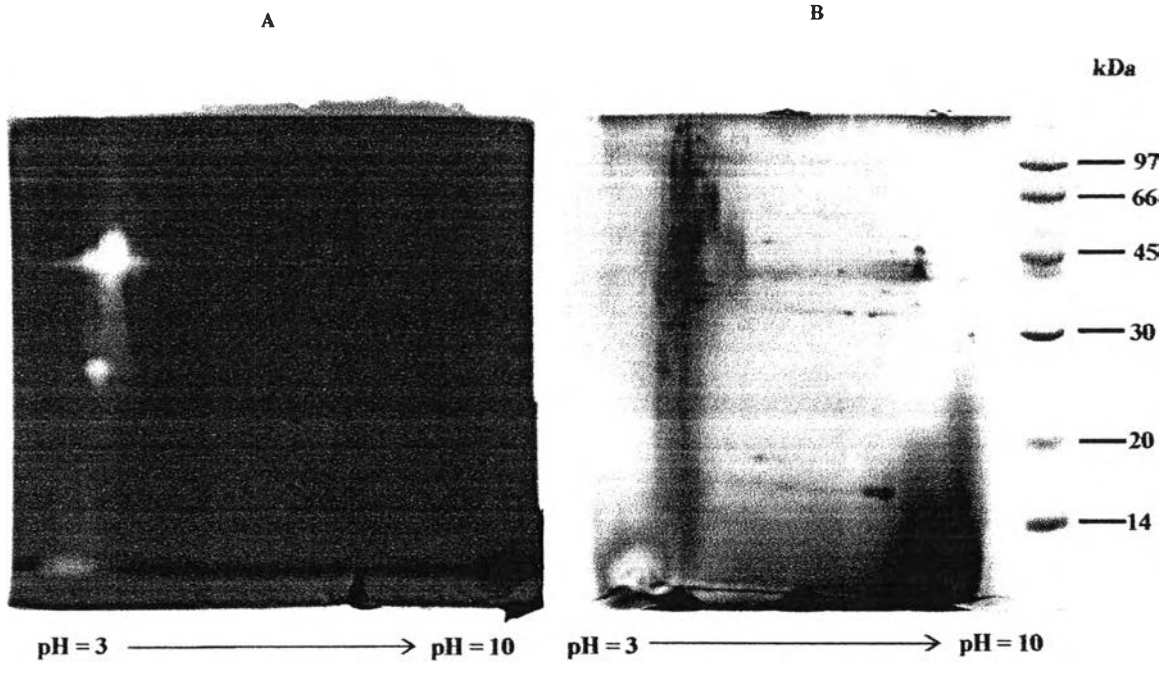

Figure 3. 3 Active SOD isozymes resolved and detected in the crude protein extract from *S. tuberosa*. Crude protein extract (150µg) after 2D-GE resolution with a broad range pH (3–10) IEF strip in the first dimension and stained for (A) SOD activity or (B) with Coomassie blue for protein.

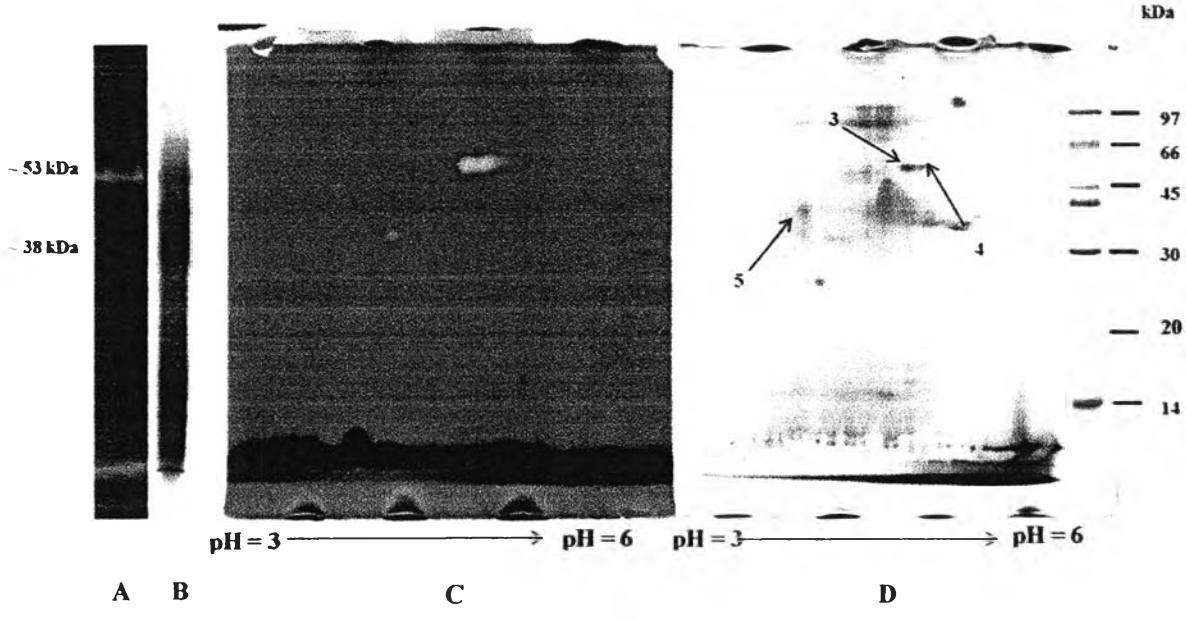

Figure 3. 4 (A, B) 1D-SDS-PAGE and (C, D) 2D-GE resolution of the crude protein extract (40 and 150μg, respectively) from *S. tuberosa* after staining for (A, C) SOD activity and (B, D) total proteins by Coomassie blue.

3.3.3. The key feature for improving the developed 2D-GE method resolution

The 2D-GE method developed for the detection and discrimination of active SOD isozymes used a non-denaturing IEF first dimension (IEF-focusing) and an apparently non-fully denaturing SDS-PAGE second dimension. For the IEF-focusing step, Tris and EDTA were used in the lysis solution and rehydration buffer instead of urea, thiourea and DTT. Since the cell membrane structure is maintained by calcium and magnesium ions, their chelation by EDTA will destabilize the cell membrane, whilst the Tris buffer maintains a stable pH for SOD activity after cell lysis. In addition, the salts obtained from the SOD extraction process can disturb the electrophoresis process giving a poor protein focusing in the IEF. Accordingly, the crude protein extract from *S. tuberosa* was dialyzed against distilled water, but this was still found to be insufficient (data not shown). Increasing the IEF time (Vh) moves the salt ions to the end of the strip and was found to improve the resultant resolution (Appendix A). Thus, the appropriate buffer and prolonged IEF electrophoresis time are important steps to improve the resolution of the non-denaturing IEF-focusing step.

Another step to increase the zymogram resolution was the gel washing steps after the SDS-PAGE to remove the bound SDS before staining for SOD activity. The presence of SOD interferes with the SOD staining activity and produces an irregular purple background on the gel. The reason for this observation remains unknown, although it is possible that SDS might inhibit SOD from binding to and reacting with superoxide radicals, thereby allowing the reduction of NBT to a deep blue-colored Formazan dye, even when SOD is present.[60]

The removal of SDS by isopropanol, as previously reported,[67, 68] was used in the first wash, followed by two $ZnCl_2$ solution washes to remove the isopropanol and then soaking the gel sequentially with 0.1 M and 0.01 M Tris-HCl (pH 7). This may also lead to renaturation of (some of) the gel-bound SOD monomers to functional homodimers and so restore (some of the) SOD activity.[69]

3.4 CONCLUSION

The developed 2D-GE method coupled with SOD staining activity was successfully applied to separate SOD isozymes that differ in pI and/or M_W from a complex protein mixture, maintaining at least some of the different isoforms of SOD in the native form of the enzyme and/or allowing renaturation of some non-permanently denatured monomers to form active homodimers, even though the second dimension was run under denaturing conditions. This developed method can be apply to many SOD-related research areas such as SOD profile from many organism and markers of plant stress responses