Chapter 4

Physical properties, internal structure, protein distribution and accumulation in the endosperm and milling quality of rice with different grain N concentration

4.1 Introduction

In Chapter 3, results had reported that N fertilization was able to improve milling quality such as head rice yield of some Thai rice varieties by increasing grain N concentration, but the effects of N on physical properties, internal structures, and storage protein accumulation and distribution in rice endosperm of the studied varieties are still unknown. Reporting of N fertilizer application could increasing head rice yield, have been done by many works. They suggested that the higher grain N increases the protein matrix around starch granules (Juliano, 1985a). However, a vary limited findings related to these works has been found in the literatures. In order to explain how high grain N concentration can effect on grain breakage during milling process, roles of grain N concentration on protein accumulation and distribution in different parts of rice endosperm should have priority to understand and verify.

Grain physical properties are grain size and shape. Price of commercial rice varieties are considered primarily according to grain size (weight), shape (physical dimensions) and uniformity of rice grains. Efferson (1985) reported that slender long grain is the most demand in the world market. Physical property improvement, therefore, are the primary objective in any breeding program (Juliano, 1985a).

The bulk of the rice grain consumed is composed of storage tissue, known as endosperm, that nourishes the embryo during germination and early seedling growth. The endosperm consists of thin-walled cells, usually elongated radially on cross-sectional view, and is filled with compound starch granules and some protein bodies (Juliano, 1985a). Very little information is available on the physical properties and internal structures of the endosperm of indica rice, which includes Thai rice. Most of the information on internal structure of rice has learned from studying of japonica rice.

Rice storage proteins localize mainly in proteinaceous organelles called protein bodies (PBs). In cereals, the PBs present in the starchy endosperm are the main site of accumulation of storage protein. The rice endosperm protein is localized mainly in two types of protein bodies: spherical (PB-I) and irregular-shaped (PB-II). These two types of protein bodies are different in density, shape and protein composition. PB-II size (2-3 μm in diameter) is larger than PB-I and can be stained uniformly with osmium tetroxide, uranyl acetate and lead citrate (Bechtel and Juliano, 1980; Tanaka et al., 1980). PB-II contains glutelin and globulin, whereas prolamin is localized in PB-I (Ogawa *et al.*, 1987). In term of soluble proteins, the major storage protein fraction of rice grain is glutelin (alkali soluble protein) which may constitute up to 75% of the grain protein. The prolamin fraction (alcohol soluble protein), which is the predominant storage protein in other cereals, comprise 5-10% and the rest is albumin and globulin fractions (acid soluble protein) (Basak *et al.*, 2002; Villareal and Juliano, 1978). Asano *et al.* (2000) reported that brown rice consisted of 18-20% albumin and globulin fractions, 12-15% prolamin and 66-68% glutelin.

The objectives of this study is to investigate how levels of grain N concentrations affect the physical properties, internal structures, concentration of soluble protein families, and the accumulation/distribution of storage protein in the different parts of the rice endosperm, on which relate to grain breakage resistance during the milling of some Thai rice varieties.

4.2 Materials and methods

4.2.1 Materials

Brown, head and broken rice of KDML105, KLG1, PTT1 and CNT1 varieties which contained low (1.3%) and high (2.0%) N concentrations, were obtained from the nil N and 120 kg N ha^{-1} applied at flowering treatments in experiment 2 (Chapter 3) and used for the study materials in this chapter. Details of study were showed in Table 4.1.

4.2.2 Physical properties of rice grain

Brown rice, 100 grains of each variety of low and high N concentrations, and 3 replications, were weighed and measured for length, width (length from dorsal to ventral sides) and thickness (length from both lateral sides) using a vernier caliper (Mitutoyo, Japan) (Figure 4.1). The length/width ratio was calculated and used to classify the physical grain properties with the following categories: extra long grain (grain length > 7.50 mm); long grain (6.61-7.50 mm); medium grain (5.51-6.50 mm) and short grain (< 5.50 mm). The length/width ratio was classified for grain shape using the following categories: slender (length/width ratio > 3.0); medium (2.0-3.0); bold (1.1-2.0) and round (< 1.1) (Jennings *et al.*, 1979).

Table 4.1 Details of materials used in the different methods and the purposes of the investigation

Method	Materials	Purpose
Physical properties	Brown rice of KDML105, KLG1, PTT1 and CNT1 with low and high grain N concentration	Determine effect of N on physical properties
Glycol methacrylate (GMA)	Brown and head rice of KDML105, KLG1, PTT1 and CNT1 with low and high grain N concentration	Determine accumulation and distribution of storage protein
Spurr's resin	Brown rice of KDML105 and CNT1 with low and high grain N concentration	Determine type of protein bodies
Scanning electron microscopy (SEM)	Head rice of KDML105 and CNT1 with low and high grain N concentration	Determine location of storage protein
Protein extraction	Brown, head and broken rice of KDML105, KLG1, PTT1 and CNT1 with low and high grain N concentration	Determine classes of soluble proteins

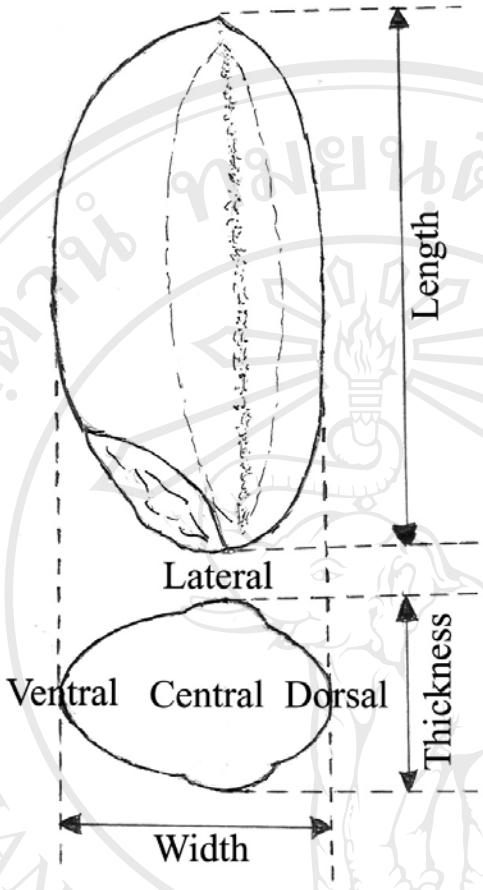

Figure 4.1 Grain dimensions (length, width and thickness) and different regions of rice grain.

4.2.3 Internal structure of rice endosperm

4.2.3.1 Sample preparation for light microscopy

4.2.3.1.1 Glycol methacrylate

Brown and head rice of KDML105, PTT1, KLG1 and CNT1 varieties with low and high N, 3 grains of each treatment, were cut into three pieces by razor blade. The central endosperm/aleurone portion, ca. 2 mm. wide, was fixed in 2.5% gluteraldehyde in 0.05 M phosphate buffer, pH 7.0 (with a brief vacuum to remove gases in tissue) for 24 hrs at room temperature (21^{o}C). The samples were bathed in 0.05 M phosphate buffer, 2 times for 30 min each at room temperature, and then dehydrated in an alcohol series (100% methoxyethanol, 100% ethanol, 100% 1-n-propanol, 100% 1-n- butanol) 1 days each, at room temperature. The specimens were infiltrated with GMA for 2 weeks at room temperature with 2 changes of resin before flat embedding in fresh GMA in an oxygen-free oven at 60^{o}C overnight (O'Brien and McCully, 1981). Sections (2.5 μm) were cut using glass knives (25 x 6.4 mm) on a Sorvall-microtome (JB-4) and stained.

The following staining procedures were carried out:

1. To detect storage protein, Amido black 10B (AB): 1% w/v in 7% v/v acetic acid. Sections were stained for 10 min at room temperature and then slides were rinsed in tap water and dried on a hot plate.

2. To detect starch, Periodic acid/Schiff's (PAS) reaction. The slides were placed in 1% periodic acid solution for 10 min, rinsed in running water for 5 min, placed in Schiff's reagent for 30 min and rinsed in running water for 3 min and dried on a hot plate.

4.2.3.1.2 Spurr's resin

Samples were obtained as for GMA procedure as mention earlier, trimmed to a small piece (approx. 3 x 3 mm) by razor blade and fixed in gluteraldehyde as for the GMA process. The specimens were washed with 0.05 M phosphate buffer, 3 times each for 30 min and subsequent fixing with 1% osmium tetroxide (O_sO_4) in 0.05 M phosphate buffer, pH 7.0, for 2 hrs and then washed twice with phosphate buffer for 30 min each. The specimens were dehydrated in an alcohol series (25, 50, 70, 90, 95 and 100% ethanol and 100% propylene oxide; 8 hrs each). The propylene oxide was substituted with Spurr's resin (10, 30, 50, 70, 90% resin in acetone; 8 hrs each). The specimens were transferred into 100% Spurr's resin, 3 times each for 8 hrs, before embedding with 100% resin and polymerizing at 60 ^{o}C overnight. Ultra-thin sections (ca. 200 nm) were cut by using a Reichert ultramicrotome (Leica Ultracut E) with a glass knife. The sections were stained with toluidine blue O, in benzoate buffer, pH 9.0.

Stained slides from both resins were examined under a compound Axioskop II plus (ZEISS, Germany) microscope by using a transmitted bright field mode. Suitable images of representative areas were captured by the attached Ziess digital camera. The number of cell layers, starch granules and cell size were quantified by using Assess software (APS Press, ISBN 0890542961).

4.2.3.2 Scanning electron microscopy (SEM)

Six brown rice grains each of KDML105 and CNT1, with low and high N concentration, were prepared for SEM by fracturing each grain centrally by hand. The pieces were fixed in 2.5% glutaraldehyde in 0.05 M phosphate buffer pH 7.0 with

vacuum infiltration for 3-5 min and left in the fixative for 24 hrs. The fixative was replaced with phosphate buffer, 2 times for 30 min each. Thereafter, the samples were dehydrated in an acetone series (25, 50, 75, 90 and 95%) for 24 hrs each, and then replaced with 100% acetone for 24 hrs, 2 times. The samples were dried in a Critical Point Dryer with liquid CO_2. The dried specimens were mounted on SEM stubs and then coated with gold-palladium (200 °A) by Polaron E5000 Sputer Coater. The specimens were examined and imaged under a LEO 1555 SUPRA Variable Pressure SEM at 10 kv with 30 μm spot size in high pressure mode.

4.2.4 Soluble protein

Extraction methods of soluble protein were adapted from Juliano and Boulter (1976) and Villareal and Juliano (1978). Brown, head and broken rice of KDML105, KLG1, PTT1 and CNT1 with low and high N, 3 replications each, were ground by using a mortar and pestle to obtain flour. For albumin-globulin determination, the powder (0.25 g) was extracted with 5 ml of 0.5 M NaCl by shaking for 1.5 hrs, the suspension was centrifuged at 734.5 g for 10 min, the supernatant was kept and the residue was extracted 2 times with 5 ml of 0.5 M NaCl for 1 hr. The extracted residue was washed 3 times with distilled water and re-extracted 2 times for 30 min with 5 ml of 70% ethanol containing 0.6% β-mercaptoethanol for prolamin and the suspension was centrifuged at 734.5 g for 10 min. The supernatant was kept and the residue was washed 2 times with distilled water. The washed residue was extracted for 2 hrs with 0.5% sodium dodecyl sulfate (SDS) containing 0.6% β-mercaptoethanol then centrifuged at 734.5 g for 10 min. The supernatant was kept. The supernatants from

all extractions were analyzed for determining protein concentration by the Bradford's method (Bradford, 1976).

4.2.5 Statistical analysis

Data were analyzed by analysis of variance (ANOVA) in a factorial in RCB and simple linear regression. Significant difference of means was separated by the least significant difference (LSD) test at $P < 0.05$. The brown rice weight and physical properties data were generated for multiple regression equations. All of statistical were analyzed by using commercial software (Statistix V. 7.1, Analytical Software, Inc.).

4.3 Results

4.3.1 Physical properties of rice grain

Brown rice dimensions were not affected by N treatments (Table 4.2) but significant differences were found among varieties. KLG1 and KDML105 had the largest and smallest grain, respectively. The extra long grain size was observed in all varieties (length >7.5 mm). The length ranged from 7.5 to 8.0 mm, width 2.0 to 2.4 mm and thickness 1.6 to 1.8 mm. The length/width ratio was highest in KDML105 (3.60) followed by PTT1 (3.54), CNT1 (3.49) and KLG1 (3.35). All varieties were classified as having slender shape because their length/width ratios were >3.

Grain N concentration did not affect individual brown rice grain weight (Table 4.3). Individual brown rice weight was highest in KLG1, intermediate in CNT1 and PTT1, and lowest in KDML105. The brown rice weight depended on grain width and

thickness but not grain length (equation 4.1). Brown rice weight was significantly correlated with the length/width ratio (equation 4.2).

Table 4.2 Effect of N concentration on brown rice dimensions of four Thai rice varieties

	Grain dimension (mm)											
	Length			Width			Thickness			L/W ratio		
Variety	Low N	High N	Mean	Low N	High N	Mean	Low N	High N	Mean	Low N	High N	Mean
KDML105	7.53	7.47	7.50c†	2.11	2.06	2.09d	1.66	1.64	1.65c	3.58	3.63	3.60a
PTT1	7.61	7.57	7.59bc	2.16	2.13	2.15c	1.75	1.74	1.74b	3.52	3.55	3.54b
KLG1	7.92	7.81	7.86a	2.38	2.32	2.35a	1.82	1.82	1.82a	3.33	3.37	3.35d
CNT1	7.74	7.73	7.74ab	2.22	2.22	2.22b	1.71	1.75	1.73b	3.50	3.49	3.49c
Mean	7.70	7.70		2.22	2.18		1.73	1.74		3.48	3.51	
	N	V	N x V	N	V	N x V	N	V	N x V	N	V	N x V
F-test	ns	*	ns	ns	***	ns	ns	**	ns	ns	***	ns
$LSD_{0.05}$	-	0.22	-	-	0.05	-	-	0.05	-	-	0.03	-

† Mean values in a column followed by different letters are significantly different by $LSD_{(P < 0.05)}$.

Table 4.3 Effect of N concentration on individual brown rice weight of four Thai rice varieties

Variety	Individual grain weight (mg)		
	Low N	High N	Mean
KDML105	19.6	19.3	19.5c
PTT1	22.1	21.6	21.8b
KLG1	25.2	25.1	25.2a
CNT1	22.9	22.8	22.8b
Mean	22.5	22.2	
	N	V	N x V
F-test	ns	***	ns
$LSD_{0.05}$	-	1.3	-

Mean values in a column followed by different letters are significantly different by $LSD_{(P < 0.05)}$.

$BW = -0.32L^{ns} + 13.52W^{*} + 11.51T^{*} - 24.92$ $(r^2 = 0.94^{***})$ (4.1)

BW = brown rice weight L = length

W = width T = thickness

$BW = -20.08LW^{***} + 92.62$ $(r^2 = 0.86^{***})$ (4.2)

BW = brown rice weight LW =length/width ratio

4.3.2 Internal structure of rice endosperm

Grain N concentration did not affect on internal structure of rice grain. The number of radial endosperm cell layers was not altered by grain N concentration (Table 4.4). The number of cell layers of the 4 varieties ranged from 10 to 14 layers in the dorsal-central axis, 11 to 13 layers in the ventral-central axis and 9 to 12 layers in the lateral-central axis. The number of radial endosperm cell layers was in the order CNT1 > PTT1 = KDML105 > KLG1.

Endosperm cell sizes varied with position, being larger in the middle of the peripheral-central region and smaller in the peripheral and central regions of rice endosperm (Tables 4.5-7, Figure 4.2a). The cells in the central region were quite small and hexagonal or polygonal in shape, whereas those in the mid-dorsal and mid-ventral axis were radially elongated, and rectangular. The length/width ratios were 2.5 and 3.0, respectively. The cell size in the mid-lateral region was polygonal shape, and the cell size was bigger than mid-dorsal and mid-ventral. In the subaleurone layer of rice endosperm, cells were rather small, roughly and polygonal in shape.

The number of cell layers in the aleurone differed between the examined regions of the rice grain, in all varieties (Figures 4.3-5). The aleurone was comprised of three to four layers on the dorsal side, one layer on the lateral side and one or two layers on the ventral side. Dorsal aleurone cells were polygonal shape whereas rectangular shape was observed on the lateral and ventral sides. The diameter of dorsal aleurone cells (20 to 35 μm) was smaller than for cells on the other two sides (30 to 40 μm).

Table 4.4 Effect of N concentration on the amount of endosperm cell layers from the central to the dorsal, lateral and ventral axis of four rice varieties

	Amount of cell layers from the centre of rice endosperm								
	Dorsal			Lateral			Ventral		
Variety	Low N	High N	Mean	Low N	High N	Mean	Low N	High N	Mean
KDML105	11.4	11.8	11.6ab	10.5	11.8	11.2a	11.0	11.2	11.1b
PTT1	11.6	13.0	12.3a	10.7	12.0	11.4a	11.0	11.4	11.2b
KLG1	10.0	10.8	10.4b	8.8	10.3	9.6b	10.8	10.6	10.7b
CNT1	14.0	12.0	13.0a	12.3	11.5	11.9a	13.3	12.0	12.6a
Mean	11.8	11.9		10.6	11.4		11.5	12.6	
	N	V	N x V	N	V	N x V	N	V	N x V
F-test	ns	*	ns	ns	**	ns	ns	*	ns
$LSD_{0.05}$	-	1.5	-	-	1.1	-	-	1.3	-

Mean values in a rows followed by different letters are significantly different by LSD ($P < 0.05$).

Table 4.5 Effect of N concentration on length of cells in different parts of the endosperm of four Thai rice varieties

Variety	N concen-tration	Length of endosperm cells (μm)							Mean
		Central	Mid-dorsal	Dorsal	Mid-ventral	Ventral	Mid-lateral	Lateral	
KDML 105	Low N	81	153	75	179	64	122	65	
	High N	84	164	83	159	61	139	85	
	Mean	83aC†	158cA	79aC	169bcA	63bC	130bB	75aC	108
KLG1	Low N	90	202	76	213	79	142	76	
	High N	81	228	107	250	96	185	107	
	Mean	86aC	215aA	92aC	231aA	88aC	163aB	91aC	138
PTT1	Low N	73	149	84	162	70	135	74	
	High N	85	198	98	156	73	144	93	
	Mean	79aC	174bA	91aC	159cA	71ab	140bB	83aC	114
CNT1	Low N	98	171	72	183	84	127	79	
	High N	88	188	84	196	76	160	90	
	Mean	93aC	180bA	78aC	190bA	80ab	144abB	84aC	121
	Low N	86aC	169aA	77aC	184aA	74aC	132aB	73aC	114
	High N	84aC	194bA	93bC	190aA	76aC	157bB	94bC	127
Mean		85	182	85	187	75	144	83	

	N	V	P	N x V	N x P	V x P	N x V x P
F-test	*	**	***	ns	*	**	ns
LDS 0.05	9	14	11	-	15	21	-

† The lower case and capital letters are used for comparison between rows and columns, respectively. The different letters are significantly different by LSD $_{(P < 0.05)}$.

Table 4.6 Effect of N concentration on width of cells in different parts of endosperm of four Thai rice varieties

Variety	N concen-tration	Width of endosperm cells (μm)							Mean
		Central	Mid-dorsal	Dorsal	Mid-ventral	Ventral	Mid-lateral	Lateral	
KDML 105	Low N	51	64	58	64	50	101	47	
	High N	47	68	58	74	57	107	58	
	Mean	49aC†	66aBC	58aC	69aB	54bC	104aA	53bC	65
KLG1	Low N	53	69	57	58	70	113	65	
	High N	46	68	66	68	69	100	80	
	Mean	50aD	68aBC	61aC	63aBC	70aBC	106aA	73aB	70
PTT1	Low N	44	75	59	68	59	75	53	
	High N	46	65	67	74	59	97	62	
	Mean	45aD	70aB	63aBC	71aB	59bC	86bA	57bC	64
CNT1	Low N	59	77	61	70	75	83	56	
	High N	44	68	58	57	58	90	67	
	Mean	52aD	72aB	60aCD	64aBC	66acBC	87bA	62bC	66
	Low N	52aD	71aB	59aCD	65aBC	64aC	93aA	55aD	66
	High N	46aD	67aBC	62aBC	68aB	61aC	98aA	67bB	67
Mean		49	69	60	67	62	96	61	
	N	V	P	N x V	N x P	V x P	N x V x P		
F-test	ns	ns	***	ns	**	***	ns		
LDS 0.05	-	-	5	-	7	9	-		

† The lower case and capital letters are used for comparison between rows and columns, respectively. The different letters are significantly different by LSD $_{(P < 0.05)}$.

Table 4.7 Effect of N concentration on length/width ratio of cells in different parts of endosperm of four Thai rice varieties

Variety	N concentration	Length/width ratio of endosperm cells (μm)							Mean
		Central	Mid-dorsal	Dorsal	Mid-ventral	Ventral	Mid-lateral	Lateral	
KDML 105	Low N	1.6	2.6	1.3	2.8	1.3	1.2	1.4	
	High N	1.8	2.5	1.5	2.6	1.1	1.4	1.5	
	Mean	1.7bB	2.5bA	1.4aBC	2.7bcA	1.2aC	1.3aBC	1.5aB	1.8
KLG1	Low N	2.6	3.0	1.4	4.0	1.2	1.4	1.2	
	High N	1.8	3.7	1.7	4.0	1.5	1.9	1.4	
	Mean	2.2aC	3.4aB	1.6aD	4.0aA	1.3aD	1.7aD	1.3aD	2.2
PTT1	Low N	1.7	2.0	1.5	2.4	1.2	1.8	1.5	
	High N	1.9	3.2	1.5	2.2	1.3	1.6	1.5	
	Mean	1.8abB	2.6bA	1.5aBC	2.3cA	1.3aC	1.7aBC	1.5aBC	1.8
CNT1	Low N	1.8	2.3	1.2	2.7	1.3	1.5	1.4	
	High N	2.0	2.8	1.5	3.5	1.5	1.8	1.4	
	Mean	1.9abC	2.6bB	1.3aD	3.1bA	1.4aD	1.7aCD	1.4aD	1.9
		N	V	P	N x V	N x P	V x P	N x V x P	
	F-test	ns	*	***	ns	ns	***	ns	
	LDS $_{0.05}$		0.3	0.2			0.4		

† The lower case and capital letters are used for comparison between rows and columns, respectively. The different letters are significantly different by LSD $_{(P < 0.05)}$.

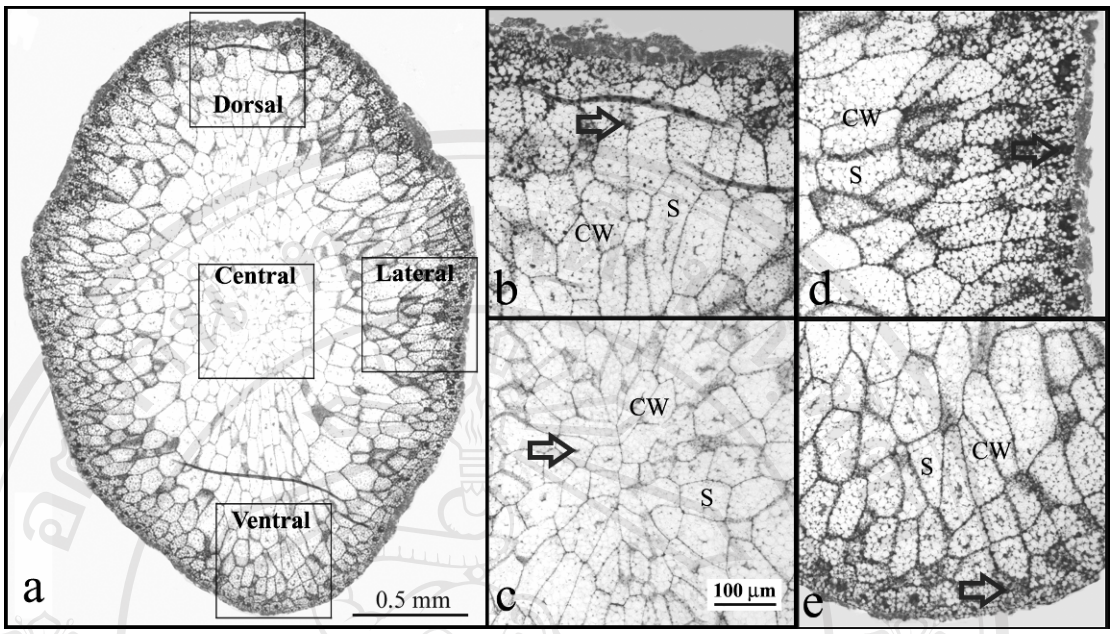

Figure 4.2 Storage protein accumulation as shown by positive staining with amido black 10B (arrow) in rice endosperm of KDML105, low N: a; transverse section of rice endosperm, b-e; higher magnification of dorsal, central, ventral and lateral regions, respectively. CW: cell wall, S: starch granules

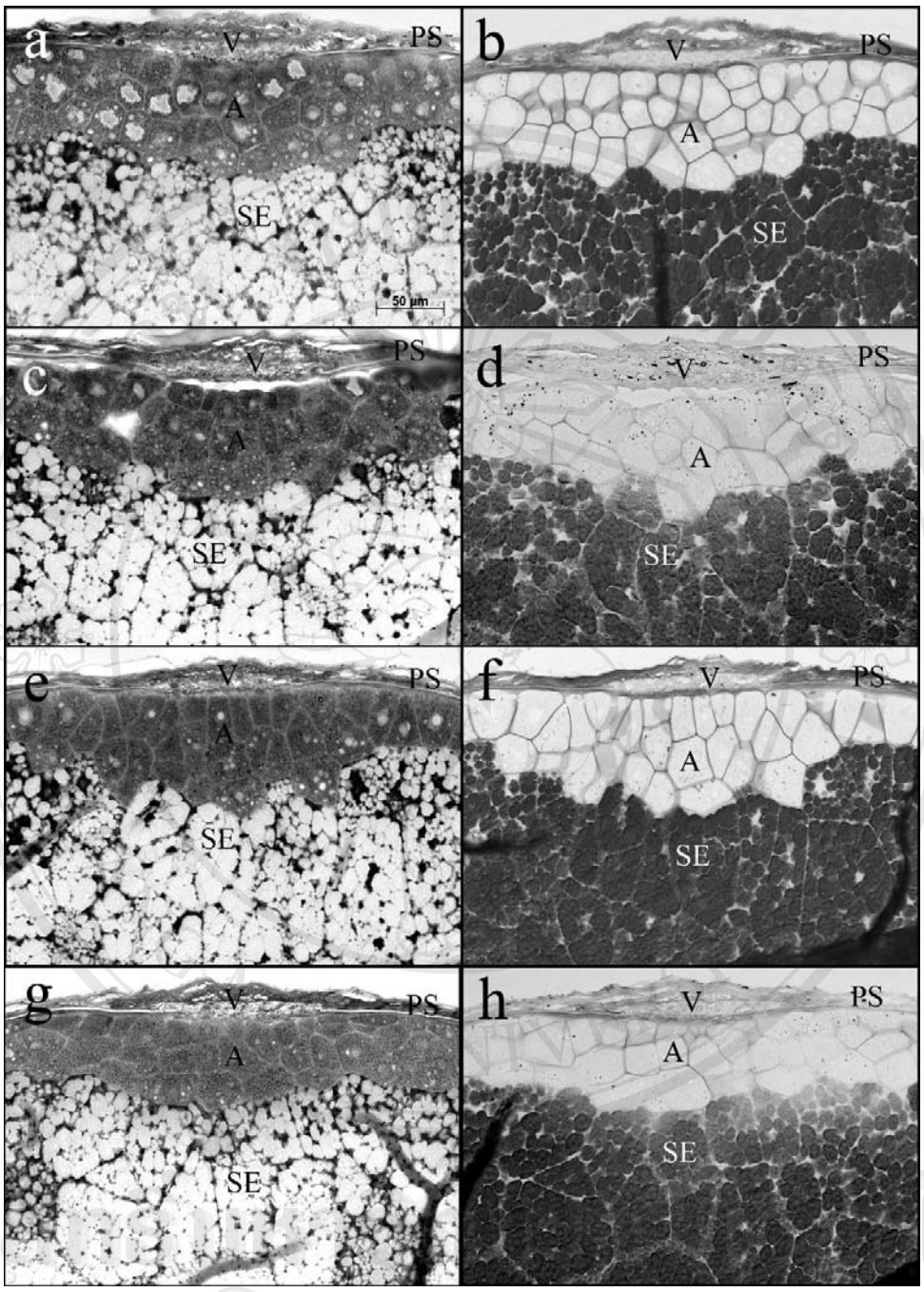

Figure 4.3 Aleurone layer in the dorsal region staining with AB (left) and PAS (right) of KDML105 (a, b), KLG1 (c, d), PTT1 (e, f) and CNT1 (g, h), low N. V: Vascular bundle, PS: Pericarp and seed coat, A: Aleurone layer, SE: Starchy endosperm

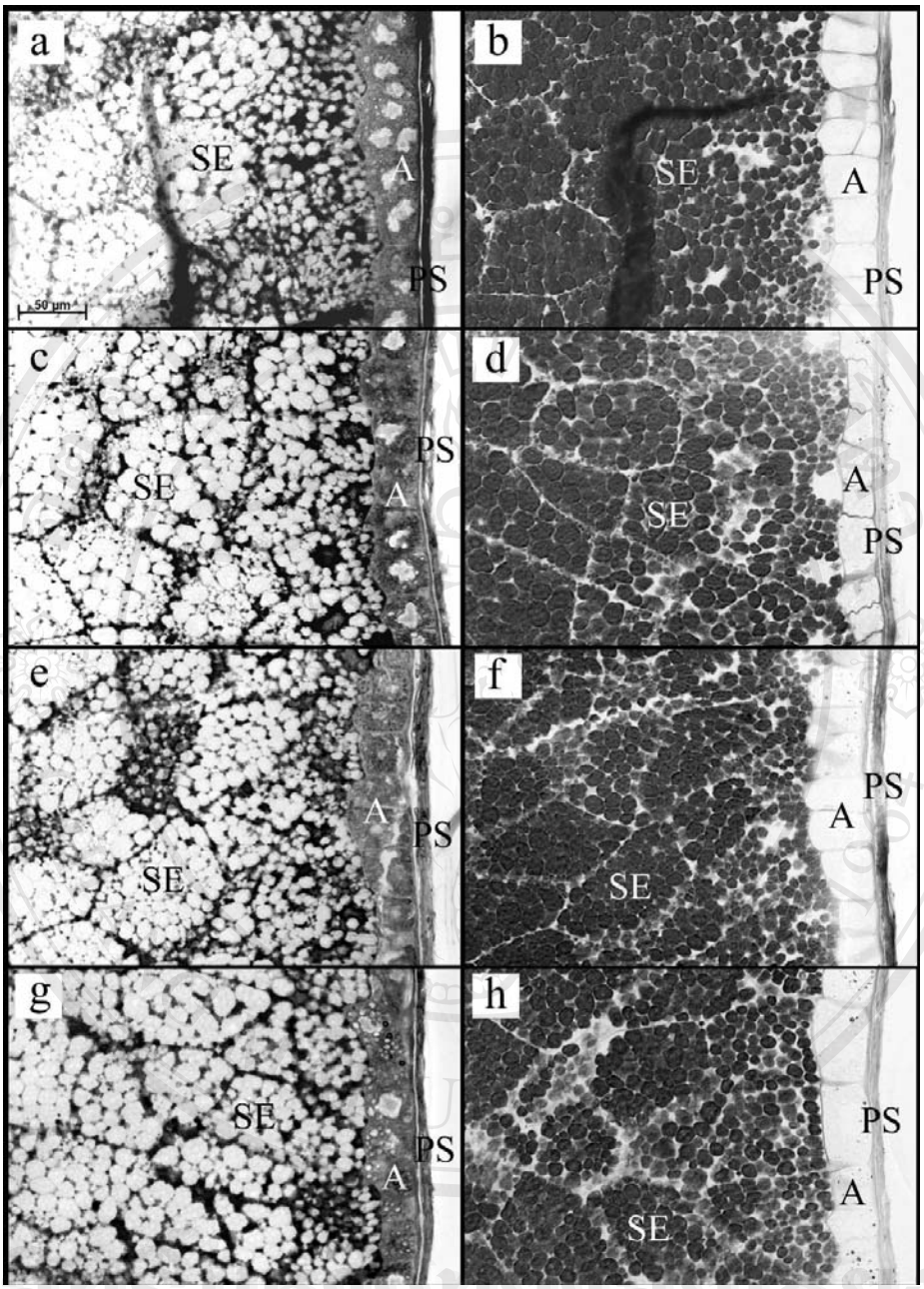

Figure 4.4 Aleurone layer in the lateral region staining with AB (left) and PAS (right) of KDML105 (a, b), KLG1 (c, d), PTT1 (e, f) and CNT1 (g, h), low N. PS: Pericarp and seed coat, A: Aleurone layer, SE: Starchy endosperm

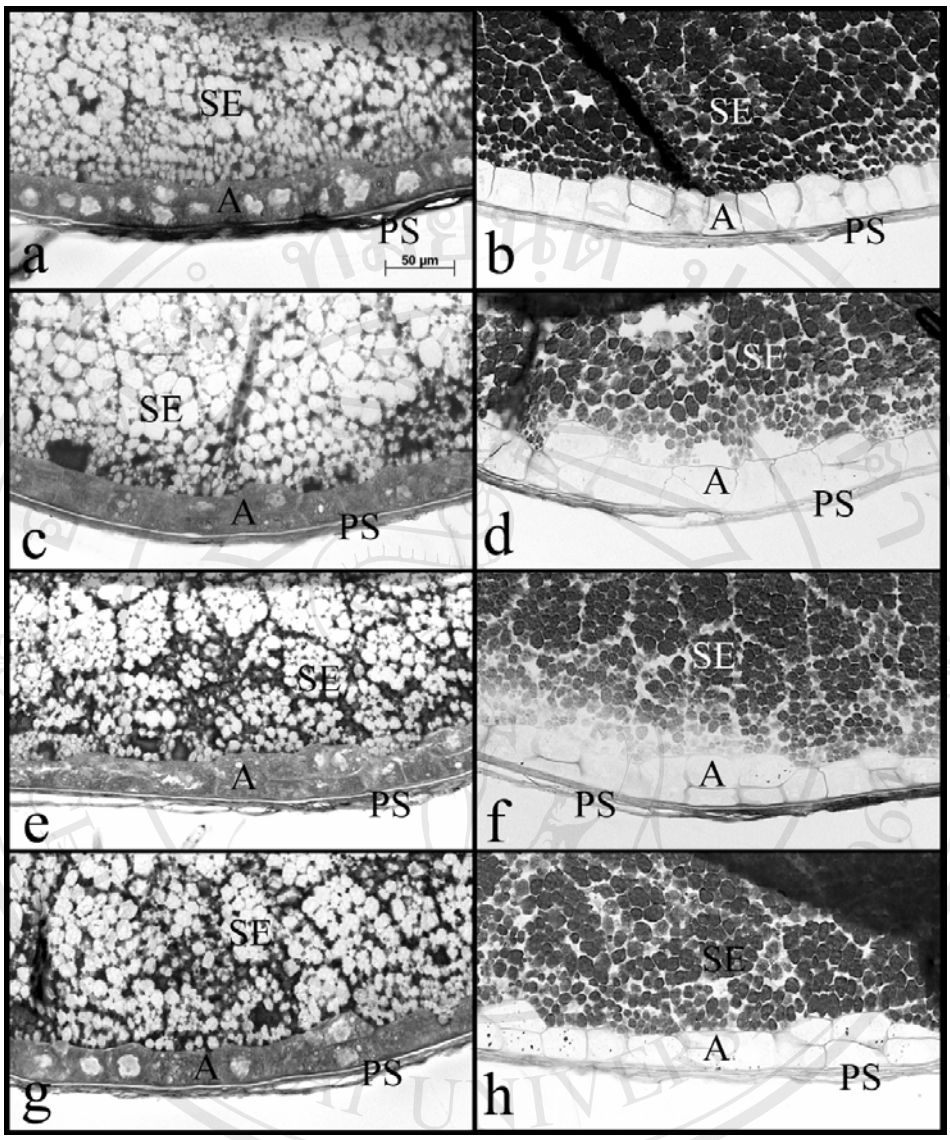

Figure 4.5 Aleurone layer in the ventral region staining with AB (left) and PAS (right) of KDML105 (a, b), KLG1 (c, d), PTT1 (e, f) and CNT1 (g, h), low N. PS: Pericarp and seed coat, A: Aleurone layer, SE: Starchy endosperm

The starch granules, or amyloplasts, consisted of compound starch (Figure 4.6). Thus, each starch granule was composed of many small granulums. Starch granule size was affected by N, variety or by endosperm location. Starch granule size was higher by 10% in KLG1 than the other varieties (Table 4.8). Starch granule size was larger in the middle region of the endosperm (21 to 23 μm) and the central region, 16 μm, was larger than the peripheral region of rice endosperm, 12 to 13 μm (Table 4.8). Furthermore, the starch granule size also differed intracellular, being smaller in the peripheral than the median areas (Figure 4.7).

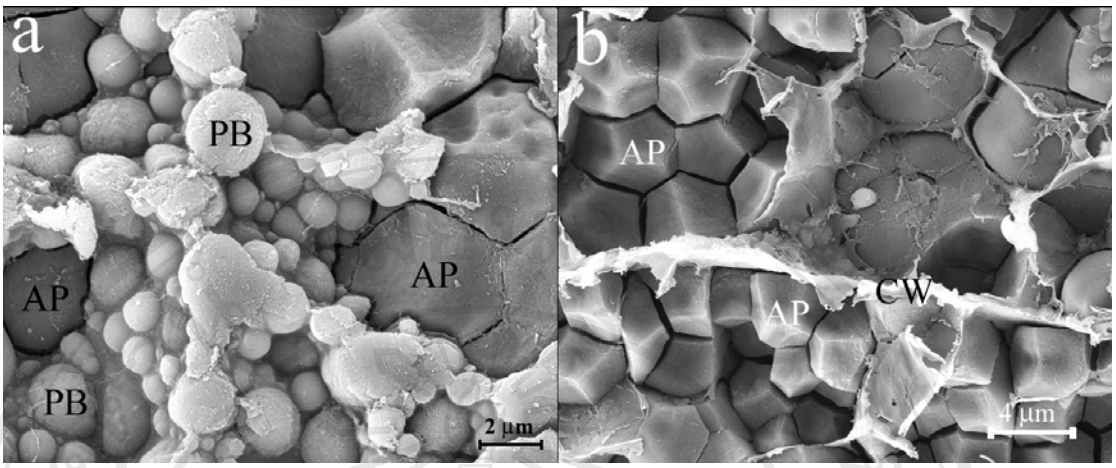

Figure 4.6 Scanning electron micrograph of starch granule and protein bodies in the periphery (a) and central (b) region of KDML105, low N. AP: Amyloplast, PB: Protein bodies, CW: Cell wall

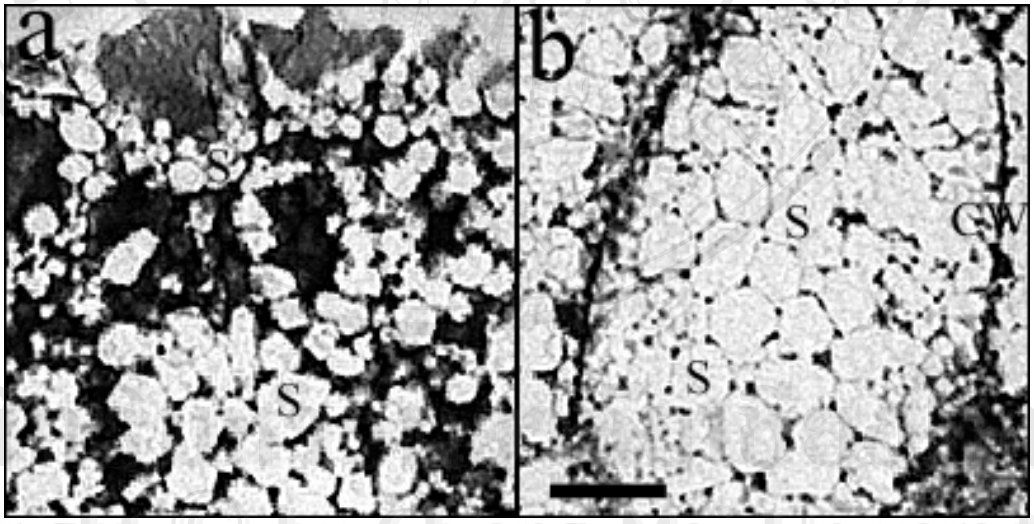

Figure 4.7 Starch granules in the peripheral (a) and interior region (b) of transverse section of KDML105, low N. CW: Cell wall, S: Starch granule, the scale bar is 2 µm.

Table 4.8 Effect of N concentration on starch granule size in seven positions of the endosperm in four Thai rice varieties

Variety	N concen-tration	Starch granule size, by position (μm)							Mean
		Central	Mid-dorsal	Dorsal	Mid-ventral	Ventral	Mid-lateral	Lateral	
KDML 105	Low N	15.3	21.8	13.0	21.9	11.4	20.5	12.3	
	High N	15.1	19.8	12.2	19.9	12.9	20.8	11.5	
	Mean	15.2	20.8	12.6	20.9	12.2	20.6	11.9	16.3b †
KLG1	Low N	19.2	23.2	13.5	23.4	14.3	21.2	13.1	
	High N	17.2	22.2	13.3	21.8	10.6	22.2	12.7	
	Mean	18.2	22.7	13.4	22.6	12.5	21.7	12.9	17.7a
PTT1	Low N	14.4	21.3	12.1	18.8	11.2	22.0	11.1	
	High N	17.0	21.4	13.5	22.0	11.1	20.6	10.9	
	Mean	15.7	21.3	12.8	20.4	11.2	21.3	11.0	16.2b
CNT1	Low N	15.5	21.9	15.1	21.0	13.5	22.0	12.0	
	High N	14.8	19.6	12.1	20.5	11.6	20.0	12.0	
	Mean	15.2	20.7	13.6	20.8	12.5	21.0	12.0	16.5b
	Low N	16.1	22.0	13.4	21.3	12.6	21.4	12.1	17.0b
	High N	16.0	20.8	12.8	21.1	11.5	20.9	11.8	16.4a
Mean		16.1B	21.4A	13.1C	21.2A	12.1D	21.2A	12.0D	

	N	V	P	N x V	N x P	V x P	N x V x P
F-test	*	*	***	ns	ns	ns	ns
$LDS_{0.05}$	0.5	1.0	0.9				

† The lower case and capital letters are used for comparison between rows and columns, respectively. The different letters are significantly different by $LSD_{(P < 0.05)}$.

The longitudinal section showed more abundance of protein bodies in the peripheral region than the inner cells, especially in the interior region of the endosperm (Figure 4.8). Furthermore, the dorsal region had more abundant storage protein than the ventral and central regions. The protein bodies were located between the starch granules, sized from 1 to 3 μm (Figure 4.6). Protein bodies were classified into two types, PB-I and PB-II, by their shape and degree of staining with toluidine blue O, pH 9 (Figure 4.9) in spurr's resin sections. PB-I was spherical in shape and was more densely stained in the central than the peripheral part of the protein body, whereas PB-II was irregular in shape and was uniformly stained with toluidine blue O. The stained sections showed that PB-II was more abundant than PB-I. PB-I mostly accumulated in the peripheral region of endosperm cell, beside of the cell wall.

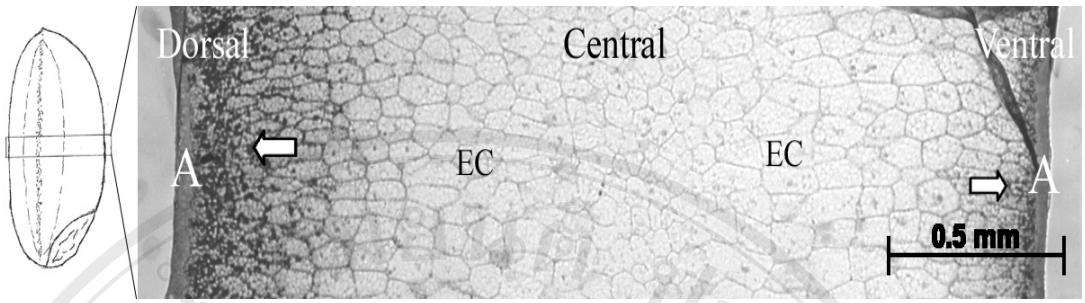

Figure 4.8 Longitudinal section of brown rice of KDML105 showing storage protein (arrows) accumulation and distribution in rice endosperm. A: Aleurone layer, EC: Endosperm cell

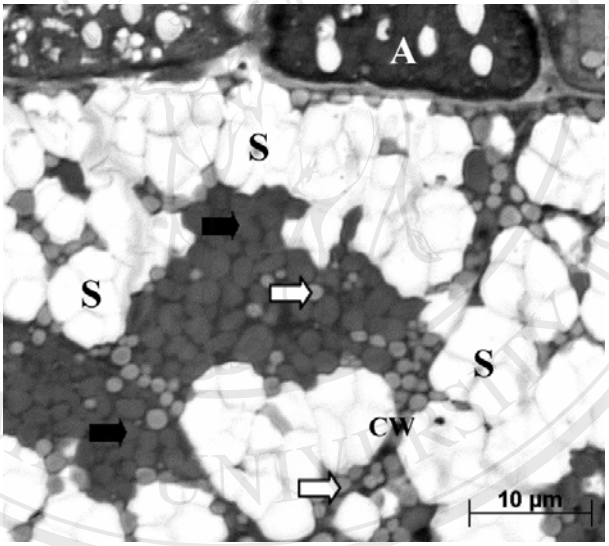

Figure 4.9 Two types of protein bodies staining with toluidine blue O, pH 9, PB-I (white arrow) and PB-II (black arrow), distributed in the peripheral region of CNT1, high N. A: Aleurone cell, S: Starch granule, CW: Cell wall

4.3.3 Soluble proteins

Soluble proteins in brown, head and broken grain were extracted into three fractions; glutelin, prolamin and albumin-globulin. Increasing N concentration increased soluble protein in rice grain. The soluble protein concentration in rice was in the order glutelin > albumin-globulin > prolamin.

Increasing N concentration increased soluble protein fractions differently, with some differences among the varieties. The glutelin concentration of grain with high N was double that of grain with low N in KDML105, KLG1 and PTT1, and 2 to 3 times in CNT1 (Table 4.9). At low N, the glutelin concentration was especially low in CNT1, being half or less of the glutelin concentration of the other three varieties. Polishing increased the glutelin concentration in PTT1, CNT1 and KDML105 but not in KLG1. The glutelin concentration was higher in broken than head rice in all four varieties at low N.

The prolamin concentration did not respond to grain N concentration in brown rice except for a small increase in KLG1 and CNT1 (Table 4.10). The effect of polishing on prolamin concentration depended on grain N concentration and variety. With low N concentration, polishing decreased prolamin concentration in all varieties. However, with high grain N concentration, polishing increased prolamin concentration in KDML105 and CNT1 but depressed in KLG1 and PTT1.

The albumin-globulin concentration was higher in brown than polished rice (Table 4.11). Brown, head and broken rice with low N concentration had depressed albumin-globulin concentrations in KDML105, but not in KLG1, PTT1 and CNT1. Glutelin concentration positively correlated with percent unbroken rice but not in prolamin and albumin-globulin concentrations (Figure 4.10).

Table 4.9 Effect of N concentration on glutelin concentration in brown, head and broken rice of four Thai rice varieties

Variety	N concentration	Glutelin concentration ($mg.g^{-1}$)		
		Brown rice	Head rice	Broken rice
KDML105	Low N	9.05bA[†]	10.07bB	21.00cC
	High N	22.14eA	23.07dA	28.63dB
PTT1	Low N	8.32bA	12.67cB	18.43cC
	High N	20.51deA	25.61dB	29.45dB
KLG1	Low N	11.27cA	10.77bcA	14.22bB
	High N	22.95eA	23.89dA	28.25dA
CNT1	Low N	3.79aA	6.62aB	10.63aC
	High N	17.51aD	21.23dB	26.88dC

	N	V	T	N x V	N x T	V x T	N x V x T
F-test[‡]	*	***	***	**	***	***	*

[†] The lower case and capital letters are used for comparison between rows and columns, respectively. The different letters are significantly different by LSD $_{(P < 0.05)}$.

[‡] Data transformed by Log_{10} for analysis.

Table 4.10 Effect of N concentration on prolamin concentration in brown, head and broken rice of four Thai rice varieties

Variety	N concentration	Prolamin concentration ($mg.g^{-1}$)		
		Brown rice	Head rice	Broken rice
KDML105	Low N	2.60bcC[†]	1.29aA	1.76cB
	High N	2.22bA	5.32dB	5.00dB
PTT1	Low N	1.90abB	1.35abA	1.35bA
	High N	2.30bcB	2.06bAB	1.75cA
KLG1	Low N	1.72aA	1.37abA	1.58bcA
	High N	2.37bcB	1.77bA	1.56bcA
CNT1	Low N	2.08bB	1.09aA	0.93aA
	High N	2.83cA	3.98cB	5.25dC

	N	V	T	N x V	N x T	V x T	N x V x T
F-test[‡]	***	***	ns	***	***	***	***

[†] The lower case and capital letter are used for comparison between rows and columns, respectively. The different letters are significantly different by LSD $_{(P < 0.05)}$.

[‡] Data transformed by Log_{10} for analysis.

Table 4.11 Effect of N concentration on albumin-globulin concentration in brown, head and broken rice of four Thai rice varieties

Variety	N concentration	Albumin-globulin concentration ($mg.g^{-1}$)			Mean
		Brown rice	Head rice	Broken rice	
KDML105	Low N	7.09	4.62	5.42	5.71d
	High N	4.07	2.75	2.95	3.26a
PTT1	Low N	6.49	4.08	4.43	5.00c
	High N	5.11	3.94	4.77	4.61c
KLG1	Low N	6.83	3.82	4.28	4.97c
	High N	6.19	4.25	4.45	4.97c
CNT1	Low N	5.67	3.34	3.42	4.14b
	High N	5.95	3.52	4.37	4.61c
	Low N	6.52bC†	3.96bA	4.39aB	
	High N	5.33aC	3.62aA	4.14aB	

	N	V	T	N x V	N x T	V x T	N x V x T
F-test‡	ns	**	***	***	*	ns	ns

† The lower case and capital letter are used for comparison between rows and columns, respectively. The different letters are significantly different by LSD $_{(P < 0.05)}$.

‡ Data transformed by Log_{10} for analysis.

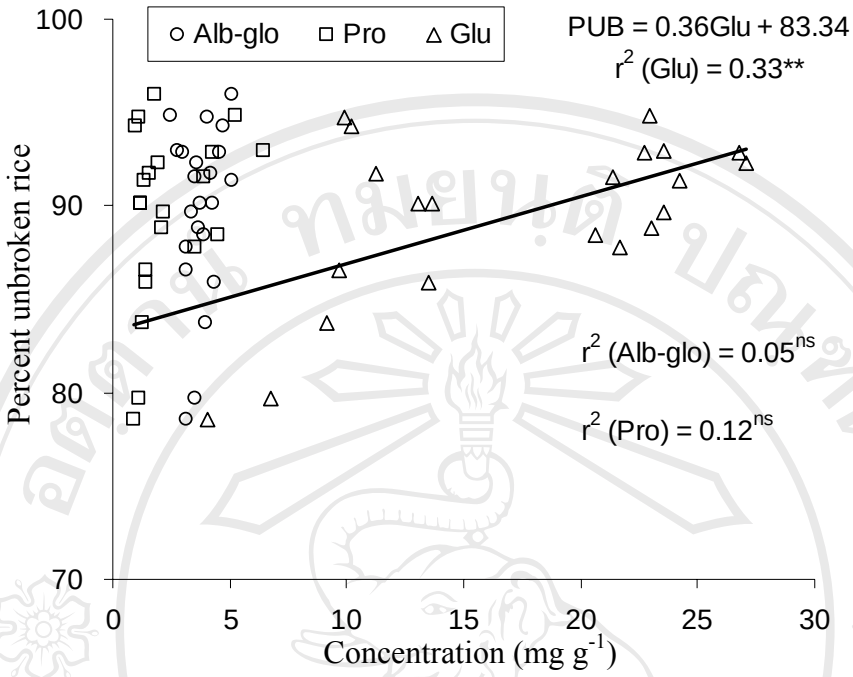

Figure 4.10 Correlation between soluble protein (Glu = glutelin, Pro = prolamin and Alb-glo = albumin-globulin) concentration and percent unbroken rice of four Thai rice varieties.

4.3.4 Nitrogen supply on protein accumulation and distribution in rice endosperm

Anatomical sections (Figure 4.2) showed that abundance of storage protein was in the order: lateral > ventral > dorsal > central regions of rice endosperm (Table 4.12). With an increase in N concentration, the density of storage protein doubled in all regions of the endosperm, except the central region. At low N concentration, except for the lateral region, the density of storage protein was not different between the different regions of rice endosperm in all varieties. Storage protein was more dense in the lateral region of KDML105 than the other three varieties (Figure 4.11). However, the storage protein was not significantly different in the lateral region at high N. Furthermore, the relative abundance of storage protein in the lateral region showed a positively correlation with percent head rice of all varieties (Figure 4.12).

Table 4.12 Effect of N concentration on storage protein distribution in four parts of rice endosperm of four Thai rice varieties

Variety	Protein distribution in rice endosperm (%)†							
	Central		Dorsal		Ventral		Lateral	
	Low N	High N	Low N	High N	Low N	High N	Low N	High N
KDML105	4	4	16aA‡	31aB	14aA	26bB	29aA	35aB
PTT1	5	5	17aA	21bA	21aA	28bA	22bA	36aB
KLG1	4	7	14aA	28aB	14aA	40aB	18bA	37aB
CNT1	4	8	18aA	27aB	13aA	26bB	19bA	33aB
Mean	4B	6A	16	27	15	30	22	35
	F-test	$LSD_{0.05}$	F-test	$LSD_{0.05}$	F-test	$LSD_{0.05}$	F-test	$LSD_{0.05}$
N	*	1.7	***	2.2	***	4.7	***	2.2
V	ns	-	*	3.1	ns	-	***	3.1
N x V	ns	-	*	4.4	*	9.4	***	4.4

† Each number is the mean of storage protein distribution rating in the specific part of 3 grains.

‡ The lower case and capital letters are used for comparison between rows and columns, respectively. The different letters are significantly different by $LSD_{(P < 0.05)}$.

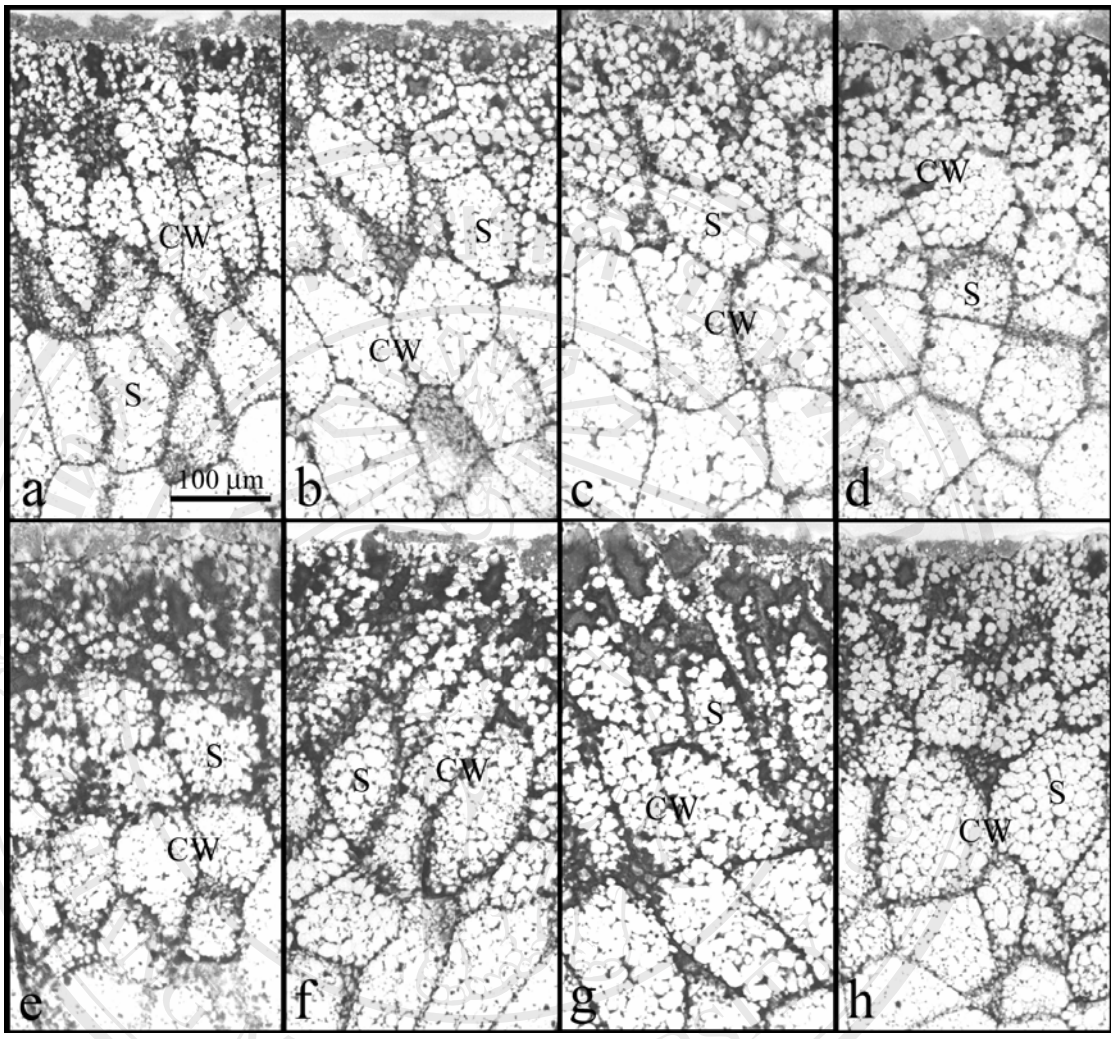

Figure 4.11 Storage protein, positive staining with amido black 10B (black color), distribution in the lateral region of rice endosperm of low grain N (upper) and high grain N (lower) concentrations of KDML105 (a, e), PTT1 (b, f), KLG1 (c, g) and CNT1 (d, h). CW: cell wall, S: starch granules

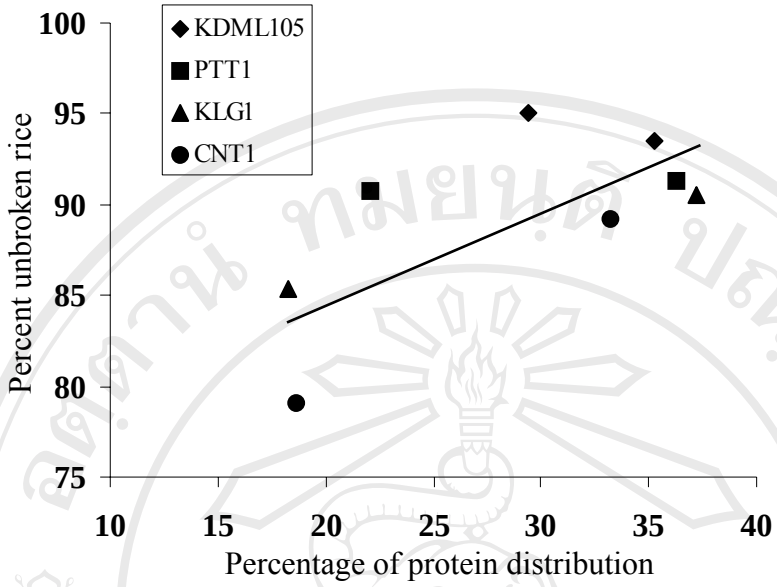

Figure 4.12 Relationship between percentage of protein distribution in the lateral portion with percent head rice of four Thai rice varieties.

4.4 Discussion

4.4.1 Rice quality characteristics associated with grain N concentration

As other workers have observed (del Rosario *et al.*, 1968; Nangju and De Datta, 1970; Seetanum and De Datta, 1973), N fertilizer did indeed increase head rice yield. Furthermore, the outcome results of this study had shown that increasing of head rice yield was positively correlated with grain N concentration. The data from this chapter clearly showed that storage protein abundance in the lateral region of the rice grain positively correlated with head rice yield, which represents resistance to break during milling. The doubling of grain N concentration doubled the density of storage protein in the peripheral region (subaleurone layer) surrounding the rice endosperm. This incidence seems to be strengthened rice grain to resist against the breakage, since this assumption is possible to coincide with the area of the grain where breakage generally occurs during milling. The increased numbers of protein bodies were packed mainly in the spaces between starch granules. Similar evidence has been reported in wheat, where grain hardness has been found to be associated with the protein matrix surrounding starch granules (Barlow *et al.*, 1973; Stenvert and Kingswood, 1977). The physico-chemical basis of endosperm hardness in wheat was reported to be associated with the interface between the protein matrix and starch granules (Greenblatt *et al.*, 1995; Greenwell and Schofield, 1986). If this also applies to rice, then increasing protein abundance may decrease breakage because protein bodies occupy the inter-spaces between unpacked starch granules and cement the starch granules to make the rice grain more hardy and resistant to breakage during milling.

Soluble protein concentration was also closely associated with grain N concentration. Increasing N concentration resulted in increased soluble protein fractions in the grain. The soluble protein fraction that was increased by N fertilizer to the greatest extent was glutelin, the main storage protein of rice. The effect of polishing on soluble protein fractions differed among varieties. Polishing decreased the albumin-globulin concentration in all varieties. However, polishing increased the glutelin concentration in PTT1 and CNT1, increased the prolamin concentration in KDML105 and CNT1, but depressed the prolamin concentration in KLG1 and PTT1. This suggests that genotypes may vary in the distribution of some protein fractions on the different locations on the grain, which is in agreement with previously published results. Works undertaken by Cagampang *et al.* (1966), Houston *et al.* (1968) and others suggested that storage protein types were not evenly distributed across the grain, with albumin and globulin were more abundant in the outer region of the grain (including the aleurone layers) and glutelin increased in proportion towards the center of the endosperm. By contrast, prolamin was more evenly distributed across the endosperm.

The significant increase in concentration of glutelin, which is relatively rich in lysine (Juliano *et al.*, 1973), with increasing N concentration will increase the nutritional value of rice by improving the essential amino acid composition. Nanda and Coffman (1979) suggested that improving milled rice protein by 2% (from 7 to 9%) will double the protein intake in the Asian diet from 10 to 20%.

4.4.2 Rice quality characteristics independent of grain N concentration

The physical properties and internal structure of rice grain were not affected by N concentration but varied with variety. This agrees with many previously published works. Borrell *et al.* (1999) reported that grain size of Lemont, Newbonnet and Starbonnet appeared to be affected more by genetics than N fertilization. Resurreccion *et al*. (1977) observed that grain size (100 grains weight) was correlated with grain breadth, while Ebata and Nagato (1967) reported that high temperature during ripening resulted in a lower final grain weight for japonica rice. The lower temperature, below 27.5°C, during the grain filling period was associated with broader grain, resulting in grain width > 2.3 mm in the Lemont variety (Borrell *et al.*, 1999). Genotypic variation for grain length, grain width and grain length/width ratio has also been found in typical commercial long, medium and short grain types in the USA (Webb *et al*., 1968; Adair *et al*., 1973; Webb *et al*., 1979).

The aleurone layer of the rice grain is important in two ways. Firstly, it is the part that is removed in polishing, and becomes the rice bran. Rice bran is valuable as animal feed, it may also be extracted for edible oil. Secondly, the aleurone layer confers to brown rice its appearance and quality. The number of aleurone cell layers was similar in all tested varieties (Figures 4.3-5). These layers are composed of three to four cells on the dorsal side, one on the lateral side and one or two cell layers on the ventral side. Hoshikawa (1967b) reported that the cause of the multi-layered dorsal side is that one or two layers of cells in the periphery begin to differentiate into special shapes about the fifth day after anthesis, each of which then divides once or twice into two to four layers of cells having a similar shape. By the sixth day, these cells show themselves to be precursors of aleurone cells. After this, the innermost cell

layer starts division and multiplication like the peripheral cells in other parts, completing division by about the tenth day. So, many aleurone layers are formed in this region only. On the ventral side, the aleurone is bi-layered because the daughter cells, which have been formed at the final cell division, are both differentiated into aleurone cells.

The starch granules in the peripheral region of the dorsal-central axis were larger than in the other peripheral regions examined. The larger granules may result from greater access to the supply of reserve substances during grain fill. In the early ripening stage of the endosperm, the reserve substances are transported through the conducting vascular bundle to the aleurone layers, then pass the dorsal-central axis to the starch storage tissue in the central part of the endosperm (Hoshikawa, 1972).

Storage protein was classified into two types, PB-I and PB-II. The PB-I mostly accumulated in the peripheral region, close to the cell wall, but PB-II accumulated predominantly in the center of the cell. Kavakli *et al.* (2000) reported that prolamin protein bodies, PB-I, were distributed predominantly in a region within 4 μm of the plasma membrane, whereas glutelin-containing protein bodies, PB-II, were localized mainly around the nucleus located near the center of the cell.

Protein bodies were more abundant in the peripheral region and less in the interior region, which is in contrast with the relative abundance of starch granules. In rice endosperm, the protein bodies occupied spaces between the starch granules, especially in the sub-aleurone layer. The sub-aleurone layer was rich in protein and had more small amyloplasts and compound starch granules than the inner region, which contains relatively pure starch. This result confirms many previous studies (e.g. Itani *et al.*, 2002; Zhou *et al.*, 2002). Furthermore, the different amount of

storage protein in the different regions of rice endosperm may be due to the different efficiency transportation of assimilates through the vascular bundle, pigment strand, nucellus and aleurone layer to the endosperm (Figure 1.4), so, the part of the endosperm that is closest to the vascular bundle having more access to assimilates.

The number of endosperm cell layers of studied varieties (10–13 layers) was lower than in japonica rice. Matsuo and Hoshikawa (1993) reported that the number of endosperm cell layers in japonica rice ranged from 19–20 layers along the dorsal-central axis, 15 – 16 layers on the ventral-central axis and 14-16 layers on the lateral-central axis. Along the longitudinal diameter, there are about 200 cells in japonica rice and about 150 cells in indica rice, but some long-grain indica rice varieties have more 20 to 100 cells (Matsuo and Hoshikawa, 1993). Hoshikawa (1968a) found that some of the indica rice has more layers on the ventral than the dorsal radii. In the studied varieties, however, there was no difference in the number of dorsal and ventral cell layers.

Conclusion could be drawn in this chapter that increasing N concentration in rice grain are able to increase head rice yields but this interesting result did not observe in rice variety which possess high percent unbroken rice already at low N concentration. Data is also clearly indicated that high available soluble proteins in rice grains is very much beneficial for consumers who eat rice as their staple food in terms of increasing values of useful nutrition.