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Determination of Extraction Buffer and Ammonium Sulfate Percentage for Pollen Crude Protein Extracts of Mangifera, Durio and Syzygium Fruit Flowers

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ABSTRACT

Flowering trees are considered the producers of allergenic agent carried by pollen protein. The isolation of protein of interest from intracellular compartment into a solution of well-defined composition especially from never isolated species involves the manipulation of several parameters. The aim of this work is to find the best isolation method for pollen protein of the widely distributed flowering fruit species in Malaysia includes *Mangifera indica, Mangifera odorata, Durio graviolens, Durio zibethinus, Syzygium aqueum (red flower), Syzygium aqueum (white flower),* to give the maximum yield extract of crude protein. The most efficient buffer to be used specifically for each species were as followed; *Mangifera indica* (Tris HCl 0.5M pH 6.8 with 1.3 mg/ml); *Mangifera odorata* (Tris HCl 0.5M pH 6.8 with 1.8 mg/ml); *Durio graviolens* (PBS 0.02M pH 7.4 with 1.6 mg/ml); *Durio zibethinus* (PBS 0.02M pH 7.4 with 2.2 mg/ml); *Syzygium aqueum* -red flower (Tris HCl 0.5M pH 6.8 with 1.0 mg/ml); *Syzygium aqueum*-white flower (PBS 0.02M pH 6.8 with 1.7 mg/ml). Pectinase activity reveals the best ammonium sulfate percentage for *Mangifera indica, Mangifera odorata, Durio graviolens*, *Durio zibethinus, Syzygium aqueum* -red flower, *Syzygium aqueum*-white flower is either 80% or 85%. Different types of buffer definitely have different ability on the protein solubilization, while the 80% of salt precipitation was the most ideal percentage for salting out the protein in every sampled species.

Key Words: Flower, Pollen, Protein, Buffer, Precipitation

INTRODUCTION

Plant pollen is one of the most common causes of seasonal allergic disease worldwide. Between 10%- 40% of the present world population is allergic to pollen [1]. Pollen allergy is caused by proteins, glycoprotein or even a single peptide which are present in the pollen wall and cytoplasm [2]. According to Allergy Centre Malaysia in 2014, pollen is the third contribution to allergic rhinitis. Although, studies on the allergenic properties of pollen from various species have been carried out by several workers [3,4,5,6], information on allergy to pollen in Malaysia is completely lacking, even though there are numerous tropical fruits trees flowering in all season of the year. Nevertheless, there was no such type of research work carried out in Malaysia for investigating the reason of pollen allergy except by [7] where he perform a survey of skin prick tests (SPT) using pollen extracts on 200 patients with clinical symptoms of asthma. The plantation with thousands acres of fruit orchards could release unlimited pollen just waiting to be inhale by people which could create asthma, bronchitis, rhinitis and lung cancer also in the severe stage [8]. In view of the extensive distribution of local plant trees in Malaysia and the prevalence of its pollen in the atmosphere, three genera and six species of fruit flowers have been chosen for this study. They are *Mangifera indica, Mangifera odorata, Durio graveolens*, *Durio zibethi-*

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nus, Syzygium aqueum -red flower, *Syzygium aqueum*-white flower. These local fruits are among the most planted in fruit orchards in Malaysia.

Since the target protein was undefined until it has been characterize, protein extract from the species must be at its optimal yield as possible. Protein extraction procedures have been reported in the literature as the first step in proteomics studies and the most important initial steps for further purification and characterization of allergenic protein [9, 10, 11]. The type, concentration and pH of buffer used may differ regarding the species.

The development of a reliable extraction method will ensure highest extraction efficiency before further purification and allergenic test could be conducted. In this study, an efficient method of cell disruption has been developed involving the manipulation of extraction buffer which release the protein in soluble form from intracellular compartment into a solution of a well-defined composition. In addition, a series of percentage of ammonium sulphate precipitation has been tested to ensure that solubility of the pollen crude protein.

METHODOLOGY

Collection and screening of flower pollen

Fruit flowers were collected from various fruit orchards in Kelantan and sun dried. To obtain pollen grains powder, the dried flowers were sieved through different grades of sieves (100, 200, and 300 mesh/cm2) and analyzed under the microscope to ensure purity varying from 85% to 90%. Pollen grain powder was further dried in an oven at 36 °C and stored at 4^0C in airtight containers for further use.

Pollen Protein Buffer Extraction

Proteins from dried pollen grain powder were extracted in two types of buffer with different concentration and pH (Tris HCl 0.2M pH 6.8, Tris HCl l 0.2M pH 7.4, Tris HCl 0.5M pH 6.8, Tris HCl 0.5M pH 7.4, Phosphate buffer saline (PBS) 0.02 pH 6.8, Phosphate buffer saline (PBS) 0.02M pH 7.4, Phosphate buffer saline (PBS) 0.05M pH 6.8 and Phosphate buffer saline (PBS) 0.05M pH 7) by continuous stirring at 4^0C for 24 hrs. Then, the pollen was macerated by mortar and pastel to form a pollen paste. The pollen paste extract was clarified by centrifugation at 15,000 x g for 20 min at 4^0C. The supernatant was separated and tested for protein concentration with Bradford method [12]. The highest crude protein concentration supernatant from a particular buffer extraction was selected for ammonium sulphate precipitation.

Ammonium sulphate precipitation

The selected supernatant with high crude protein concentration was gently stirring in 50% and 90% concentration at interval of 5% and 10% for fractional precipitation with ammonium suphate to screen for the best percentage that could dissolved the salts completely. The dissolved solution was kept overnight in a chiller and was centrifuged at 10,000g for 15 minutes [13]. The precipitate was re-suspended in specific buffer and desalted by dialyzing against distilled water for 48 hrs at 4^0C by frequent changes of the distilled water using dialysis sacs (MW cut off 9 kDa) [5]. The protein crude extracts were subjected to pectinase enzyme assay to determine its activity.

Pectinase enzyme assay of protein crude extracts

Pectinase assays were carried out by using modified DNS method [14]. 1 ml of the reaction mixture containing equal amount of substrate (0.5%) prepared in suitable diluted enzyme was incubated at 50^oC for 30 min in a water bath. After incubation, 3.0 ml DNS solution was added to stop the reaction and the tube was kept in boiling water for another 10 min. Once cooled, the developed colour was read at 575 nm using UV-visible spectrophotometer. The amount of released sugar was quantified using D-galacturonic acid as standard. The enzyme activity was calculated as the amount of the enzyme required to release one micromole equivalent of D-galacturonic acid per min under assay condition. Finally, the supernatant that was proven to have enzyme activity was passed through a Millipore filter membrane (0.45µm), lyophilized in small aliquots, and stored at -20^0C until further purification steps before it allergenic properties could be ascertained. The crude pectinase without addition of ammonium sulphate (0%) was used as control.

RESULTS

Figure 1 shows the crude protein supernatant concentration of the three genera of common fruit flowers. Generally, genus *Mangifera* showed high protein concentration yield by both PBS and Tris HCl buffers. Protein concentration for *Durio* genus could only be detected for PBS buffer. The two varieties from *Syzygium aqueum* show contrasting result; the red flower gave better yield in Tris-HCl buffer and low yield in PBS buffer, and the result was vice versa for white flower. Extracts for *Mangifera indica* and *Mangifera odorata* contained 1.3 mg/ml and 1.8 mg/ml protein concentration respectively. While for *Durio*, PBS 0.02M pH 7.4 gave 1.6 mg/ml protein concentration for *Durio graveolens* and 2.2 mg/ml in *Durio Zibethinus*, which was the highest compared to the other extraction buffer. The highest protein concentration obtained for *Syzygium aqueum* (red flower) was 1.0 mg/ml in Tris HCl 0.5M pH 6.8 while *Syzygium aqueum* (white flower) was 1.7 mg/ml showed by PBS 0.02, pH 6.8.

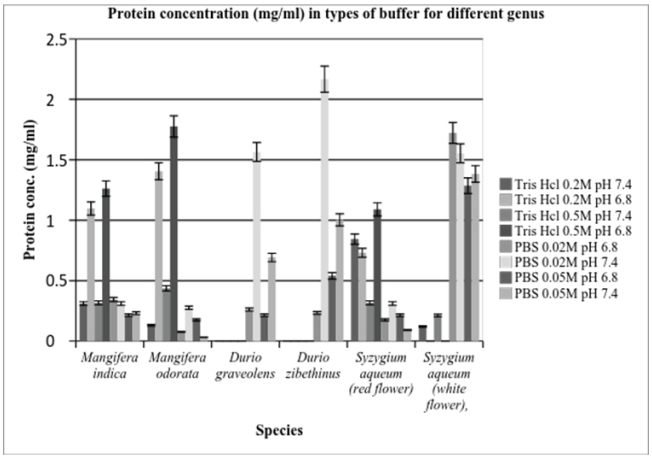

Figure 1: Crude protein supernatant concentration of the three genera *Mangifera*, Durio and *Syzygium* extracted with Tris Hcl and PBS buffers at different concentration and pH. Error bars indicate the standard deviation values. This experiment was carried out in triplicates and there are significant different between means (Duncan, $p<0.05$).

Mangifera odorata and *Syzygium aqueum* (white flower) protein crude extract precipitation with 85% percent of ammonium sulphate gave the highest pectinase activity at 40.67 U/ml and 39.07 U/ml respectively as shown in Figure 2. Meanwhile, 80% of ammonium sulphate precipitation was the best percentage for *Mangifera indica, Durio graveolens, Durio zibethinus* and *Syzygium aqueum* (red flower) crude protein extract giving activity at 35.68, 38.47, 48.37, 33.47 U/ml respectively.

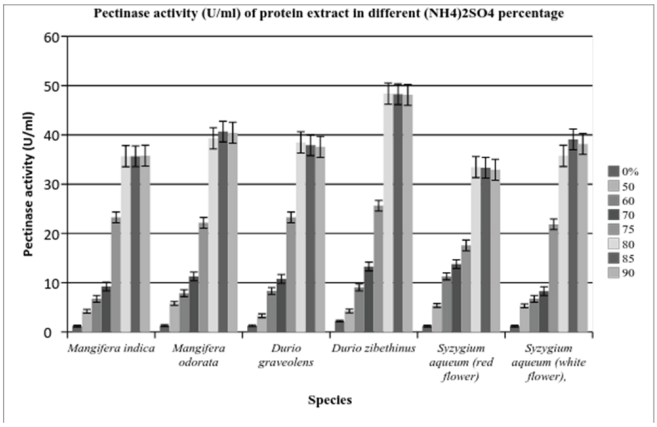

Figure 2: Pectinase enzyme activities of protein crude extract from genera *Mangifera, Durio and Syzygium* protein precipitated by range of different percent of ammonium sulphate. Error bars indicate the standard deviation values. This experiment was carried out in triplicates and there are significant different between means (Duncan, $p<0.05$). Table 1 provides recommended type of buffer and percentage of ammonium sulfate to be used for each species for efficient yield of pollen crude protein for the three species.

Table 1: Type of extraction buffer and percentage of Ammonium sulphate precipitation recommendation for each genera *Mangifera, Durio* and *Syzygium*

Species	Extraction Buffer	Ammonium sulphate precipitation (%)
Mangifera indica	Tris-HCl 0.5M pH 6.4	80
Mangifera odorata	Tris-HCl 0.2M pH 7.8	85
Durio brunei	Phosphate buffer saline (PBS) 0.05 pH 6.8	80
Durio zibethinus	Phosphate buffer saline (PBS) 0.02 pH 7.4	80
Syzygium aqueum (red flower)	Tris-HCl 0.5M pH 6.4	80
Syzygium aqueum (white flower)	Phosphate buffer saline (PBS) 0.02 pH 6.8	85

DISCUSSION

The first step to isolate this protein is to dissociate and solubilize the protein as the polypeptide chains in inclusion body protein are trapped in a number of partially or incompletely folded conformations that are stabilized by association with other polypeptide chain [15]. The selection of an appropriate buffer is important in order to maintain a protein at desired pH and to ensure reproducible experimental result. An appropriate buffer solution also can help improve the stability of protein molecules as these molecules are subjected to various forces designed to isolate them for study. A buffer solution can protect the integrity of the proteins while separating them from other integrated cell components [4, 9 & 16]. The ammonium precipitation step was performed to concentrate the protein from dilute solution. This step is important since most of allergenic protein is hydrophobic and the saturation must be up to 80%. When high concentration of salt is present, protein tend to aggregate and precipitate out of solution. Since different proteins (especially for different species) precipitate at different salt concentration, salting out is often used during protein purification and this is actually the initial step of protein purification. Most protein will precipitate at 55% ammonium sulphate, thus seven concentrations with interval 10% and 5% saturation percentage up to 90% has been chosen to maximize the protein precipitation.

CONCLUSION

Choosing the right buffer is important to ensure reliable result of protein extraction. This study has shown that different buffer concentration and pH is critical in determining highest protein concentration yield for pollen protein from different genus. This study has also able to make recommendation for type of extraction buffer and percentage of Ammonium

sulphate precipitation for each genus *Mangifera, Durio* and *Syzygium*. Different types of buffer definitely have different ability on the protein solubilization, while the 80% of salt precipitation was the most ideal percentage for salting out the protein in every species.

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