

**PROTEOMIC EXPRESSION OF PHYSIOLOGICAL DISORDER
SYNDROME IN LONGAN (*DIMOCARPUS LONGAN* L. "DAW") ON
INITIAL STAGE USING ONE-DIMENSIONAL ELECTROPHORESIS
COUPLED WITH LC-MS/MS**

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ABSTRACT

Comparative study on leaves, peels and seeds of longan (*Dimocarpus longan* Lour. var. Daw) at Prao and Mae Taeng district in Chiang Mai, was compared between normal and physiological disorder syndrome in longan on initial fruit by non-native conditions in electrophoresis gel with staining gel by Coomassie brilliant blue R-250 following liquid chromatography mass spectrometry (LC-MS/MS). The results showed that plasma membrane-associated cation-binding protein 1 (19.5 kDa) was found in decreased protein on abnormal longan at Prao district at 20 weeks after fruition. The vegetative storage protein (25.2 kDa) was also found at the seed in Prao district similar to the results from Mae Taeng district presented biomarkers at leaves, peel and seed since disorder on initial stage (20 weeks after fruition) to harvest stage (26 weeks after fruition). It was noted that leaves and seeds of abnormal longan is higher in protein than normal longan but lower in protein than in peel. The abnormal longan in seed at 24 weeks after fruition was not found three biomarkers, triosphosphate isomerase-like protein type 1 (27.4 kDa), hypothetical protein

(24.8 kDa) and peroxidase 4 (39.7 kDa). These proteins may be important protein in part of the recovery process of abnormal longan.

KEYWORDS: Longan, Leaves, Peel, Seeds, Physiological Disorder Syndrome, Electrophoresis

1. Introduction

Physiological disorder syndrome in longan peel of 'Daw', native name "red skinned longan", is classified into two types. The first type of syndrome on mature stage, is studied regarding fruit cracking during fruit transportation on low temperature to consumer [1]. Characteristics of this syndrome in fruit cracking are different from fruit cracking cause from climatic factors such as drought, high temperature and excessive water [2] because of no representation of fruit cracking in tree and weight and size. This type is not different from normal fruit except color of peel skin that is more reddish or brownish than normal fruit. Cracking incidence of longan peel in the physiological disorder syndrome on mature stage implicated in quantity of biomarker proteins as same as protein changing in harvest period of longan fruit [3]. Previous study [4] showed vegetative storage protein (25.2 kDa), an important protein on storage metabolism in the plant's food, and cytosolic class I small heat shock protein (15.6 kDa), having important implications in plant stress tolerance, represented in physiological disorder syndrome on mature stage at Hod district. The second types of syndrome on initial stage, was studied about no growth in fruit during fruit development on initial stage (20 weeks after fruition) to mature stage (26 weeks after fruition). Proteomic methods were applied to indicate protein profiles in between physiological disorder syndrome and normal longan fruit in leaves, peel and seed and to predicate biomarker protein. These biomarkers may disturb normal growth and development of longan fruit at Prao and Mae Taeng district. It would be possible with protein identification in biomarkers on leaves, peel and seed by using suitable protein extraction methods of leave [5], peel [6] and seed [7] in previous studies. These approaches were determined using qualitative and quantitative gel electrophoresis methods including sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). This experiment compared the leaves, peel and seed proteins extracted by each suitable extraction method for using in protein identification of biomarker for physiological disorder syndrome on initial stage.

2. Materials and Methods

2.1 Samples of Leaves, Peels and Seeds of Longan

Physiological disorder syndrome and normal longan fruit on initial stage (syndrome occurring at 20 weeks after fruition) in leaves, peel and seed were collected for five samples from Prao district (no fruit maintenance after this syndrome occurring, only at 20 weeks after fruition) and Mae Taeng district (fruit maintenance on 8 weeks after this syndrome occurring, at 20 to 26 weeks after fruition). These components were investigated in physiological measurements such as weight (g), size (wide & long), and colour (L, a and b values). Then leaves, peel and seed were blended into small pieces with liquid nitrogen using a prechilled mortar and pestle for sample grinding and stored at -20 ° C to enter the next protein extraction.

2.2 Protein Extraction

Prior to each type extraction of longan, 1.00 g powder of longan was transferred to a 15-mL tube containing the respective extraction solution and mixed as described below. Suitable protein extraction methods of leave (extraction solution of PVPP-Tris-HCl buffer solution in pH 6.8), peel (100 mM Tris-HCl pH 8.0, 50 mM L-ascorbic acid and 100 mM KCl) and seed (extraction solution of to 100 mM Tris-HCl pH 8.0, 50 mM L-ascorbic acid, 100 mM KCl, 50 mM disodium tetraborate decahydrate, 1% (v/v) Triton X-100, 2% (v/v)β-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride) were extracted and mixed as described following Kongkaew [5], Reinthong [6] and Chamnan [7], respectively. Solution of three protein extractions was stored at -20 °C to enter the next protein electrophoresis.

2.3 One-Dimensional Gel Electrophoresis

Each extraction type was used for quantitative analysis by modified Lowry's method [8] and was used SDS-PAGE for protein band-picking. The extraction was mixed with a sample buffer containing 0.0625 M Tris HCl, pH 6.8, 10% SDS, 10% glycerol, 0.1% 2-mercaptoethanol and 0.01% bromophenol blue. In brief, the final concentration of each protein extraction was adjusted to 100 µg, boiled for 5 min and stored at -70°C until electrophoresis. SDS-polyacrylamide denaturing gels, stacking gels (4%) and separating gel (12.5%) were used for separating different range of protein extraction. Samples of 100 µg

were applied to gels in different experiments. Molecular weight marker was in broad range (Bio-Rad CL161-0318). Electrophoresis was carried out using a vertical slab gel electrophoresis apparatus (AE-6530 mPAGE, Atto Corporation, Tokyo, Japan) with electrode buffer solution (0.025 M Tris, 0.192 M glycine, 0.1% SDS at pH 8.3). Constant current (20 mA/gel) was applied to the electrophoresis gel. After electrophoresis, gels were stained with 0.25% Coomassie brilliant blue R250 in 90 ml of methanol: acetic acid: water (5.7: 1: 7.5). In Coomassie blue R-250 staining, gels were fixed in a solution of 50% methanol and 10% acetic acid for 1 h, stained for 15 min in conventional Coomassie blue R-250 solution, and developed in de-staining solution (25% methanol and 7% acetic acid) for overnight prior to scanning.

2.4 Gel Analysis

Each gel was scanned on the image scanner (Gel Doc XR System, Bio-Rad, USA), gel documentation system consisting of a high sensitivity multichrome camera according to the manufacturer's specifications. Differential protein bands between physiological disorder syndrome and normal longan fruit were excised for next step on mass spectrometry identification.

2.5 Mass Spectrometry and Protein Identification

Approximately, 6 bands of selected protein were picked manually from only preparative gels in Coomassie blue R-250 staining using sterile lancet. Selected bands were investigated protein analysis and peptide mass profiling using liquid chromatography tandem mass spectrometry (LC-MS/MS) in Research Instrument Center of Khon Kaen University, Thailand. Mass spectrometry data were automatically registered, analyzed, and searched by using National Center for Biotechnology Information public protein databases (NCBI databases). MASCOT (Matrix Science Ltd., London, UK) search engines were used for peptide mapping. Data identifications were registered when searching results of protein score greater than 52 were significant ($p < 0.05$).

3. Results and Discussion

3.1 Physiological Differences

Determination of physiological difference between physiological disorder syndrome and normal longan fruit on initial stage (after 20 weeks after fruition) in fruit and leave at Prao and Mae Taeng district was shown in Figure 1. Morphology and characteristic phenotype showed that group of physiological disorder syndrome was red skinned fruit. These values of weight and size on fruit, seed and leave (data not shown) were no significant difference between both groups. Physiological disorder syndrome had effects on some color values, especially in a and hue values at 20 weeks after fruition on both Prao and Mae Taeng district (Table 1). Data revealed that these values had efficiency to promote red skinned fruit production as same as previous study (4) in physiological disorder syndrome on mature stage (after 30 weeks after fruition) at Hod district. However, fruit garden in Mae Taeng district had processes of fruit maintenance after this syndrome occurring. Therefore, color values did not show any difference from normal group on 22, 24 and 26 weeks after fruition.

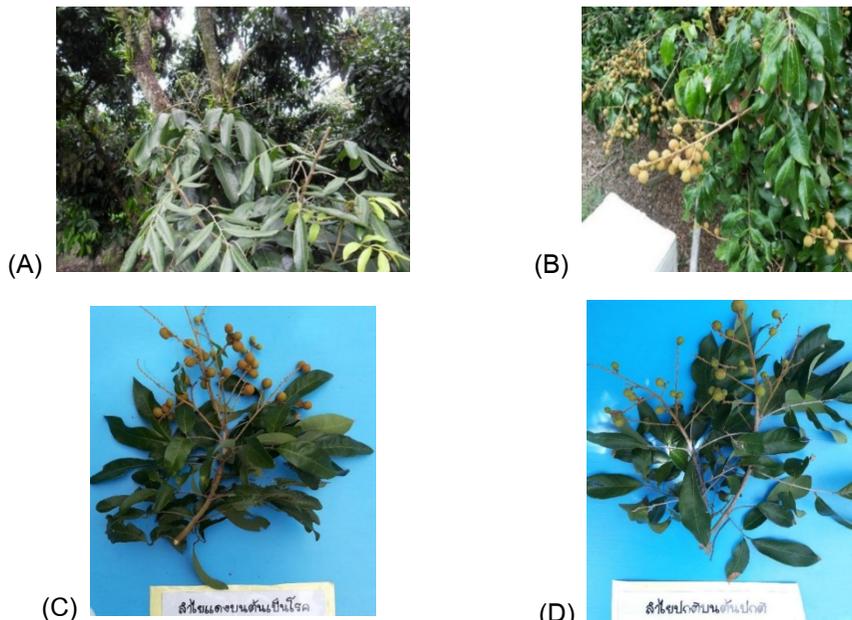


Figure 1 The presence of physiological disorder syndrome of fruit and leave on initial stage on fruit garden at Prao (A) and Mae Taeng (B) district, Chiang Mai province, after 20 weeks after fruition, morphology of fruit on physiological disorder syndrome (C) was reddish than normal group (D).

Table 1 Color values in longan peel were determined in physiological disorder syndrome (PDS) and normal longan fruit on fruit growth (20 samples/group) at Prao and Mae Taeng district, Chiang Mai province.

Weeks after fruition	Color values in peel of longan fruit				
	(L)	(a)	(b)	Chroma	Hue
Prao district					
20 (Normal)	41.55 ± 2.54	5.11 ± 0.98	34.06 ± 2.15	36.88 ± 3.19	81.91 ± 1.33
20 (PDS)	42.49 ± 2.55	8.89 ± 1.28*	30.99 ± 2.88	33.11 ± 3.92	73.59 ± 1.53*
Mae Taeng district					
20 (Normal)	42.87 ± 2.15	5.52 ± 1.03	34.96 ± 2.21	35.34 ± 2.19	80.93 ± 1.96
20 (PDS)	43.56 ± 2.14	8.65 ± 1.06*	29.78 ± 2.94	30.11 ± 4.92	74.02 ± 1.37*
22 (Normal)	42.55 ± 2.70	5.44 ± 1.80	33.95 ± 2.31	34.89 ± 3.67	79.65 ± 1.86
22 (PDS)	43.88 ± 2.96	8.00 ± 2.79	30.44 ± 3.01	30.82 ± 3.31	74.23 ± 4.26
24 (Normal)	42.10 ± 3.22	5.20 ± 1.15	33.80 ± 3.31	33.48 ± 2.02	77.01 ± 6.99
24 (PDS)	44.01 ± 3.96	7.99 ± 2.55	31.00 ± 3.26	30.99 ± 3.76	74.87 ± 8.12
26 (Normal)	46.59 ± 1.99	6.45 ± 1.47	32.66 ± 2.65	31.93 ± 2.18	77.11 ± 3.07
26 (PDS)	48.32 ± 1.47	7.25 ± 2.14	32.58 ± 1.22	33.15 ± 1.32	75.07 ± 1.81

* Significantly different from normal.

3.2 Quantitative Differences

Determination of protein quantitation from leave, peel and seed on initial stage on fruit garden at Prao and Mae Taeng district, Chiang Mai province, was shown in Table 2. The results showed that the protein extraction of peel and seed on physiological disorder syndrome at 20 weeks after fruition (In this time, peel could be not separated from seed to extract protein) had higher protein content than normal in both districts. In Mae Taeng district, there was fruit maintenance after this syndrome occurring (at 26 to 26 weeks after fruition) and this syndrome might have the potential to significantly differ the protein content over

normal. It is possible that internal metabolism of this syndrome can be involved in some proteins.

Table 2 The concentration of the protein solution (Mean \pm SD) from physiological disorder syndrome (PDS) on initial stage compared with normal longan on SDS-PAGE analysis of leave, peel and seed was investigated by modified Lowry 's method with the BSA standard curve and the absorbance at a wavelength of 480 nm.

Fruit garden	Weeks after fruition	Components of longan	The concentration of the protein solution ($\mu\text{g}/\mu\text{l}$)	
			Normal	PDS
Prao district	20	leave	36.37 \pm 1.82	33.77 \pm 1.69
		peel and seed	9.18 \pm 4.95	15.36 \pm 0.77*
Mae Taeng district	20	leave	10.66 \pm 0.53	10.47 \pm 0.52
		peel and seed	5.90 \pm 0.30	10.53 \pm 0.53*
		22	leave	7.23 \pm 0.36
	24	peel	13.39 \pm 0.42	16.25 \pm 0.32
		seed	28.88 \pm 0.58	29.65 \pm 0.59
		24	leave	6.37 \pm 0.32
	26	peel	5.42 \pm 0.27	10.41 \pm 0.52*
		seed	23.90 \pm 0.48	25.65 \pm 0.51
		26	leave	15.32 \pm 0.77
	peel	19.33 \pm 0.39	6.76 \pm 0.34*	
	seed	19.33 \pm 0.19	38.85 \pm 0.39*	

* Significantly different from normal.

3.3 Gel Analysis of Protein in SDS-PAGE

The protein expression profiles of leave, peel and seed in physiological disorder syndrome and normal longan fruit on initial stage were examined in Figure 2 at Prao district and Mae Taeng district in Figure 3, 4 and 5 (leave, peel and seed, respectively). In Mae Taeng district, there were physiological disorder syndrome and normal longan fruit on initial stage (26 to 26 weeks after fruition) because of fruit maintenance after this syndrome occurring.

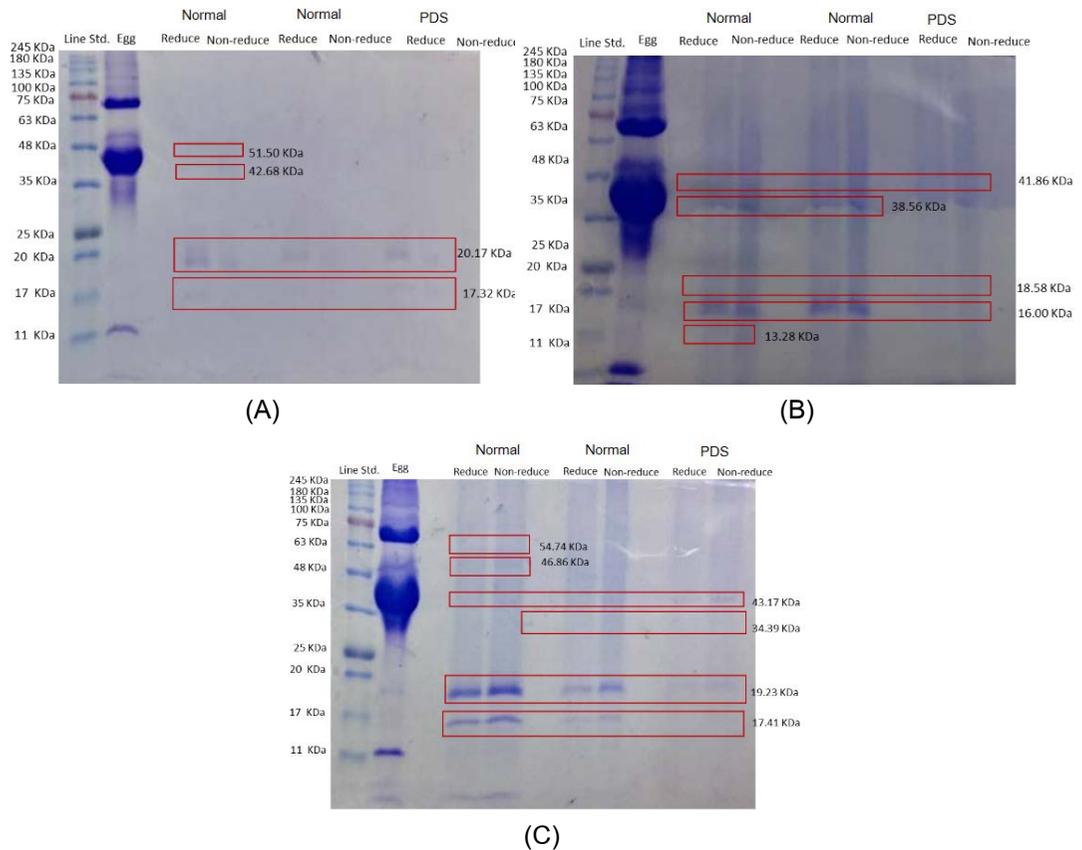


Figure 2 The presence of gel analysis of protein in longan extracted between physiological disorder syndrome (PDS) and normal longan fruit on initial stage at 20 weeks after fruition in three compositions; leaves (A), peel (B) and seed (C) at Prao district with Coomassie brilliant blue R-250 staining on 12.5% gel at 100 µg/well gel. Notice; Line Std. = Standard protein marker, Line Egg = Egg albumin, Notice: P1, P2, P3 and P4 are 20, 22, 24 and 26 weeks after fruition, respectively.

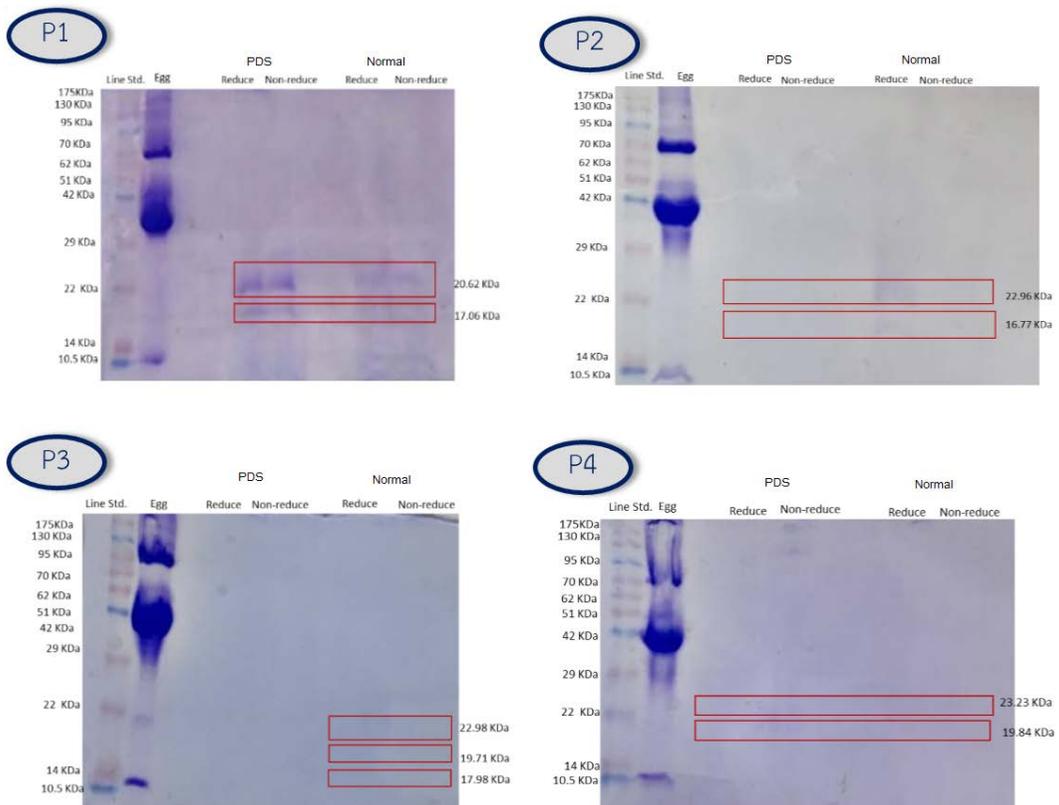


Figure 3 The presence of gel analysis of protein in longan extracted between physiological disorder syndrome (PDS) and normal longan fruit on initial stage (20 to 26 weeks after fruiting) in leave at Mae Taeng district with Coomassie brilliant blue R-250 staining on 12.5% gel at 100 µg/well gel. Notice; Line Std. = Standard protein marker, Line Egg = Egg albumin, Notice: P1, P2, P3 and P4 are 20, 22, 24 and 26 weeks after fruiting, respectively.

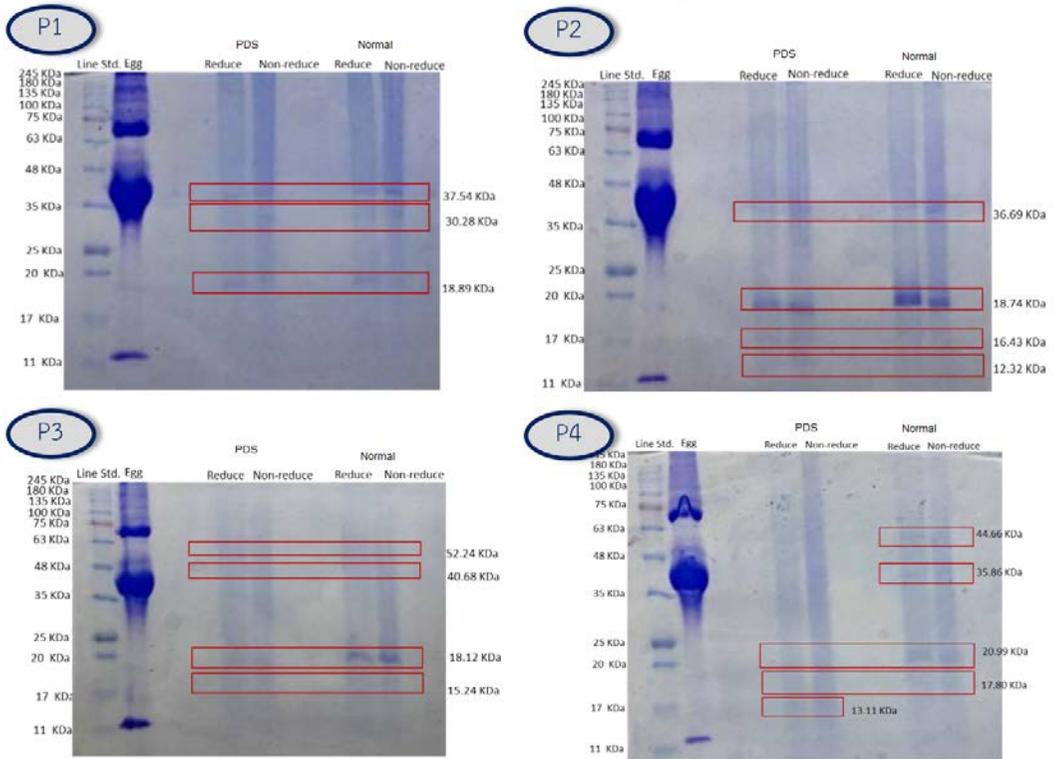


Figure 4 The presence of gel analysis of protein in longan extracted between physiological disorder syndrome (PDS) and normal longan fruit on initial stage (20 to 26 weeks after fruition) in peel at Mae Taeng district with Coomassie brilliant blue R-250 staining on 12.5% gel at 100 µg/well gel. Notice; Line Std. = Standard protein marker, Line Egg = Egg albumin, Notice: P1, P2, P3 and P4 are 20, 22, 24 and 26 weeks after fruition, respectively.

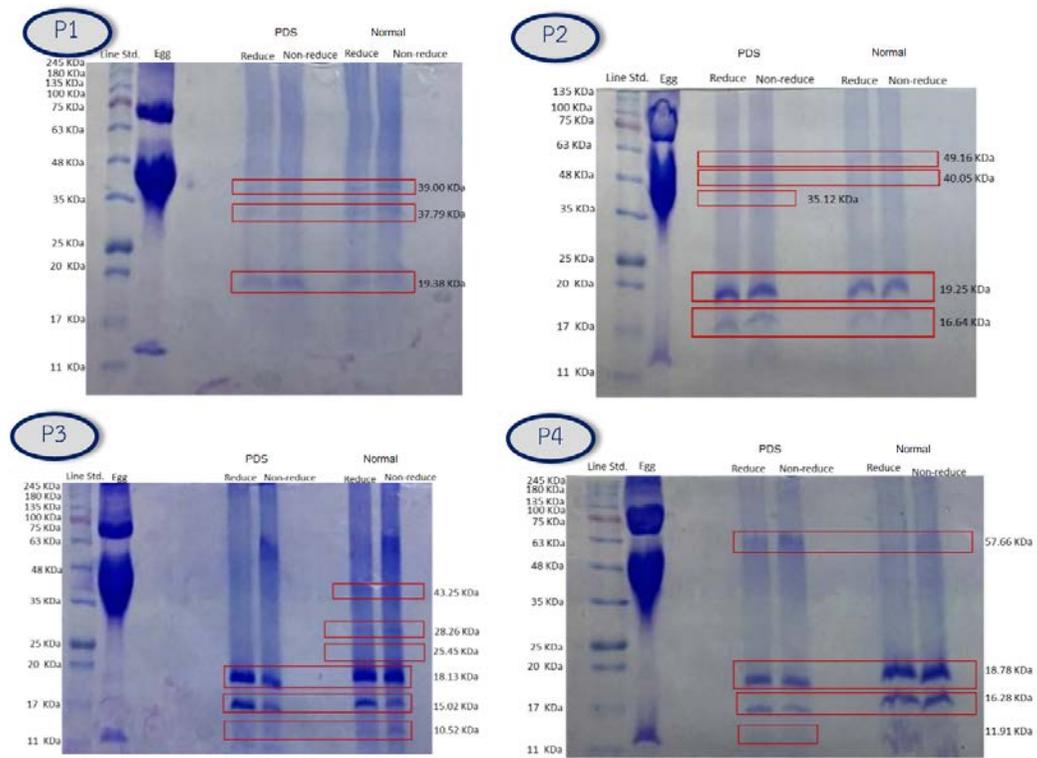


Figure 5 The presence of gel analysis of protein in longan extracted between physiological disorder syndrome (PDS) and normal longan fruit on initial stage (20 to 26 weeks after fruition) in seed at Mae Taeng district with Coomassie brilliant blue R-250 staining on 12.5% gel at 100 µg/well gel. Notice; Line Std. = Standard protein marker, Line Egg = Egg albumin, Notice: P1, P2, P3 and P4 are 20, 22, 24 and 26 weeks after fruition, respectively.

To determine in the protein identification by using LC-MS / MS, some selected proteins were extracted from preparative gels stained with Coomassie brilliant blue R-250. A summary of selected abundant proteins of biomarker proteins extracted was demonstrated in Table 3. The MS analysis confirmed that physiological disorder syndrome on initial stage at 20 weeks after fruition had protein expression of vegetative storage protein (25.2 kDa), a bioactive in herbaceous plants or trees, has distributed in mature leaves, bark tissues of branches, trunk and large roots, on leave, peel and seed of longan at both Pao and Mae Taeng district. Moreover, it was found at the seed in Prao district similar to the results from Mae Taeng district presented biomarkers at leaves, peel and seed since disorder on initial stage at 20

weeks after fruition to harvest stage at 26 weeks after fruition. It has been reported that this protein is reduced during fruiting [9] and responding to specific stimuli (photoperiod, chilling accumulation, rest-breaking treatment, and wounding) [10]. It may be involved with biochemical function in physiological disorder syndrome on initial stage as well as mature stage in previous study [4]. Plasma membrane-associated cation-binding protein 1 (19.5 kDa), a protein involved on intracellular signaling in peels [11], was found in decreased protein on abnormal longan at Prao district. In addition, triosphosphate isomerase-like protein type 1 (27.4 KDa), hypothetical protein (24.8 kDa) and peroxidase 4 (39.7 kDa), were identified to biomarker protein in longan seed corresponding to this syndrome in 24 weeks after fruition. Thus, these proteins play many important roles in this experiment that are basic to biomarker function.

Table 3 Some Mascot databases of MS/MS score identified with ≥ 52 confidence protein within individual protein bands of physiological disorder syndrome compared with normal longan on SDS-PAGE analysis of leave, peel and seed in reducing condition, 7 cm, 12.5% gel staining techniques by Coomassie brilliant blue R-250 at 100 μ g of protein. Notice: P1, P2, P3 and P4 are 20, 22, 24 and 26 weeks after fruition, respectively.

District	Composition of longan	MW (kDa)	Types	Score	Type of expression
Prao	Leaves	N/A	N/A	N/A	N/A
	Peels	19.5	Plasma membrane associated cation-binding protein 1 [Theobroma cacao]	56	P1 (paler)
	Seeds	25.2	Vegetative storage protein [Litchi chinensis]	75	P1 (paler)
Mae Taeng	Leaves	25.2	Vegetative storage protein [Litchi chinensis]	60	P1 (darker), P2 (paler) and P3 (not appear)
			Vegetative storage protein [Litchi chinensis]		
	Peels	25.2	Vegetative storage protein [Litchi chinensis]	65	P1, P2 and P3 (paler)

Table 3 (continued) Some Mascot databases of MS/MS score identified with ≥ 52 confidence protein within individual protein bands of physiological disorder syndrome compared with normal longan on SDS-PAGE analysis of leave, peel and seed in reducing condition, 7 cm, 12.5% gel staining techniques by Coomassie brilliant blue R-250 at 100 μ g of protein. Notice: P1, P2, P3 and P4 are 20, 22, 24 and 26 weeks after fruition, respectively.

District	Composition of longan	MW (kDa)	Types	Score	Type of expression
Mae Taeng	Seeds	25.2	Vegetative storage protein [Litchi chinensis]	62	P1 and P2 (darker), P3 and P4 (paler)
		39.7	Peroxidase 4 [Litchi chinensis]	210	P2 (appear) and P3 (not appear)
		24.8	Hypothetical protein PHAVU_009G035900g [Phaseolus vulgaris]	56	P3 (not appear)
		27.4	triosphosphate isomerase- like protein type I [Dimocarpus longan]	71	P3 (not appear)

4. Conclusion

In conclusion, there are two points highlighted by these data. First, protein quantitation from leave, peel and seed on initial stage on fruit garden at Prao and Mae Taeng district, Chiang Mai province, has the potential to significantly differ the protein content over normal in no physiological differences. Secondly, It is possible that vegetative storage protein (25.2 kDa), plasma membrane-associated cation-binding protein 1 (19.5 kDa), triosphosphate isomerase-like protein type 1 (27.4 kDa), hypothetical protein (24.8 kDa) and peroxidase 4 (39.7 kDa) have involved biochemical function in physiological disorder syndrome on initial stage. Finally, aim of this study is to screen biomarker proteins in physiological disorder syndrome so as to be able to demonstrate their biological functions, and to identify their properties with improving on longan gardening.

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