Faculty of Resource Science and Technology

Protein profiling of mutant type and wild type of *Leucaena leucocephala*

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PROTEIN PROFILING OF MUTANT TYPE AND WILD TYPE OF *Leucaena leucocephala*

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A final report submitted in partial fulfilment of the Final Year Project 2 (STF 3015)

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Resource Biotechnology

Faculty of Resource Science and Technology

Universiti Malaysia Sarawak

9th June 2018

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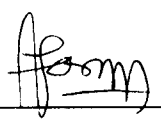
Student signature  (9 June 2018)

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Protein profiling of mutant type and wild type of *Leucaena leucocephala*

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Abstract

Leucaena leucocephala is also known as Petai Belalang tree. This leaf of *L. leucocephala* has many benefits toward human and animals such as fodder, fuel and charcoal. The proteins were extracted by using the three different methods; phenol, TCA and imidazole for mutant and wild type of *L. leucocephala.* The extraction methods have different substances in the extraction buffer that can give the process of extraction become more efficient. The purpose of this study is to identify the most suitable methods for extraction the proteins between phenol, TCA and imidazole methods by observing the band intensity on the gel of SDS-PAGE. TCA method has shown the clear and bright bands for wild type and mutant of *L. leucocephala.* Therefore, the TCA is the most suitable method method among phenol and imidazole has proceeded into 2-D PAGE whereby there is difference in spot intensity for wild type is 0.557 while mutant is 0.363 by using Delta2D software.

Keywords: Extraction Buffer, Imidazole, Protein extraction, SDS-PAGE, TCA

Abstrak

Leucaena leucocephala juga dikenali sebagai pokok Petai Belalang. Daun L. leucocephala ini mempunyai banyak faedah terhadap manusia dan haiwan seperti pakan, bahan bakar dan arang. Protein telah diekstrak dengan menggunakan tiga kaedah yang berbeza; phenol, TCA dan imidazole untuk jenis leucocephala mutan dan liar. Kaedah ekstraksi mempunyai bahan yang berlainan dalam penampan pengekstrakan yang dapat memberikan proses pengekstrakan menjadi lebih efisien. Tujuan kajian ini adalah untuk mengenal pasti kaedah yang paling sesuai untuk pengekstrakan protein antara kaedah fenol, TCA dan imidazole dengan memerhatikan intensiti band pada gel SDS-PAGE. Kaedah TCA telah menunjukkan jalur jelas dan terang untuk jenis liar dan mutan L. leucocephala. Oleh itu, TCA adalah kaedah terbaik di antara fenol dan imidazol yang telah diteruskan ke PAGE 2-D di mana terdapat perbezaan intensiti tempat untuk jenis liar adalah 0.557 manakala mutan adalah 0.363 dengan menggunakan perisian Delta2D.

Kata kunci: *Penampan Pengekstrakan, Imidazole, Pengekstrakan protein, SDS-PAGE, TCA*

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List of Abbreviation

DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EMS	Ethyl methanesulphonate
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
TCA	Trichloroacetic acid
TEMED	Tetramethylethylenediamine
β-ME	β-mercaptoethanol
APS	Ammonium persulfate
BSA	Bovine Serum Albumin
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
kDa	Kilodalton
rpm	Revolutions per minute

1.0 Introduction

Leucaena leucocephala (Lam.) de Wit belongs to leguminosae family that are mostly known as *Petai Belalang*. This tree is originated from Central America and Mexico. It also a fast growing legume tree in tropics and subtropics (Ho et al., 2014). Meanwhile, this tree may have many usages of applications; food, fuel, fibre, timber, resin, tannin and referred as the "miracle trees" according to its medical efficiency and other applicable (Kuppusamy et al., 2014).

L. leucocephala is broadly used in reforestation, livestock fodder and timber (Nazri et al., 2011). Economic value could be raised by having this *L. leucocephala* tree because it can be a good in degrading soils in terms of green fertilization (Parrotta et al., 1997) and act as modern soil degradation (Balota & Chaves, 2010). Good in fodder, fuel, charcoal and pulp makes this tree known as multipurpose uses (Lefroy et al., 1992). The leaves are high sources of carotenoids and vitamins which are functioning in photosynthesis by absorbing the light energy from sunlight and also photo protection while under the stress level (Adams et al., 1996).

Protein profiling is actually to bring out the proteins that are available in the tree according to the right method and extraction buffer. While doing this, it can observe the proteins in *L. leucocephala* tree that may give much information afterward in protein field. The best protein profiling is based on their methods how to extract the protein. The protein profiling results will show the difference protein expression between wild and mutant type of *L. leucocephala* clearly by using these methods TCA extraction, phenol extraction and imidazole extraction. The protein expression of mutant type and wild type of *L. leucocephala* can be observed by conducting SDS-PAGE. This may help a lot in observing the differences protein expression between these samples from *L. leucocephala.*

In addition, this study is conducted in order to reduce some problems that had faced in protein profile of *L. leucocephala* for example mimosine in *L. leucocephala* is a substance which is a toxic non-protein amino acid that can harm the consumers. By observing their differences protein expression between mutant type and wild type of *L. leucocephala*, there is a lot of knowledge can be used in science field in order to reduce amount of mimosine content in the leaves of *L.* leucocephala. Other than that, this study is conducted to determine the protein content in *L. leucocephala* because in science field this study still in poorly studied.

The objectives of this research are:

1. To determine the most suitable method of protein extraction for mutant type and wild type of *Leucaena leucocephala* among Phenol, TCA and Imidazole.
2. To compare the protein bands between mutant type and wild type of *Leucaena leucocephala* by conducting SDS-PAGE and 2-D PAGE.

2.0 Literature Review

2.1 *Leucaena leucocephala*

Leucaena leucocephala is known in other names of *Petai Belalang* or the money tree as which this plant is belong towards Fabaceae family. While this species is a native plant from Southern Mexico and Northen Central America and the West Indies (Kadir et al., 2013). Characteristic of *Leucaena leucocephala* are small size, shrubby and branched (Orwa et al., 2009).

L. leucocephala has several properties that a suitable for a plant crops which is act as source for green manure, firewood and plant material (Kang et al., 1981). This plant is managed to be an alternative for replacing the traditional bush fallow system. By acting as bush fallow system it can lead to control the erosion control, shade and nitrogen fixing (Orwa et al., 2009). Erosion control can be done by planting *L. leucocephala* because the taproot systems can break the compacted subsoil layers and reduce the surface runoff. The *L. leucocephala* plant can act as shade tree for others plant such as coffee, tea and cocoa.

2.2 Mutant type of *Leucaena leucocephala*

Mutated of *L. leucocephala* also can be one of the important roles that involving the crop improvement either by directly or conventional breeding. In order to obtain the mutated *L. leucocephala* is by using ethyl methanesulphonate (EMS) whereby this method actually can improve few of characters of the plants in a well-adapted variety. Therefore, EMS has been reported as the most effective mutagen reagent in producing point mutations (Okagaki, 1991). Afterwards, EMS also the mostly used as mutagen in plants which is gives result of nucleotide substitutions even without substantial killing (Talebi, 2012).

Mimosine is a chemical that are toxic when consumed for human either or to animals in *L. leucocephala*. In fact this *L. leucocephala* tree has many benefits and the higher value of mimosine in *L. leucocephala* tree could eliminate all the benefits as livestock fodder (Chanchay & Poosaran, 2009). This mimosine is existed mostly in leaves and seed of *L. leucocephala* and may give the effects of in non- functioning of thyroid and contribute to poor growth. Besides mimosine is toxic in *L. leucocephala* tree, there is another toxic compound which is the degradation of mimosine products; 3-hydroxy-4(1H)-pyrodine (DHP). Both of these are toxic once the consumer has consumed. In order to remove these toxic, the leaves of *L. leucocephala* will leached in 100% of seawater and other acidic and basic solutions for 24 hour at 25°C as the mimosine is insoluble in distilled water but soluble in ionic solutions (Tacon, 1979). By reduce the amount of mimosine in *L. leucocephala* can make the benefits of this tree become wider in livestock fodder field.

2.3 Proteome

Proteome is a set of proteins whereby can observe when the proteins extracted, the rates of proteins and interaction of protein itself. Study of proteome has three categories; expression, structural and functional. The expression between the proteome and quantitative methods can be difference based on their experimental condition (Graves & Haystead, 2002). Proteins profiling is take action on the expression level that happens on the different of the samples.

2.4 Protein extraction

Protein extraction is a method where the proteins can be extracted out from the organism whether animals or plants. This extraction depends on how the method is applied on the organism. Different organism has different protein extraction method. In this study, there are three methods of protein extraction that will be used to identify difference expression between mutant and wild type of *L. leucocephala*. While for this era, there are three also

method that most suitable used for extraction protein from plant; phenol, trichloroacetic acid/acetone/SDS/phenol (TASP) and borax/polyvinvyl-polypyrrolidone/phenol (BPP) extraction (Jiang et al., 2017). These methods are produced high quality protein extraction.

2.5 Quantification

In science field, there are mostly five protein quantification assays that available which are Bicinchoninic Acid (BCA), Bradford, Folin- Lowry, Kjeldahl and Ultraviolet Absorption. While in this study, the Bradford assay has been preferred method because this method is simpler, faster and sensitive rather than Lowry method (Bradford, 1976). The Bradford assay is a binding of protein with dye Coomassie Blue G250 (Chial et al., 1993). The more cationic which in red and green have 470 nm and 650 nm maximum absorbance. In contrast, the more anionic blue has maximum absorbance at 595 nm with binding to proteins. Total amount of protein can be estimated by calculating the dye in blue ionic form by reading the absorbance of the protein samples.

2.6 SDS Page

Mostly for getting high resolution separation of complex mixtures of proteins is by using sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Nowakowski et al., 2014). This method can denature the proteins while undergo electrophoresis. Electrophoresis are functioning in separation mixtures of proteins based on molecular mass, observe subunit compositions and homogeneity of protein samples (Gallagher, 2006). Migration of proteins is regarding to size, shape, and combination of pore size and protein charge. Next level of SDS-PAGE is 2-D PAGE which is to identify and separate protein. While SDS-PAGE only separate the protein based on their molecular weight. Differences between these two methods are SDS-PAGE is rapid or save many time because only need one hour to run in order to complete the process. Therefore, 2-D PAGE is

requiring a lot of time as their rehydration time and focusing time about 24 hours or one day to complete for whole process. SDS-PAGE methods are easily to conduct and handle while 2-D PAGE is complicated and not easy to handle as it has a lot of things to consider such as the voltage, temperature and operation hour of the machines. Furthermore, 2-D PAGE can load more amount of sample until 125 µl (169 µg) but for SDS-PAGE only can load small amount of sample because the voltage also lesser than 2-D PAGE. On the other hands, each method shows benefits such as SDS-PAGE is easier to conduct and cheap while for 2-D PAGE is complicated but it plays a big role in identify and separation of the proteins that are available in the samples easily.

3.0 Materials and Methods

3.1 Sample Preparation

Both of the leaf samples of *L. leucocephala* were collected at Dr. Ho Wei Seng's laboratories. Only the leaves parts of the *L. leucocephala* were used in this study. By using cold mortar and pestle with added liquid nitrogen, these leaves were grinded into fine powders and stored in the 1.5 mL falcon tube in -20°C freezer with separating tubes for mutant and wild type of *L. leucocephala.*

3.2 Protein Extraction

3.2.1 Phenol Extraction

Phenol extraction method was used in this study with slightly modification (Isaacson et al., 2006). Using ration of 1:3, the 0.3 g of powdered samples were mixed with 900 µl ice-cold extraction buffer (0.7 M sucrose; 0.1 M KCl; 0.5 M Tris-HCl, pH 7.5 and 50 mM EDTA) for the proteins to be solubilized in the buffer. Then equal volume of phenol saturated with Tris-HCl, pH 7.5 was added and the mixture was shaken about 30 minutes at 4°C. The mixture was centrifuged at 10,000 g for 15 minutes at 4°C. The phenol phases were collected and discarded the lower aqueous phase. The extraction buffer was added again into the phenol phase that has been collected and the step repeated for vortex and centrifuge for two times. For getting the phenol phases to be precipitate, 5 volumes of cold 0.1 M ammonium acetate in methanol was added into the collected phenol phase. The solutions were stored at -20°C for overnight. The precipitate should be in white flakes. Then, the solutions were centrifuge for 10 minutes at 16,000 g at 4°C and discarded the supernatant by using pipette. Two times volume of ice cold methanol was added to the wash the pellet formed and mixed well the solution by using vortex. The solutions were centrifuge for

10 minutes at 16,000 g at 4°C and the supernatant was removed by using pipette. Did the washes within two times to get rid of the ammonium acetate and phenol, lipids and other pigments and then also washes twice by using acetone instead of methanol for getting faster drying. Then, the pellet was dried up and stored in the freezer -20°C.

3.2.2 TCA Extraction

Using modified method from Rodrigues et al (2009) the powdered samples were added with ice-cold 10% TCA in acetone added with 0.07% 2-mercapthoethanol (β-ME) and were incubated for one hour. The samples were centrifuge at 16,000 g for 10 minutes at 4°C and were discarded the supernatant. The tissue pellet then re-extracted by adding extraction buffer (7 M urea, 2 M thiourea, 4%SDS, 1%DTT) in the ratio 1:3. Then, the solutions were centrifuge at 16,000 g for 10 minutes at 4°C to collect the supernatant. Ice-cold 10% TCA in acetone with 0.07% 2-mercapthoethanol were added in equal volume of the collected supernatant and allowed to precipitate overnight. The solutions were centrifuge at 16,000 g for 10 minutes at 4°C and were discarded the supernatant. The pellets were washed twice in ice cold acetone added with 0.07% 2-mercapthoethanol and centrifuge for 10 minutes at 4°C for removing any pigments. The dry pellet were suspended in 100 µL extraction buffer (7 M urea, 2 M thiourea, 4%CHAPS, 1%DTT). Dried up the pellet then stored in freezer -20°C.

3.2.3 Imidazole Extraction

Referring to Nakamura (2012) extraction method with modification the powdered samples were homogenized in ice-cold extraction solution (50 mM imidazole-HCl, pH 7.4, 8 mM $MgCl_2$, 50 mM 2-mercapthoethanol, and 12.5% (v/v) glycerol) in the ration 1:3 at 4°C for 1 hour shaken. The homogenize solutions were centrifuge at 16,000 g at 4°C for 10 minutes. The supernatant was collected and added with equal volume of 20% TCA in acetone. Then,

the solution was allowed to precipitate overnight at freezer -20°C. The mixture solutions were centrifuge at 16,000 g at 4°C for 10 minutes to form pellet. The supernatant was discarded and the pellet was washed with ice cold acetone. This step was repeated three times in order to get the pure pellet without any impurities. Lastly, the pellet was dried and stored in the freezer -20°C.

3.3 Quantification

3.3.1 Bradford assay

Protein concentration is quantified by using Bradford protein assay (Bradford, 1976). The pellet samples were dissolved in 100 mL of solubilize buffer. The sample dilutions were prepared in 100 x, 25 x and 10 x. For standard graph, the bovine serum albumin (BSA) was pipetted in volumes of 10, 20, 40, 60, 80 and 100 µl of 100 mg/mL y-globulin standard solution into the 1.5 mL tubes then filled up with ultrapure water until it reach 100 µl. The 100 µl of distilled water was pipetted into further test tube to provide the reagent blank. 1 mL of Bradford reagent (Coomassie Brilliant Blue G-250, methanol, phosphoric acid) was added into the sample dilutions and vortex for 1 min. The spectrophotometer was set into A595 then the samples were measured and recorded the data in tabulate form.

3.4 SDS Page

3.4.1 1-Dimensional SDS PAGE

The polyacrylamide gel was prepared according to the standard protocol (Laemmli, 1970). The samples were loaded into the well about 10 µl in SDS running buffer (15 g Tris, 72 g Glycine, 5 g SDS) in 1 litre of distilled water. Then, the gel was run for 1 hour and 30 minutes at 120 V. The gels were stained with Coomassie blue stain and placed in a plastic container on shaker for overnight. The solution of Coomasie blue was pour off into recycle

bottle of CBB. The gels were washed with ultrapure water for 10 minutes in two times. Then, the destain solution was pour into the gel and let it for 20 minutes on shaker at 70 rpm. Lastly, the gels were washed once and the results were analysed by observing their bands on the gel.

3.4.2 2-Dimensional SDS page

Isoelectric focusing (IEF) method was followed by 2D – SDS PAGE whereby the protein samples were loaded about 125 µl into the ceramic strip holder according to IEF – 2DE protocols (Wu et al., 2013). 7 cm of IEF strip was taken out and removed the thin plastic seal from the gel side of the stripe. The mineral oil was pipetting about 2.5 mL into the ceramic strip holder. Therefore, the IEF strip was placed on the mineral oil. The samples were run for 12 hours. After that, the IEF strip was transferred into another lane of the ceramic strip holder and pipette another 2.5 mL of mineral oil. At the same time, the wick was added into the each end of the strip and let the samples ran about another 5 hours. While waiting the samples ran, the separating gel was prepared for saving more time. Then, the strips was taken into another lane in order to suspend with equilibrium buffer for 10 minutes and shaken at 70 rpm. The overlay gel was used on the separating gel to become as indicator of the samples to reach at the bottom of the gel. The strip was transferred onto the prepared gel. Casting the glass plate in the container and pour off the running buffer and ran about 1 hour and 30 minutes as usual SDS-PAGE at 120 V. The gel was stained with Coomassie blue stain for overnight. Then the gel was washed with ultrapure water for twice and put the destain solution for 20 minutes on shaker at 70 rpm. Lastly, the image of gel was captured by using BioRad machine at Faculty Medicine, Universiti Malaysia Sarawak.

3.4.3 Analysis of 2D – SDS PAGE

By using software of Delta2D, the image of the gel from TCA extraction methods was imported into this software. The group was created to differentiate between mutant type and wild type image of gel. The images need to warp image manually to ensure both of the images were connected. Hence, in order to detect and quantify spots, the images were fused in dual view that can detect the spots easier. Then, the fused image was analysed the expression profiles followed by observing to the quantitation table, expression profiles and analysis. Lastly, the result can be presented in project summary, spot album, spot quantities and labels. But somehow, this software needs to buy the license to have the permissions to use all the features that are available in this software.

4.0 Results

4.1 Sample Preparation

The figure 4.1 shows the leaves of *L. leucocephala* were taken freshly from the plant and weighed about 3 g for each samples. The mortar and pestle was chilled first with liquid nitrogen then start grinded until it forms fine powder tissues.

Figure 4.1: The leaves samples were grinded with liquid nitrogen until form fine powder tissues by using mortar and pestle.

4.2 Protein Extraction

4.2.1 Phenol extraction

The figure 4.2 shows the pellets were formed after phenol extraction was done by 2 days. The pellets size was referred to amount of leaves were grinding which is 3 g for wild type and mutant type of *L. leucocephala*.