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Seed Storage Protein Extraction and Gel pH gradient for Cucumber Varietal Identification via an Ultrathin-Layer Isoelectric Focusing Technique

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ABSTRACT

A suitable seed protein extract solution and gel pH gradient for cucumber varietal identification via an ultrathin-layer isoelectric focusing (UTLIEF) technique were studied. Four seed protein extract solutions, namely; water, phosphate buffer, Na_2EDTA and NaCl in combination with 2 gel pH gradients of 2-11 and 4-5/3-10 were used with UTLIEF carried out on four commercial F-1 hybrid cucumber cultivars namely; Micro C, Big C, Chok-Dee and Bussaba. The results showed that the phosphate buffer solution dissolved most cucumber seed protein, resulting in the highest total protein concentration, followed by water solvent. Most polymorphic cucumber seed protein bands were focused in the pH range of 7-10. The pH gradient of 2-11 showed sharper and clearer protein bands than that of pH gradient 4-5/3-10. Water-extracted seed protein run on in a gel pH gradient of 2-11 had the most polymorphic protein band markers that could completely differentiate the four cucumber cultivars from each other.

Keywords: cucumber, seed storage protein, electrophoresis, isoelectric point

INTRODUCTION

Thailand has a tropical climate, where crops can be cultivated all year round because of the availability of sufficient fertile land with a supply of water and skilled agricultural labor. These beneficial factors strengthen Thailand's goal to become the center of seed production for tropical crops in Asia. In 2009, Thailand exported 15,605 tons of seed with a value of USD 91.56 million. Cucumber (*Cucumis sativus* L.) is one of the major horticultural crop seeds being produced in Thailand, with its seed production worth approximately USD 12.8 million annually. Approximately 113.3 tons of cucumber seed, of which 90% is an F-1 hybrid, are produced in Thailand each year by more than 20 major seed companies, who supply both the domestic and international markets (Seed Association of Thailand, 2009). Therefore, varietal identification and hybrid purity testing play a significant role in Thailand's cucumber seed production to protect the rights of breeders and seed producers.

Cucumber seed for both open-pollinated and F-1 hybrid varieties is produced in open fields by contracted seed growers. To control the genetic

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purity, an individual seed lot from each grower must be subjected to a genetic purity test before payment. A seed quality test that is effective is needed, to help determine the seed preparation time and cost. The grow-out test method, which is currently used by most seed companies, is less effective, as it is dependent on the environment and is time consuming (Arus, 1983). In contrast, seed storage proteins, which are environmentally independent, have been used as biochemical markers to identify many plant varieties (Wang *et al*., 2000; Syros *et al*., 2003; Zhao *et al*., 2003; Noli, 2004; Yan *et al*., 2006). Storage proteins are found in the seed of many species. They are classified into four groups by their solubility and their sedimentation coefficient (S_{20w}). Prolamins dissolve in aqueous alcohols, while the 2S albumins dissolve in water and 7S and 11S globulins dissolve in diluted salt solutions (Shewry and Casey, 1999). Gel electrophoresis has been known as a technique for protein separation in an electric field, based on differences in physical or chemical properties, such as the molecular weight or size, or the electrical charge (Cooke, 1995). In isoelectric focusing (IEF) gel electrophoresis, proteins are separated on polyacrylamide gel by the differences in their electric charges. Under the electric field, these proteins migrate through the pH gradient in gel and settle where the pH of the gel is equal to their isoelectric point (pI), which is the pH at which the net charge of the protein becomes zero. Different proteins that have different pI values will settle at different positions on the gel and form a protein band pattern. Theoretically, different cultivars contain different seed proteins and have different protein band patterns under IEF. Therefore, varieties can be differentiated from each other by comparing their protein band patterns (Dunn, 1993).

A modification of IEF on polyacrylamide gel with a thickness of 0.15 mm is called ultrathin-layer isoelectric focusing (UTLIEF) and offers a faster, safer and cheaper technique for protein separation (Van den Berg, 1990). This technique is currently accepted by the International Seed Testing Association for varietal verification and hybrid testing of maize (*Zea mays* L.) and sunflower (*Helianthus annuus*), as described in the ISTA Rules for Seed Testing (ISTA, 2007). However, UTLIEF has not been applied for varietal identification of cucumber (*Cucumis sativus* L). Thus, it is necessary to optimize the conditions affecting the separation of cucumber seed protein in UTLIEF. The first two main factors to be determined are the appropriate protein extract solvent and the range of pH gradient in the gel.

First, proteins must be extracted in the appropriate solvent that is able to dissolve the marker proteins of individual plant varieties that distinguish one variety from the others. In the ISTA set of rules, 2-chloroethanol is generally recommended as the standard protein extract solution, because of its high dissolving ability for many seed protein types, which increases the possibility of separation. However, the use of this solvent in Thailand is not appropriate, because this highly toxic chemical is classified as an explosive agent and is restricted by the Ministry of Defence.

The separation of proteins in UTLIEF is caused by differences in the pI values of the proteins. Under an electric field, individual proteins migrate toward the electric pole with the opposite charge to their own and settle at a position on the gel where the gel pH is equal to the protein's pI value. Therefore, the pH gradient on the gel plays a significant role in the separation of proteins on the gel. The appropriate gel pH gradient varies with plant species that possess different protein types. Zhao *et al*. (2003) reported that a gel pH gradient of 5-8 gave the best results in the separation of hybrid-rice seed proteins, while Wang *et al*. (2000) successfully used gel with a pH gradient of 4-8 to separate seed proteins of tomato for variety discrimination.

Therefore, the objectives of this study were to determine the appropriate solution for

extracting cucumber seed proteins and the appropriate gel pH gradient for protein separation by UTLIEF for varietal identification.

MATERIALS AND METHODS

The experiment was conducted at the Center for Agricultural Biotechnology, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom, Thailand during October 2008 and March 2009.

Plant materials

Seeds were used from four local commercial F-1 hybrid cucumber cultivars; namely, Micro C, Big C, Chok-Dee and Bussaba.

Solvent preparation and protein extraction

Four solvents: namely; 18 MΩ distilled water, phosphate buffer (0.194 g K_2HPO_4, 0.528 g KH_2PO_4, 0.38 g EDTA, 1 g dithioerythritol (DTE) and 25 mL glycerin in 1 L distilled water), 5 mM Na_2 EDTA solution and 5 mM NaCl solution were used to determine a suitable solvent for cucumber seed protein extraction.

Bulked cucumber seeds of each variety were crushed by a grinder and 200 mg of crushed seed was put in 1.5 mL tubes containing 500 µL of each solvent for 2 h and centrifuged at 2,000xg for 5 min; all steps were carried out at 25±2°C. The concentration of total protein in each seed protein extract was determined by Bradford's protein-dye binding assay (Bradford, 1976).

UTLIEF gel and pH gradient preparation

The UTLIEF gels were prepared according to the flap technique, described in section 8.8.5 of International Rules for Seed Testing (ISTA, 2007). The polymerization solution for 10 gels was prepared by mixing 50.0 mL acrylamide (%T=6.8%, %C=2.5%) with 1.60 g taurine, 50.0 µL N N N'N'-tetramethylenediamine (TEMED) and 350 µL of 20% (w/v) ammonium peroxydisulphate. The two gel pH gradients of pH 2-11 and pH 4-5/3-10 used in this study were generated by adding 4.40 mL of either pH 2-11 or 4-5/3-10 ampholytes (SinuLyte™) to the polymerization solution. A few drops of water were applied onto a glass plate before a Gel-Grip™ film (240 × 180 × 0.12 mm, Sinus) was placed on top, taking care not to cause any air bubble. An aliquot of 6.5 mL polymerization solution was dropped onto the film. The cover glass plate was secured with two pieces of adhesive tape (0.15 mm thick) on both the long sides of the plate, as the spacer was gently placed on top to evenly spread the aliquot over the film and allow it to polymerize for 1 h at 26°C.

Ultrathin-layer isoelectric focusing

IEF was carried out on an IEF-SYS™ horizontal electrophoresis unit (Scie-Plus). One anodal electrode was placed on the top and one cathodal electrode was placed on the bottom of the gel. The anode solution contained 0.33% (w/v), L-aspartic acid and 0.37% (w/v) L-glutamic acid, while the cathode solution contained 0.47% (w/v) L-arginine, 0.36% (w/v) L-lysine and 12% (v/v) ethylenediamine. An application strip was placed on top of the gel surface about 5 mm away from the anode strip. Then, for each sample, 8 µL of seed protein was dropped into each well of the application strip and l2 µL of water was added. The unit was connected to a cooling apparatus to cool the gel surface down to 8°C. A power supply unit (Consort E833) provided a current of 5 mA and 15 W at 1,500 V. After 1 h of focusing time, the gel was fixed in 12% (W/V) trichloroacetic acid for 20 min, then stained in Coomassie Brilliant Blue solution for 15 min and de-stained in de-staining solution (30% (v/v) ethanol, 5% acetic acid and 65% (v/v) water) for 10 min. After rinsing with water, the gels were air dried overnight at room temperature and covered with transparent adhesive film before interpretation of the results.

RESULTS AND DISCUSSION

In order to use UTLIEF effectively for varietal verification and hybrid purity testing of cucumber seeds, it is necessary to determine the appropriate seed protein extraction solvent and gel pH gradient. In the current study, four solvents and two gel pH gradients were tested for varietal identification of four commercial F-1 hybrid cucumber cultivars. The seed protein concentration determined by Bradford's protein-dye binding assay revealed that phosphate buffer solvent could dissolve the seed proteins of all of four cultivars better than the other solvents, followed by water, while the lowest protein concentration was found when 5 mM NaCl was used (Table 1).

The UTLIEF electrophoretograms of the seed protein of four cucumber varieties carried out with each combination of solvent and gel pH gradient are shown in Figures 1-8.

Water-extracted proteins separated in the gel with the pH gradient of 2-11 had eight polymorphic protein bands, which was the highest number compared to the other combinations of solvents and gel pH gradients (Figure 1, bands # 1-8). With this combination, the Micro C variety contained all polymorphic bands except band # 4, while Big C contained only bands # 4 and 7, Chok-Dee contained bands # 1,3,5,6 and 7 and Bussaba contained bands # 1,3,5,6 and 8. This polymorphic band variation (PBV) allowed complete differentiation among the four cucumber cultivars.

The phosphate buffer extraction of seed proteins separated on a gel pH gradient range of 2-11 provided four polymorphic protein bands (Figure 2, bands # 1-4). The Big C cultivar contained no polymorphic protein bands, while the Chok-Dee cultivar contained bands # 2, 3 and 4, and the Micro C and Bussaba cultivars contained common bands # 1, 2, 3 and 4. This PBV allowed the identification of the Big C and Chok-Dee cultivars, but was unable to differentiate between the Micro C and Bussaba cultivars.

The 5 mM Na_2EDTA solution extracted seed proteins separated with a gel pH gradient of 2-11 provided two polymorphic protein bands (Figure 3, bands # 1 and 2). Big C and Chok-Dee contained no polymorphic bands, whereas Micro C and Bussaba contained the common bands # 1 and 2. This PBV differentiated the four cucumber cultivars into two groups, with the Big C and Chok-Dee cultivars in one group and Micro C and Bussaba in the other group, but it failed to discriminate between cultivars within a group.

The extraction using 5 mM NaCl and a gel pH gradient of 2-11 gel provided five polymorphic protein bands (Figure 4, bands # 1-5). Big C contained only band 4 and the rest of the cultivars contained the common bands # 1, 2, 3 and 5. Therefore, this PBV could only differentiate the Big C cultivar from the remaining three cultivars.

The water-extracted seed proteins separated in pH 4-5/3-10 gel showed two polymorphic protein bands (Figure 5, bands # 1 and 2). The Big C and Chok-Dee cultivars contained both bands # 1 and 2, while Micro C contained only band # 1 and the Bussaba cultivar contained no polymorphic bands. This PBV could identify Micro C and Bussaba cultivars, while Big C and Chok-Dee could not be differentiated from each other.

Table 1 Concentrations of total seed protein of four cucumber cultivars extracted by different solvents.

Cultivar	Concentration (μg/μL) of total seed protein			
	Water	Phosphate buffer	5 mM Na_2EDTA	5 mM NaCl
Micro C	2.67	2.77	2.65	2.59
Big C	3.13	2.67	2.39	2.26
Chok-Dee	2.51	3.93	2.27	2.32
Bussaba 2005	2.25	4.60	2.57	1.92

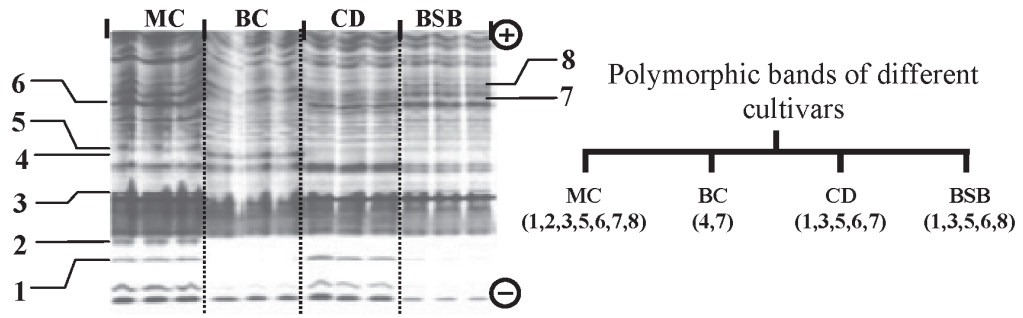

Figure 1 UTLIEF electrophoretogram of water-extracted seed proteins of four cucumber cultivars (MC = Micro C, BC = Big C, CD = Chok-Dee and BSB = Bussaba) carried out on gel pH gradient 2-11 (⊕ = anode, ⊖ = cathode; number in brackets () = existing polymorphic protein bands).

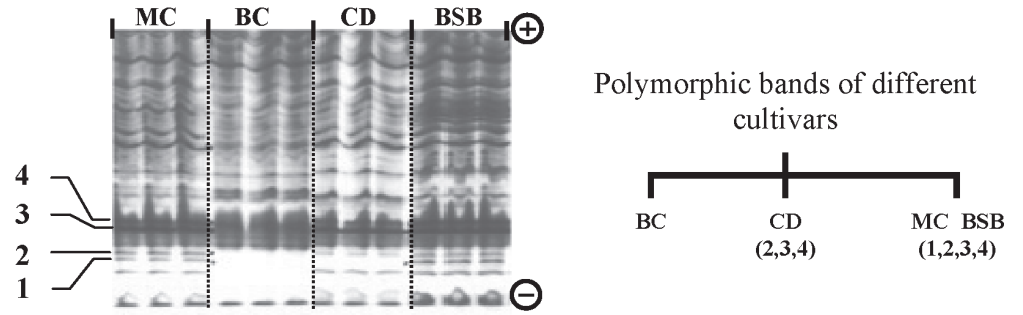

Figure 2 UTLIEF electrophoretogram using phosphate solution to extract seed proteins of 4 cucumber cultivars (MC = Micro C, BC = Big C, CD = Chok-Dee and BSB = Bussaba) carried out on gel pH gradient 2-11 (⊕ = anode, ⊖ = cathode; number in brackets () = existing polymorphic protein bands).

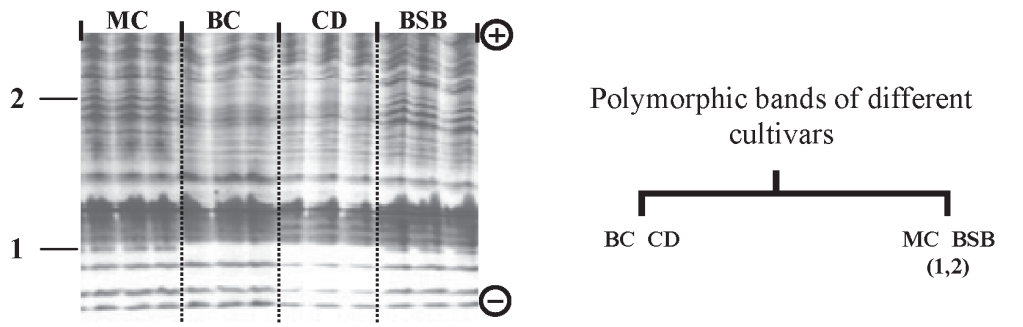

Figure 3 UTLIEF electrophoretogram using 5 mM Na_2EDTA solution to extract seed proteins of four cucumber cultivars (MC = Micro C, BC = Big C, CD = Chok-Dee and BSB = Bussaba) carried out on gel pH gradient 2-11 (⊕ = anode, ⊖ = cathode; number in brackets () = existing polymorphic protein bands).

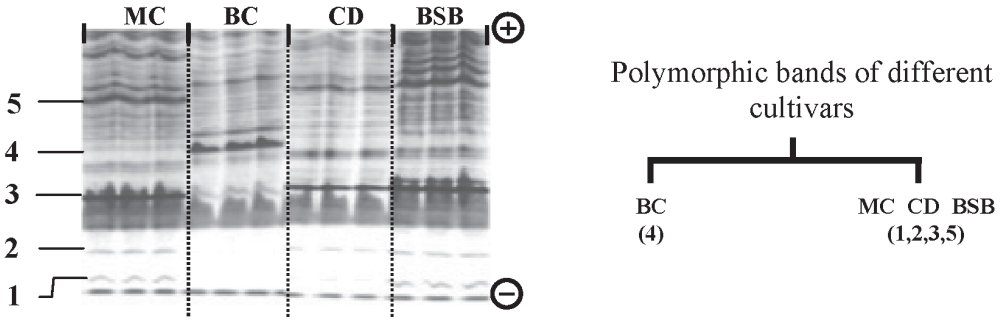

Figure 4 UTLIEF electrophoretogram using 5 mM NaCl solution to extract seed proteins of 4 cucumber cultivars (MC = Micro C, BC = Big C, CD = Chok-Dee and BSB = Bussaba) carried out on gel pH gradient 2-11 (⊕ = anode, ⊖= cathode; number in brackets () = existing polymorphic protein bands).

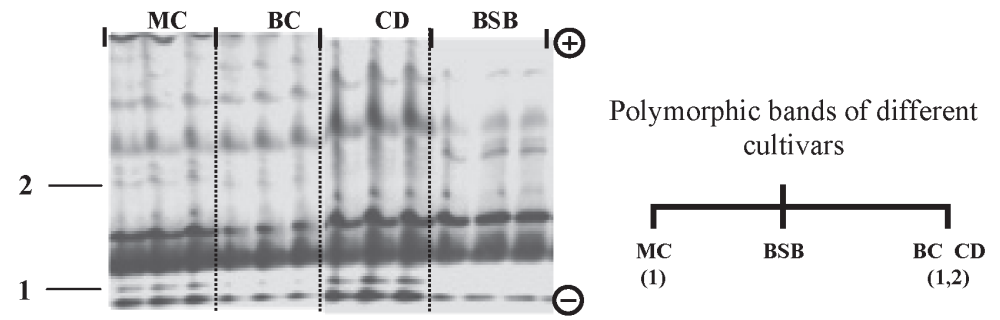

Figure 5 UTLIEF electrophoretogram using water to extract seed proteins of four cucumber cultivars (MC = Micro C, BC = Big C, CD = Chok-Dee and BSB = Bussaba) carried out on gel pH gradient 4-5/3-10 (⊕ = anode, ⊖ = cathode; number in brackets () = existing polymorphic protein bands).

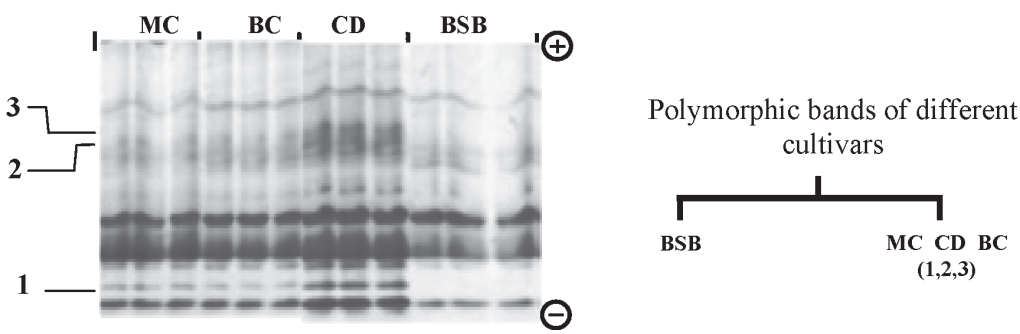

Figure 6 UTLIEF electrophoretogram using phosphate solution to extract seed proteins of four cucumber cultivars (MC = Micro C, BC = Big C, CD = Chok-Dee and BSB = Bussaba) carried out on gel pH gradient 4-5/3-10 (⊕ = anode,⊖= cathode; number in brackets () = existing polymorphic protein bands).

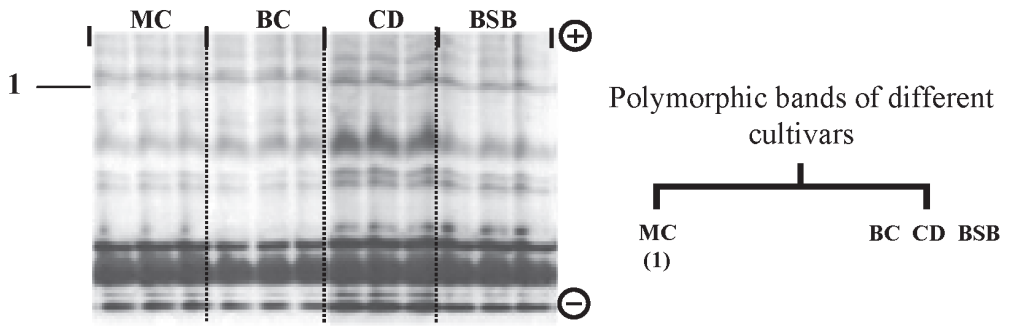

Figure 7 UTLIEF electrophoretogram using 5 mM Na_2EDTA solution to extract seed proteins of four cucumber cultivars (MC = Micro C, BC = Big C, CD = Chok-Dee and BSB = Bussaba) carried out on gel pH gradient 4-5/3-10 (⊕ = anode, ⊖ = cathode; number in bracket () = existing polymorphic protein bands).

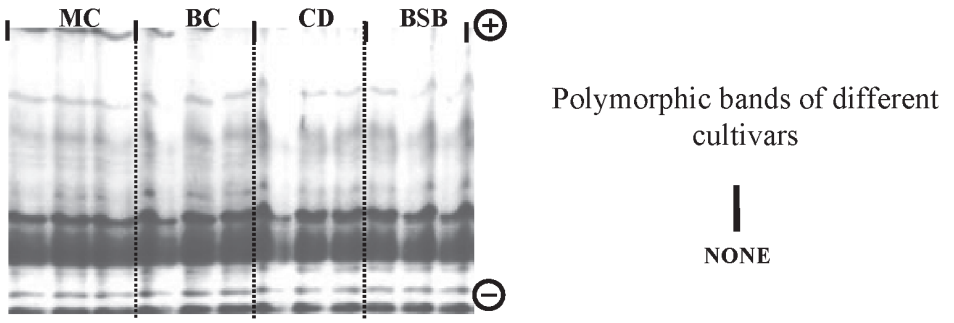

Figure 8 UTLIEF electrophoretogram using 5 mM NaCl solution to extract seed proteins of four cucumber cultivars (MC = Micro C, BC = Big C, CD = Chok-Dee and BSB = Bussaba) carried out on gel pH gradient 4-5/3-10 (⊕ = anode, ⊖ = cathode).

The phosphate buffer used to extract seed proteins separated in pH 4-5/3-10 gel provided three polymorphic bands (Figure 6, bands # 1, 2 and 3). Micro C, Chok-Dee and Bussaba contained all three polymorphic bands, while these three bands were missing in Big C. As a result, only the Bussaba cultivar could be differentiated from the other three cultivars.

The 5 mM Na_2EDTA solvent extracted seed proteins separated in pH 4-5/3-10 gel that provided only one polymorphic protein band (Figure 7, band #1) which existed only in cultivar Micro C and thus, allowed it to be differentiated from the other three cultivars.

Using the 5 mM NaCl solvent for protein separation in a gel pH gradient of 4-5/3-10 gel, yielded no polymorphic protein bands (Figure 8). Therefore, none of the four cultivars could be differentiated using this combination.

The most suitable protein extraction solution for varietal identification by the UTLIEF technique was not the one that could dissolve the most proteins from the seed, but rather the one that could dissolve the polymorphic proteins that were able to be used to differentiate among varieties (Arus, 1983). The results of this study revealed that the phosphate buffer solvent could dissolve the most cucumber seed proteins.

However, the most polymorphic protein bands were found in the water-extracted seed sample separated on gel with a pH gradient of 2-11. This implies that the major protein type in cucumber seed may be the salt-soluble proteins, globulins (Vickery *et al.,* 1941), however, the marker seed proteins for varietal identification are found in the albumins, which are water soluble proteins. This result was consistent with Degtyarenko *et al*. (1986), who investigated protein heterogeneticity in cucumber seeds using electrophoresis to separate the globulin and albumin fractions from over 20 cucumber varieties and reported that the protein variation for variety identification was found in albumin protein.

Albumins are a group of water-soluble storage proteins, mostly found in the food-reserving tissue parts of seeds, such as the endosperm in the monocotyledon, or in the cotyledon of dicotyledonous species (Shimada *et al.*, 2003). Therefore, for varietal identification of cucumber by the UTLIEF technique, water is the most appropriate solvent for seed protein extraction, since it can dissolve albumins, which are the proteins that can be used to detect genetic variation among different cucumber cultivars. Moreover, water is cheap and safe to work with compared to other solvents. Since, albumin proteins are mostly found in the cotyledon, it might be possible to use only this part of the cucumber plant to carry out varietal identification by the UTLIEF technique. This suggests that further study is required to find out whether only the upper part of the cotyledon in the embryo of a cucumber seed can provide sufficient protein for varietal identification, allowing the lower part with the embryo to be used for grow-out testing, to reconfirm the genetic purity of the results.

A comparison of the UTLIEF electrophoretograms of the two pH gradients with the same extraction solvent showed that the gel pH gradient also had a strong affect on the polymorphic protein bands across the four cultivars with all extraction solvents. The gel pH gradient of 2-11 showed 6, 4, 2 and 5 polymorphic bands, while the pH gradient 4-5/3-10 gave 2, 3, 1 and 0 bands for water, phosphate buffer, 5 mM Na_2EDTA and 5 mM NaCl solvents, respectively. Theoretically, the amounts and types of proteins obtained from seed samples extracted with the same solvent should be similar, but their separation via the UTLIEF technique with different gel pH gradients revealed a difference in protein band patterns. Comparing the gel with a pH gradient of 2-11 to the gel with a pH gradient of 4-5/3-10 revealed some missing protein bands, including polymorphic ones, when samples were extracted with the same solvent. This indicated the effect of pH gradient on the appearance of the protein bands on the gels.

In the IEF technique, the gel pH gradient is generated by the inclusion of a pH level that corresponds to that of the ampholytes in the polyacrylamide solution, as the ampolytes present in the gel mixture migrate in the electric current and establish a pH gradient in the range corresponding to their pI. The pH in the IEF gel increased linearly from the anode pole (top) to the cathode pole (bottom) in the range of the pI of the added ampholytes. On the gel with a pH gradient of 2-11, each pH position was equally spaced between the anode and cathode. However, with the gel pH gradient of 4-5/3-10, where the ampholytes pH 4-5 and 3-10 were mixed in the same gel, a wider space was generated at the upper part of the gel where ampholytes with pH 4-5 were positioned under the electric field. The wider space between the protein bands at the upper part and the narrower space at the bottom of electophoretograms in Figures 5-8 confirmed this result. The narrow space between positions of high pH on the gel gradient with pH 4-5/3-10 most likely affected the resolution of proteins with high pI values.

It was also observed in this study using pH indicators that the most polymorphic bands for

cucumber seed protein were found in the high pH range of 7-10 (data not shown). Thus, the missing protein bands, including some of the polymorphic bands, may have been caused by the close stacking of proteins with very close pI values at the high pH position on the gel, with the pH gradient 4-5/ 3-10 that resulted in the darker and thicker protein bands at the bottom part of the gel in Figures 5-8. This result also suggested that further study is required to determine whether the mixing of the narrow-range high pI value (7-10) ampholytes in the polyacrylamide gel would improve the resolution of the cucumber seed proteins.

CONCLUSION

It was possible to differentiate cucumber varieties by comparing the variation in seed protein bands using the UTLIEF technique. The cultivar variation in cucumber seed proteins could be detected in seed protein albumins, and thus, water should be used for protein extraction. For the best resolution of the separation of cucumber seed proteins using UTLIEF, a gel pH gradient of 2-11 should be used.

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LITERATURE CITED

Arus, P. 1983. Genetic purity of commercial seed lots, pp. 415-423. *In* S.D. Tanksley and T.J. Orton (eds.). **Isozymes in Plant Genetics and Breeding (Part A).** Elsevier. Amsterdam.

Bradford, M.M. 1976. A rapid and sensitive method for the quantization of microgram quantities of protein utilizing the principle of protein-dye binding. **Anal. Biochem.** 72: 248-254.

Cooke, R.J. 1995. Gel Electrophoresis for the Identification of Plant Varieties. **J. Chromatography A** 698: 281-299.

Degtyarenko L.V., P.F. Kononkov and T.I. Odintsova. 1986. An investigation of protein heterogeneity in cucumber seeds using electrophoresis. **VestnikSel'skokhozya-stvenno- Nauki** 12: 51-57.

Dunn, M.J. 1993. **Gel Electrophoresis: Proteins.** 1st ed. BIOS Scientific Publishers Limited, Oxford.

International Seed Testing Association (ISTA). 2007. **International Rules for Seed Testing Edition 2007.** 1st ed. International Seed Testing Association. Bassersdorf.

Noli, E. 2004. Analysis of Genetic Quality of Tomato, Pepper and Eggplant Seeds by Means of Seed Storage Protein Electrophoresis. **Sementi Elette** 50: 67-74.

Seed Association of Thailand. 2009. Import-export of seeds Thailand in 2009. **Seed Newsletter** 16: 7-9.

Shimada, T., K. Fuji, K. Tamura, M. Kondo, M. Nishimura and I. Hara-Nishimur. 2003. **Vacuolar sorting receptor for seed storage proteins in *Arabidopsis thaliana*.** [Web site: http://www.pnas.org/], [available from: www.pnas.org_cgi_doi_10.1073_pnas.2530568100], [cited: March 10, 2010].

Shewry, P.R. and R. Casey. 1999. **Seed Protein.** Kluwer Academic Publishers. Drenthe.

Syros, T., T. Yupsanis and A. Economou. 2003. Fractionation and electrophoretic patterns of storage proteins of *Ebenus cretica*. A preliminary survey as a tool in taxonomy. **Biologia Plantarum** 46: 435-443.

Van den Berg, B.M. 1990. Inbred testing of tomato (*Lycopersicon esculentum* L.) F1 varieties by ultrathin-layer isoelectric focusing of seed protein. **Electrophoresis** 11: 824-829.

Vickery, H.B., E.L. Smith, R.B. Hubbell and L.S. Nolan. 1941. Cucurbit seed globulins. **The J. Biol. Chem.** 28: 613-624.

Wang, X.F., R. Knoblauch and N. Leist. 2000. Varietal discrimination of tomato (*Lycopersicon esculentum* L.) by ultrathin-layer isoelectric focusing of seed protein. **Seed Sci. Technol.** 28: 521-526.

Yan, M., H. Ye and X.F. Wang. 2006. Investigation into the feasibility of assessing the hybridity of immature rice grains. **Seed Sci. Technol.** 34: 1-8.

Zhao, T., M.Yan, Y.P. Lu, F. Yang, J. Huang and X.F. Wang. 2003. Genetic purity testing of two-line hybrid rice seeds by ultrathin-layer isoelectric focusing of proteins. **Seed Sci. Technol.** 33: 45-52.