

Proteomic analysis of *Aquilaria malaccensis* seed in relation to seed viability

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Research Article

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Abstract

Aquilaria malaccensis is one of the woody species and its economic value, high demand in the market, overexploitation, and difficulty in propagation due to the low shelf life of the seed cause the species to face the threat of extinction. The main objective of the study is to understand the role of protein in seed viability. In this experiment, the protein was extracted using tris- HCl/trichloroacetic acid-acetone (TCA-acetone) from the immature seed, premature seed, fresh mature seed, 7 days stored seed, 14 days stored seed, and 30 days stored seed. The extracted protein concentration was estimated using the Bradford method and separated using 1-D SDS PAGE. With the maturity of the seed concentrations of protein increase (immature seed 18.11 µg/ml, premature seed 37.43 µg/ml, and fresh mature seed 68.92 µg/ml) and it reduces with storage (7 days 23.92 µg/ml, 14 days 20.14 µg/ml, and 30 days 16.08 µg/ml). Proteomic analysis using SDS PAGE, revealed that with the maturity of the seed, development protein was observed, immature seeds show no band, premature seeds showed four bands, and freshly matured seeds show 5 dark bands. The 7 days stored seeds show two bands 14 days stored seeds showed only one band, and no band was observed in 30 days stored seeds. Furthermore, the loss of seed proteins in seed storage might induce a loss of their functional properties in seed viability.

Introduction

A. Malaccensis is one of the potential woody plants that boost the livelihood of farmers and traders. The presence of unique organic molecules has caused this species into threatening stages from the environment. The species normally and naturally propagate through seeds. *A. malaccensis* seeds have a low shelf life, as they decline sharply with an increase in storage. Seed used for sowing has a significant impact on both field establishment and crop performance. Carryover seed storage is an absolute necessity for the sowing of any crop the following year. When seeds are stored for an extended period of time, they inevitably lose some of their vitality and vigor (McDonald 1999). Decreased seed vitality and viability can have an impact on future crop performance and yields in the field (TeKrony and Egli, 1991). Seed viability is influenced by factors such as temperature and humidity during storage (related to seed moisture content). It has been demonstrated that storing seeds at low temperatures and with a low moisture content increases their storage life (Walters, 1998; Walters et al, 2005). Accelerated aging, where seeds are stored at high temperatures and relative humidity, can also be used to study seed longevity in addition to long-term storage (natural aging) (Tesnier et al, 2002; Rajjou et al, 2008).

Reactive oxygen species (ROS) are unstable molecules produced by various cellular organelles such as chloroplast, mitochondria, and peroxisomes and are considered as by-products of plants' aerobic metabolism (Dietz et al, 2016; Huang et al, 2016; Sandalio and Roero-Puertas, 2015). Accumulation of species in the cell organelles causes damage to various macromolecules such as DNA, protein, and RNA and it also plays a vital role in signaling molecules to regulate germination, dormancy, health, and stress condition (Schieber and Chandel, 2015). The molecular mechanism of seed deterioration remains a mystery, and there is as of yet no evidence to support any hypotheses. However, increasing evidence suggests that the formation of free radicals during storage is the biggest reason for membrane

disruption, protein and nucleic acid damage, and eventual cell organelle deterioration and seed aging (Bailly 2004; Kibinza et al, 2006). Due to their prevalence in living things, proteins have been shown to be the primary targets of free radicals (Davis, 2005). Miura et al, (2002) studied the quantitative trait loci (QTLs) controlling seed longevity in rice were recognized using 98 backcross inbred lines (BILs) which was obtained from the breeding between a *japonica* variety Nipponbare and an *Indica* variety Kasalath. The BILs seeds were stored at 30 °C for 12 months with an addition of two months at 30 °C to maintain the moisture content of the seeds at 15% and their germination percentage at 25 °C for 7 days as the degree of longevity. They also investigate the chromosomal location of QTLs for seed longevity and seed dormancy, comparatively observing the trait that seems to be controlled by various genetic factors. The BILs of Milyang 23 (Indica type) and Akihikari (Japonica-type) were stored under the normal room temperature for 1, 2, and 3 years, the QTLs region seed longevity was detected on chromosome 7 and chromosome 9 and these regions control seed longevity (Sasaki et al., 2004). Current research in *Arabidopsis thaliana* has identified 18 differentially expressed proteins in dry mature seeds following a governed disintegration treatment (85% relative humidity, 20°C) (Rajjou et al, 2008). However, there is a lack of data on how the proteome of dry seeds alters over time.

Shankar (2012) studied the seed viability of *A. malaccensis* using the germination method was reported that the weight of the seeds plays an important role in germination. The seed coat is hard and the seeds with a lighter weight give a low germination rate compared to the higher weight. It was also reported that the seed can be stored for a minimum of 14 days and a maximum of 40 days. Tabin and Shrivastava, (2014) designed an experiment to study seed viability and the effect of light intensity on seed germination. They obtained that the seed sown in double-layer polynet shade (DLPS) give a higher percentage of germination compared to single-layer polynet shade (SLPS), direct sunlight (control), and stored seeds. The viability of the seed drop drastically after 10 days of storage no germination was obtained. The *A. malaccensis* seed is recalcitrant, with an increase in storage reduces the moisture content and germination percentage (Venugopal and Marbaniang, 2017). Therefore the current study is to study the effect and relationship of protein on the viability of *Aquilaria malaccensis* seeds.

Material And Methods

Sample collection

The *Aquilaria malaccensis* seeds were collected on 18 June 2022, from the agarwood plantation of Assam Don Bosco University. The sample was surface sterilized in running tap water for 30 minutes followed by rinsing 3–5 times with sterilized double distilled water. The sample was then soaked in 0.1% Bavistin for 10 minutes and then rinsed with sterile distilled water 3–5 times. The sample was then treated with 70% ethanol for 40–60 seconds and then washed with sterile distilled water 3–5 times.

Protein Extraction

The protein was extracted using tris- HCl/trichloroacetic acid-acetone (TCA-acetone) (Zhang et al, 2012). 2 gm each of the six stages of *A. malaccensis* that is an immature seed, premature seed, matured seed, 7 days stored seed, 14 days stored seed, and 30 days stored seeds, completely frozen and grounded separately using a chill mortar and pestle with 10% PVPP into a fine powder. The fine powder was suspended in the extraction buffer containing 100mM Tris-HCl (pH 7.5), 50 mM ethylenediamine tetraacetic acid (EDTA), 100 mM potassium chloride (KCl), and 2 mM dithiothreitol (DTT) and incubated at 4 °C for 1 hour. After incubation, the liquid suspension was then centrifuged at 12,000 g at 4 °C for 15 minutes. The supernatant was collected into a fresh and sterile Eppendorf tube, and an equal volume of 10% TCA/acetone was added and incubated overnight at -20 °C for precipitation. It was then centrifuged at 12, 000 g for 15 minutes at 4 °C, the supernatant was discarded and the obtained pellet was washed three times with chill acetone to remove the salt and remaining chemicals, followed by centrifugation at 12, 000 g for 15 minutes at 4 °C, and allowed the pellet to dry at -20 °C. The dried powder was stored at -20 °C for protein estimation and SDS page.

Protein Solubilization

The dried powder was dissolved in a rehydration buffer containing (7 M urea, 2 M thiourea, 4% CHAPS, 65 mM DTT, and 0.8% pH 3–10 ampholyte). The mixture solution was incubated in a water bath sonicator for 1 hour followed by centrifugation at 12,000 g for 15 minutes at normal room temperature.

Estimation Of Protein

For the estimation of protein concentration, a Bradford method was performed.

Reagent Preparation

100 mg of Coomassie Brilliant Blue G-250 was dissolved in 50 ml of 95% ethanol and was continuously stirred for 3 hours using a magnetic stirrer, followed by the addition of 100 ml of orthophosphoric acid and was kept in a magnetic stirrer for 5 hours. The mixture was then added with sterile double distilled water to bring the volume to 1 L and then filtered using Whatman No. 1. The solution was stored in an amber bottle at 4 °C for further use.

Sample Preparation

Bovine serum albumin was used as a standard protein. A stock solution was prepared by dissolving 100 µg/ml of BSA in a rehydration buffer. Various concentration of a protein standard working solution was prepared (20, 40, 60, 80, and 100 µg/ml). In a 6 Eppendorf tube 0 to 250 µl of BSA working standard was added and make up the final volume to 250 µl with sterile double distilled water as shown in Table 1. 250 µl of the six unknown samples (test solution) was added to the Eppendorf tube separately. 250 µl of

working Bradford reagent was added and mixed gently. The tubes were incubated at normal room temperature for 5 minutes. After incubation, absorbance was taken at 595 nm using a spectrophotometer. The concentration of the unknown sample was calculated by plotting the standard curve of the concentration of the standard in micrograms versus the absorbance to determine the slope and the intercept value from the standard curve. Therefore the unknown sample concentration was determined using the following formula.

Unknown concentration (µg/ml) =

Sample O.D – intercept value

Slope value

Table 1: Absorbance of BSA protein dilution and samples for Bradford assay standard curve at 595 nm.

BSA (µg/ml)	Volume of working BSA (µl)	Distilled water (ml)	Bradford reagent (ml)	Incubation for 5 minutes at room temperature	OD values (Absorbance at 595)
0	0	250	250		0
20	50	200			0.215
40	100	150			0.350
60	150	100			0.526
80	200	50			0.666
100	250	0			0.794
Unknown T1	250	0			0.222
Unknown T2	250	0			0.344
Unknown T3	250	0			0.604
Unknown T4	250	0			0.545
Unknown T5	250	0			0.357
Unknown T6	250	0			0.330

Sample Loading

The protein samples extracted from the immature seed, premature seed, mature seed, 7 days stored seed, 14 days stored seed, and 30 days stored seed were separated based on their molecular weight on 12% Bis-tris gel. 10 µl of protein ladder and samples were loaded in each well separately. The gel was running continuously for 30 to 40 minutes at 150 V. After removal, the gel was fixed in a gel-fixing solution containing 50% ethanol and 10% acetic acid, and was incubated in the oven at 100 °C for 40–60 seconds. The gel was immersed in a staining solution (Coomassie brilliant blue solution R-50) for three to six hours at room temperature and gently rock using a rocking shaker. The stained gel was immersed in a destaining solution (50% methanol and 10% acetic acid) and gently agitated in a rocking shaker until the background of the gel becomes clear and a clear band was observed. The gel was stored in a gel storage solution containing 5% acetic acid.

Result

Estimation of the concentration of protein

Figure 1, it was observed the changes of color from brown to blue, because of the chemical reaction of protein with coomassie blue. The chemical reaction of protein extract from immature seed with the dye (coomassie blue) a bluish-green color developed and its protein concentration is 18.11 µg/ml (Fig. 2 and Table 2). The reaction of protein extract from premature seed with the coomassie blue, blue color formation was obtained and the protein concentration is 37.43 µg/ml (Fig. 1 and Table 2). The reaction of protein extract from fresh matured seed with the coomassie blue dark blue color formation was observed and the protein concentration is 58.92 µg/ml (Fig. 1 and Table 2). The reaction of protein extract from 7 days stored seed, 14 days stored seed, and 30 days stored seed with the coomassie blue, a bluish-green color formation was observed and the protein concentrations are 22.43 µg/ml, 20.14 µg/ml, and 16.08 µg/ml (Fig. 1 and Table 2). The chemical reaction of various concentrations 0 µg, 40 µg, 60 µg, 80 µg, and 100 µg of standard protein (BSA) with the coomassie blue, a dark blue color was observed (Fig. 1 and Table 2).

Table 2

Absorbance of BSA protein dilution and samples for Bradford assay standard curve at 595 nm.

Sl. No.	Volume of working BSA and Unknown (μl)	Distilled water (ml)	Bradford reagent (ml)	Incubation for 5 minutes at room temperature	OD values (Absorbance at 595)	Concentration of known (BSA) and Unknown samples (μg/ml)
1	0	250	250		0	0
2	50	200			0.215	20
3	100	150			0.350	40
4	150	100			0.526	60
5	200	50			0.666	80
6	250	0			0.794	100
Unknown T1	250	0			0.222	18.11
Unknown T2	250	0			0.344	37.43
Unknown T3	250	0			0.604	58.92
Unknown T4	250	0			0.545	23.92
Unknown T5	250	0			0.357	20.14
Unknown T6	250	0			0.330	16.08

SDS PAGE

SDS-PAGE separation of protein from various stages of *A. malaccensis* seed was observed that no band was observed from the immature seed protein extract, four light bands were observed in premature seed protein extract with a molecular weight of ~ 18 kDa to ~ 48 kDa. The protein extracted from the fresh matured seed shows five bands with a molecular weight of ~ 18 kDa to ~ 110 kDa (Fig. 3)

The protein extracted from the stored seeds of 7 days, 14 days, and 30 days were separated and purified using SDS PAGE. It was observed that protein extracted from the 7 days stored seed, two bands were observed with a molecular weight of ~ 22 kDa and ~ 28 kDa whereas protein extracted from the 14 days stored seed only one band was observed with a molecular weight of ~ 28 kDa and no band was observed on 30 days stored seed (Fig. 4).

Discussion

A seed is a reproductive tissue, in which the storage protein accumulates during the stages of seed development. Seed storage protein (SSP) acts as a nutritional source for the seed during germination and the development of the seedlings (Fujiwara et al., 2002). The most abundant SSP are proteins with low molecular weight proteins, as in wheat one-third of the total seed protein is low molecular weight (LMW) subunits of glutenin and are the second most abundant after gliadins (Singh 1991). According to research conducted by Rajjou et al. (2008) using the model organism *Arabidopsis thaliana*, it was discovered that germination rates were reduced when seeds were subjected to a controlled degradation treatment or when seeds were treated for varying amounts of time. Proteomic tests revealed that the vigor seed was unable to express its usual proteome during germination, which could explain the reduction in seed vigor.

Determination of the concentration of protein of various stages (immature seed, pre-mature seed, fresh mature seed, 7 days stored seed, 14 days stored seed, and 30 days stored seed) of *A. malaccensis* seed was observed the changes in the development and degradation of the color and concentration of the protein. Development of color and concentration of protein was observed from bluish-green (immature seed) to blue (premature seed) to dark blue (fresh mature seed). The concentration of protein detected in immature seed (18.11 µg/ml) is below the minimum concentration of standard BSA (20 µg/ml). The concentration of the protein obtained in the pre-mature seed is 37.43 µg/ml which falls in the range of 35–40 µg/ml of the standard. The concentration of the protein extracted from the fresh mature seed (58.92 µg/ml) falls in the range of 60–70 µg/ml of the standard (Table 2 and Fig. 3). The concentration of protein detected on a storage seed was observed that seed after 7 days of storage dropped to 23.92 µg/ml, at 14 days of stored seed is 20.23 µg/ml, and after 30 days of storage, the concentration declined to minimal concentration of the standard which is 16.08 µg/ml.

For the confirmation of the degradation of protein and purification of protein 1D-SDS- PAGE was performed. No band was observed in the immature seed and four light bands were observed in a premature seed with molecular weight ranges approximately from 18 kDa to 63 kDa. Five bands were observed from fresh mature seed samples in SDS PAGE with a molecular weight ranging approximately from 18 kDa to 110 kDa, the bands are thick and dark (Fig. 3). Two bands were observed in 7 days of stored seed, the band with a molecular weight of ~ 22 kDa shows a light band whereas the band with a molecular weight of ~ 28 kDa is thick. The 14 days stored seed show only a band with a molecular weight of ~ 28 kDa and no band was observed in the 30 days stored seed. Hence, it was observed that with maturity development of protein was observed, but with an increase in storage, the protein gets degraded. Various researchers reported that the desiccated seed has a low shelf life, due to the effect of storage as it reduces moisture content (Walters, 1998; Walters et al, 2005) and increases the accumulation of the reactive oxygen species (ROS) which affect the seed viability and germination as it damages the macromolecules (DNA, RNA, and protein) (Kurek et al, 2019). There is mounting evidence that free radical formation during storage is the primary cause of membrane disruption, protein and nucleic acid damage, cell organelle deterioration, and eventual seed aging (Bailly 2004; Kibinza et al,

2006). Seed storage protein (SSP) buffered the seed from the oxidative stress that deteriorates the seed and protects the required proteins from degradation which are essential for germination and seedling development (Nguyen et al, 2015). Lack or absence or degradation of the protein during storage might affect the seed germination and viability of the seed.

Conclusion

Proteomics is the most useful tool to study the biological roles and functions of individual proteins and to figure out the molecular processes that control seed germination, vigor, and viability as a result of aging. Seed storage proteins are proteins that are developed in the developing seed, to maintain the oxidative stress of seeds to prevent other proteins from degradation, and also act as a nutritional source of seed germination. In this current study, it was observed that the concentration of protein increases with maturity but with an increase in storage, the concentration of the protein reduces. In 1D-SDS PAGE proteins were separated based on the molecular weight, it was obtained that the protein extracted from the immature seed no band was observed. The protein extracted from the premature seed show four light bands with a molecular weight of approximately 18 kDa to 48 kDa, whereas the fresh mature seed show five thick bands with a molecular weight of approximately 18 kDa to 110 kDa. The changes of a protein band in the seed which stored for 7 days, 14 days, and 30 days were observed. The 7 days stored show only two bands with a molecular weight of approximately 22 kDa to 28 kDa, whereas 14 days stored seed show only one band (MW ~ 28 kDa) and no band was observed in 30 days stored seed. Therefore the changes or loss of protein in the storage seed might be the reason that affects the viability and germination of the *A. malaccensis* seed. Further studies on seed viability can be done to prevent the protein from degrading, and loss of moisture content and to control and to control the high accumulation of free radicals in cell organelles during storage.

Declarations

Ethical Approval

Not applicable as the study did not involve any animals or human.

Conflict of interest

The authors declare that they do not have any conflict of interest.

Author's contribution

Both authors contributed to the study's conception and design. Material preparation, data collection, and analysis were performed by SK. Planning, supervision of the experiment, and correction of the manuscript by ST.

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Not applicable

Availability of Data and materials

The availability of data and materials for the study is “Not applicable”.

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Figures

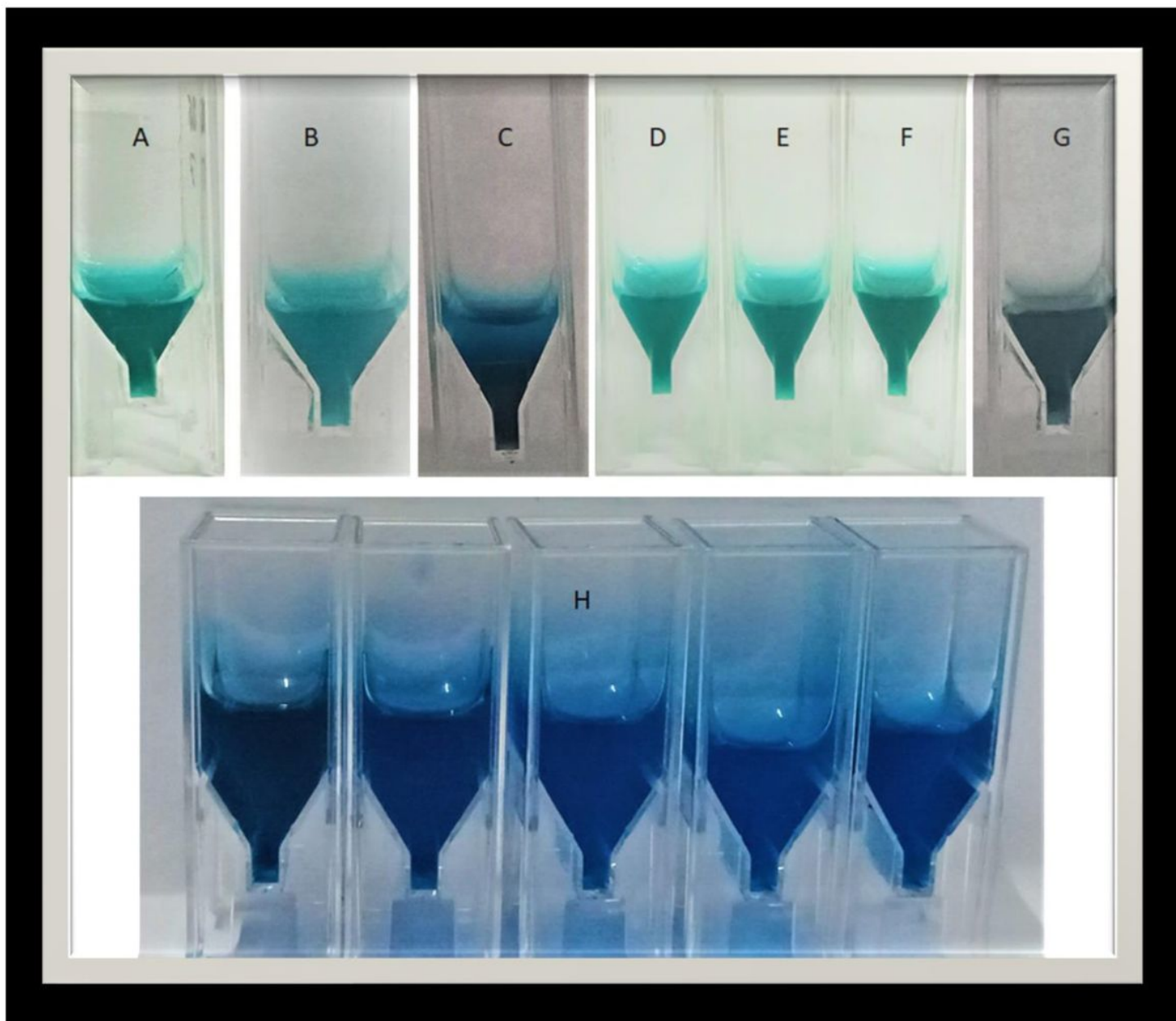


Figure 1

Estimation of protein extracted from various stages of *A. malaccensis* (A) immature seed (B) premature seed (C) Fresh seed (D) 7 days stored seed (E) 14 days stored seed (F) 30 days stored seed (G) control (H) positive control (BSA).

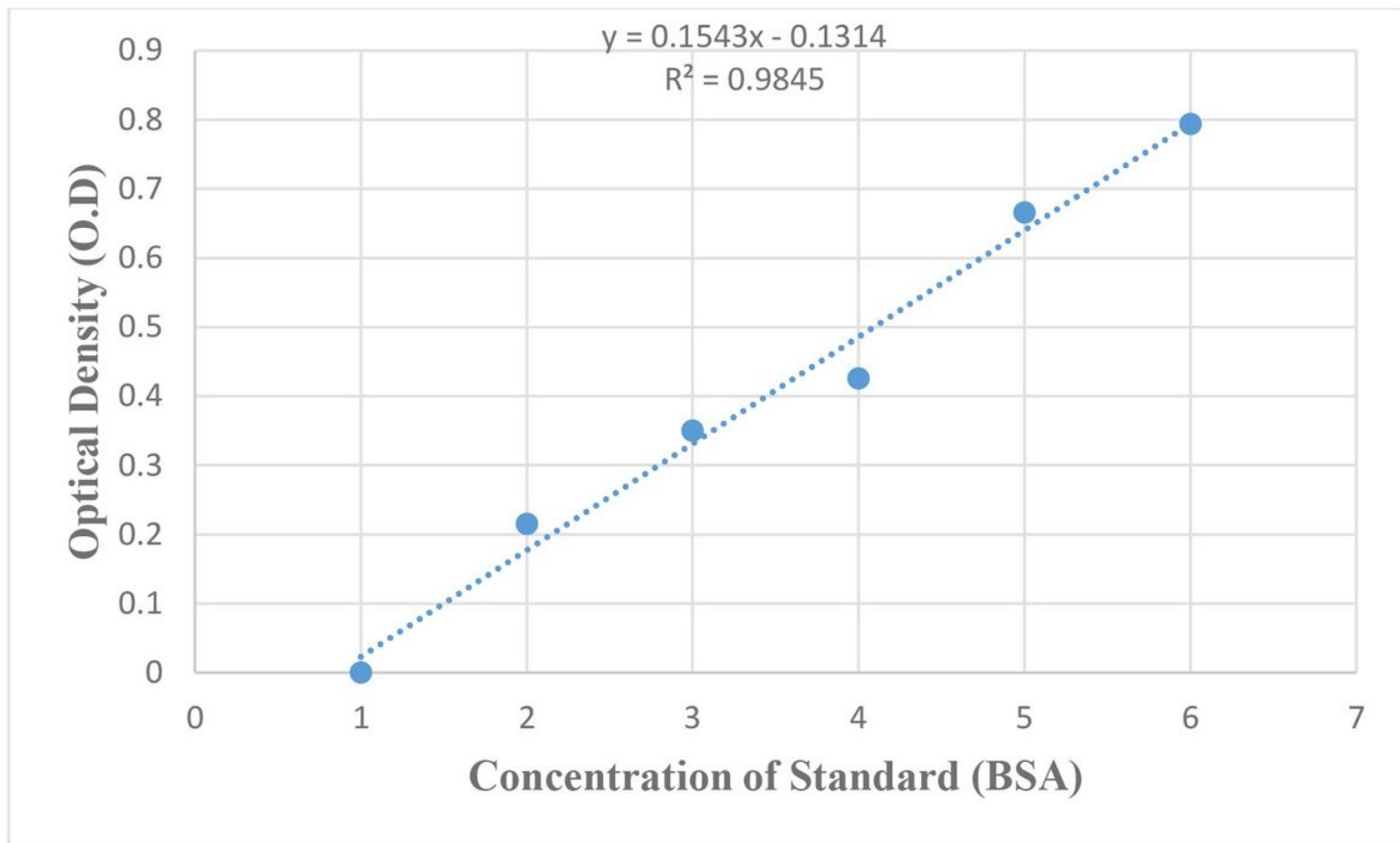


Figure 2

Protein standard curve

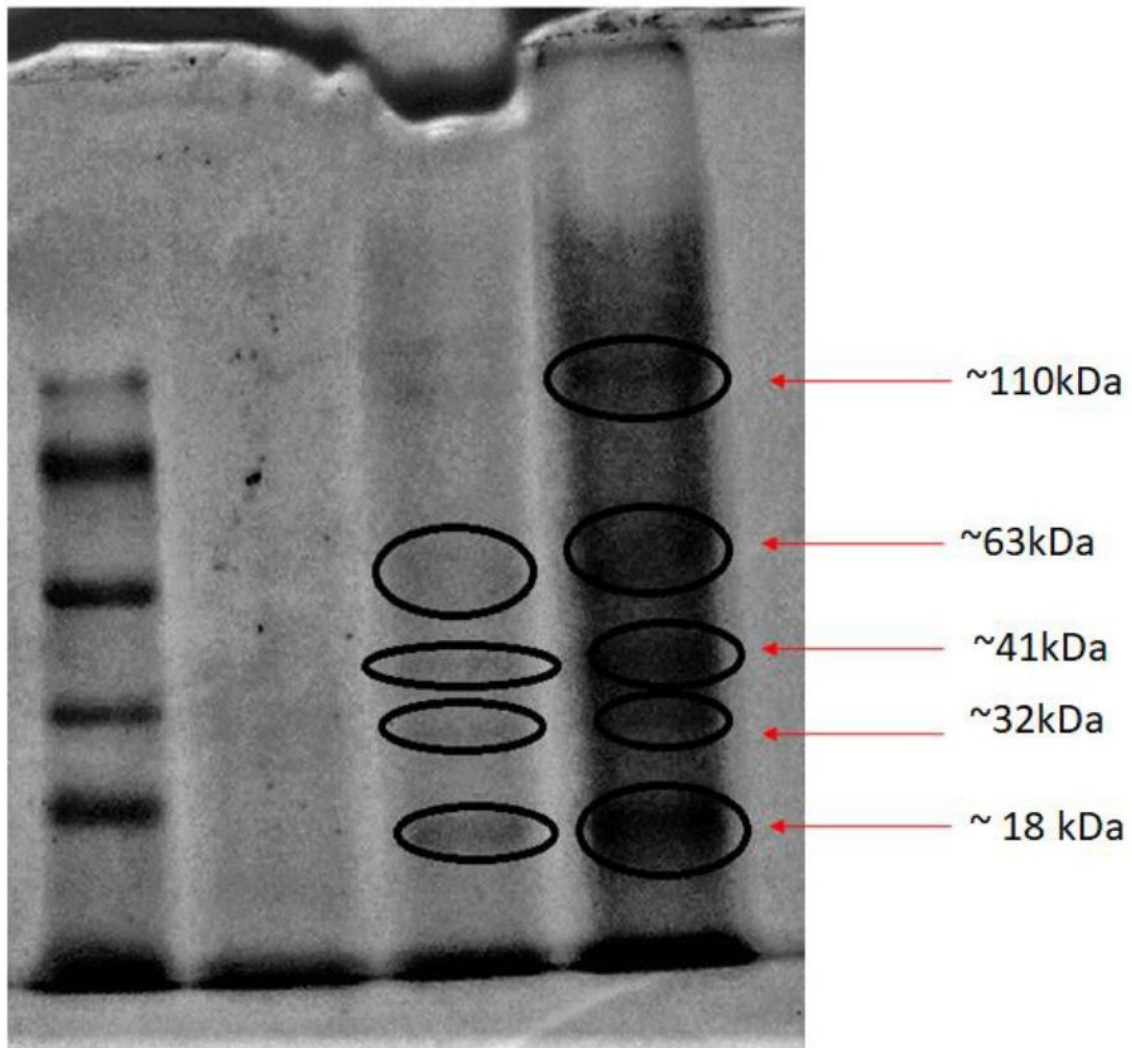


Figure 3

SDS PAGE analysis of *A. malaccensis* (A) protein molecular marker (B) protein extracted from immature seed (C) protein extracted from premature seed (D) protein extracted from the fresh mature seed.

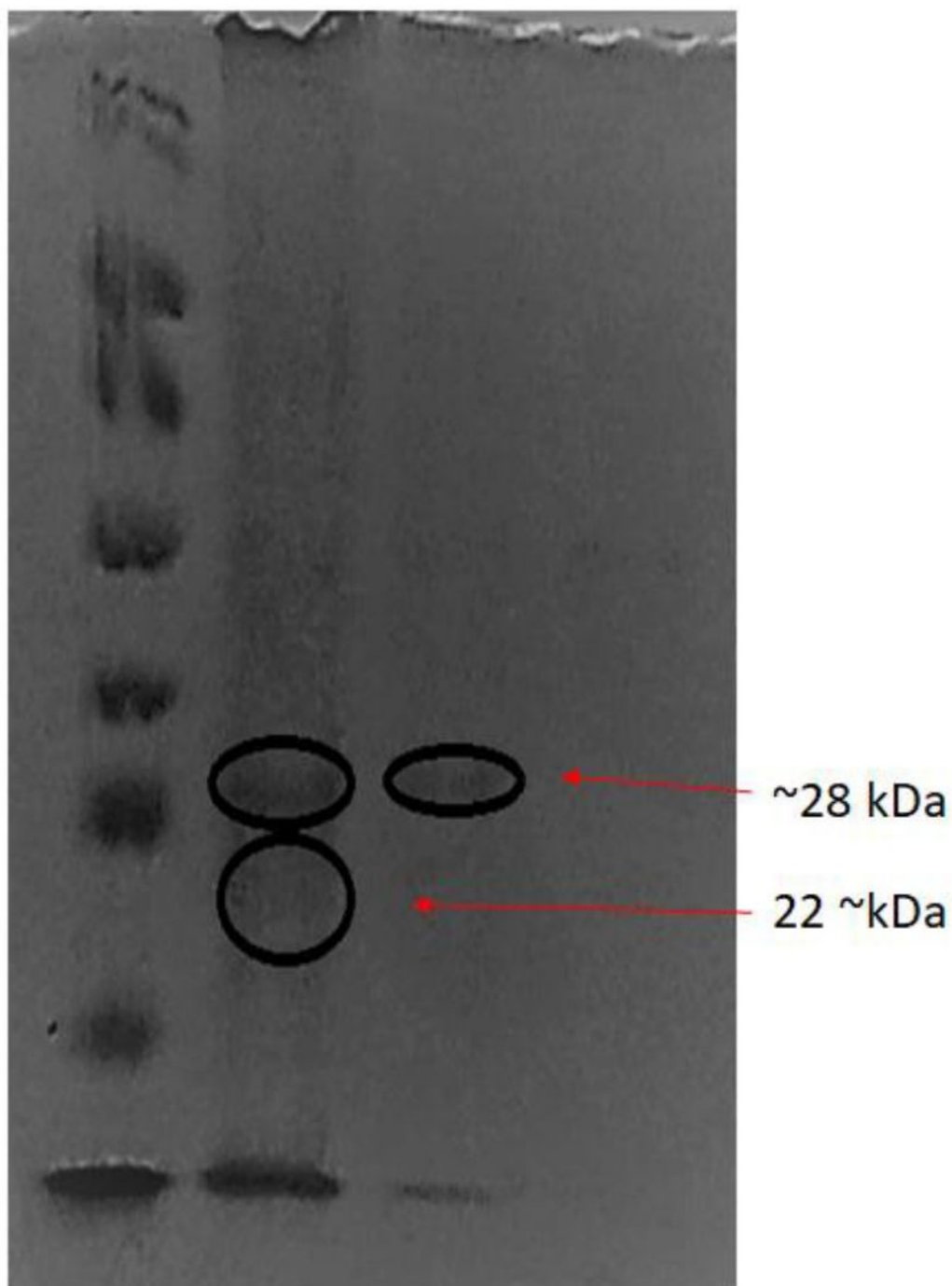


Figure 4

SDS PAGE analysis of *A. malaccensis* (A) protein molecular marker (B) protein extracted from 7 stored seed (C) protein extracted from 14 days stored seed (D) protein extracted from 30 days seed