A Study of the Protein Profile Related to Sweetness during the Developmental Stages of the Nam-Dokmai Mango (Mangifera indica)

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Abstract

Sugar production levels and protein profiles of *Mangifera indica* were examined in order to study their relationship during development. Sugar levels were analyzed over eight developmental stages using an ATAGO hand refractometer. The results showed that sugar was detected at stage two and remained at the same level through stage five, increased at stage six and seven, and reached a maximum at stage eight. Protein profiles, determined by proteomics, indicated 98 variably expressed proteins. These were found to respond to stress (16%), signal (13%), transport (11%), respiration (4%), carbohydrate metabolisms (8%), lipid metabolisms (6%), protein metabolisms (3%), secondary metabolisms (2%), cell division (3%), ripening (1%), transcription (28%) and translation (5%). Eleven proteins showed a similar trend in expression to sugar production suggesting an involvement in the development of sweetness.

Keywords: Nam-Dokmai mango, sweetness, Proteomics, protein profile.

INTRODUCTION

Mango, *Mangifera indica* L., is originated in Burma and India and is commonly grown in most tropical regions of the world. Thailand ranks fourth in the world for their production after India, China, and Mexico (Tharanathan et al., 2006). Chachoengsao is one of Thailand's chosen provinces to cultivate mangoes for marketing and export. The majorities of mangoes in Chachoengsao province are grown mostly in Bang Khla district. The mango season is usually abundance in March and a mango festival is being held annually in Chachoengsao province.

To date, there are over 200 commercial vernacular names of cultivars of mango in Thailand, i.e., Nam-Dokmai, Raed, Chok-Anan, Chao-Khoon-Thip, Falan, Ok-Rong, Thong-Dam, and etc. (Eiadthong et al., 1999). 'Nam-Dokmai' is the most popular one among those mangoes of Thailand, thus this cultivar is chosen as a representative mango to study in this investigation. It has an exceptional appearance and taste. The fruits are long, slender and sigmoid in shape. The young fruit has a creamy-green skin and its flesh is crispy with sour taste, while the ripe ones change to a soft yellow. The flesh of the ripe mango is soft and juicy, with an extreme sweetness and aromatic flavor (Ketsa et al., 1999). The Office of Agriculture Economics, Ministry of Agriculture and Cooperatives had recently reported that there was an increasing tendency of exporting mango in three consecutive years from 2010 to 2012, where the costs of export were around five hundred millions baht in 2010, seven hundred millions baht in 2011, and reached the maximum plateau in 2012 at nine hundred millions baht (equivalent to 30 millions US Dollars).

The mechanisms of sugar production during ripening stages are of interesting. Many studies in recently years have focused on single or a few genes related to the ripening of mango (Li et al., 2012; Shalom et al., 2011). It has been shown that those gene expressions were still far from comprehensively elucidating of their mechanisms. It was also shown that studying the Proteomic Code of those genes could solve this problem. The Proteomic Code is a set of rules by which information in genetic material is transferred into the physicochemical properties of amino acids and determines how individual amino acids interact with each other during folding and in specific protein–protein interactions. This is an approach that comprehensive survey of all proteins expressed at an interesting time, at any conditions, in any organism. Komatsu and Ahsan reported that the major advantage of proteomic code is that it focuses on the functional translated portion of the genome (2009).

An analysis of proteomes is a powerful tool for determining the roles and functions of individual proteins in plants. Several studies on proteomics concerning many types of plant tissue have been carried out. By proteomics approach in previous works, stem cell wall proteins of alfalfa were extracted and more than 100 proteins were identified and used to generate proteomic reference maps for cell walls of alfalfa (Watson et al., 2004). Furthermore, some researches used 1D and 2D electrophoresis to size-separated protein from apricot and identified proteins by MALDI-TOF-PMF and nanoLC-ESI-LIT-MS/MS to found out proteins that related with its palatable and nutrition (Ambrosio et al., 2013). Our lastest work about Proteomics is in physic nut, a biodiesel economic crops. We found 4 proteins during seed development were altered between the developmental stages of the kernels in a broadly similar pattern as the level of most fatty acids (Booranasrisak et al., 2013). The lastest work about proteomic in mango, protein was extracted by phenol and separated by 2D gel before identified protein spots by LC-MS/MS. Proteins related to fruit quality, putative protein involved in color development and pulp softening, were found (Andrade et al., 2012).

However, working with peptides is highly complicated, where several steps, i.e., gel electrophoresis, excision of the individual protein bands, and in-gel digestion with a protease can reduce sample complexity (Delahunty and Yates, 2005). A Proteomics approach in combination with the SDS-PAGE observation is modified to use in this study, which in-gel digestion with trypsin and LC-MS/MS technique help to understanding related mechanisms. The Proteomics code illustrates the protein expression pattern associated with the mechanisms of sugar production during ripening stages in mango flesh.

This study was aimed at determining sugar production levels and protein profiles of *Mangifera indica* in order to study their relationship during development.

MATERIALS AND METHODS

Plant materials

All fruit samples of mango were collected from an orchard in Chachoengsao province, Thailand during March to April, 2012. All fruits were segregated into eight stages of the development according to the time intervals or days after flowering and the morphological appearance of the fruits. The characteristics of the fruits are summarized as shown in Table 1. All fruits were stored at -80 ºC to preserve proteins until use.

Table 1. The eight developmental stages of mango fruits defined by the developmental time intervals (days after flowering) and morphological appearance of the fruits.

Stage of development	Time interval (days)	Fruit texture	Fruit color
1	15	hard	Green
2	30	hard	Green
3	45	hard	Green
4	60	hard	Green
5	75	hard	Green
6	90	soft	Brown spots at the upper pole, yellowish-green
7	105	soft	Brown spots at the upper pole, yellowish-green and orange at the lower pole
8	120	soft	Brown spot all over the fruit, yellow peel and orange at the lower pole

Mango flesh sugar determination by refractometer

The skin of fresh fruit was peeled off and discarded, where only flesh from the individual developmental stages of mango fruits was milled using a laboratory mortar and pestle. The solution was subsequently analyzed by ATAGO hand refractometer in triplicate manner.

Mango flesh protein analysis by proteomics

Protein extraction

The flesh tissues from the individual stages were ground into fine powder in liquid nitrogen with pre-cooled mortars and pestles, and were subsequently dissolved in one ml of 0.1%

(w/v) SDS. Each protein mixture was precipitated with cold acetone overnight at -20 ºC. The protein concentration of each sample was determined by the method of Lowry *et al*. (1951) using serial dilutions of bovine serum albumin (BSA) as the protein standard.

SDS-PAGE Analysis

Each 10 g protein sample was mixed with a loading buffer and loading dye, and was subsequently heated at 95 ºC for 10 min before loading. The sample was resolved through an acrylamide resolving SDS-PAGE. Gel electrophoresis was carried out at 30 Volts for the stacking gel and 50 Volts for the separating gel. The gel was stained by Coomassie Brilliant Blue.

Protein Identification

Protein bands from the Coomassie Brilliant Blue stained SDS-PAGE gels were excised manually according to the molecular mass range. Each selected protein band was cut from the gel and then the slice was cut into small pieces and was isolated by in-gel digestion. The peptides were extracted from the gel plugs after digestion, and then were injected into the Ultimate 3000 LC system (Dionex) coupled to ESI-Ion Trap MS (HCT ultra PTM Discovery System, Bruker Daltonik) with electrospray. The MS/MS spectra were analyzed with DeCyder MS 2.0 differential analysis software (GE Healthcare). The analyzed MS/MS data from DeCyder MS was submitted to a database search against the NCBI database using Mascot software (Matrix Science, London, UK, (Perkins et al., 1999). All proteins were functionality identified by GoCat (http://eagl.unige.ch/GoCat).

RESULTS

Sugar determination

The detected sugar profiles of mango fleshes of individual eight developmental stages are summarized in Table 2. It was noticed that the sugar content was gradually increased from stage II (8.00 degree Brix) to stage VII (12.40 degree Brix), and finally at 25.97 degree Brix in the last stage. One degree Brix was defined by one gram of sucrose in 100 grams of solution or as percentage by weight (Das et al., 2012).

Table 2. The quantity of sugar detected in all individual developmental stages and their standard deviation values. All data were the average values derived from triplicate experiments.

Developmental stage	Sugar (degree Brix)	Sucrose (%w/w)	Standard Deviation
1	0.00	0.00	0.00
2	8.00	8.00	0.00
3	8.07	8.07	0.12
4	8.33	8.33	0.12
5	8.33	8.33	0.12
6	10.40	10.40	0.53
7	12.40	12.40	0.17
8	25.97	25.97	0.06

Protein analysis

Protein concentration determination

The total proteins, which were extracted from 500 mg of mango flesh and the protein concentration was determined according to the method of Lowry. BSA standard curve, which was plotted between OD_{750} on Y-axis, while BSA concentrations (µg/ml) were plotted on X-axis, had R^2 = 0.9757. Protein concentrations (µg protein/µl) of eight ripening stages of mango were calculated from an equation y = 0.0216x + 0.0832 and are summarized in Table 3.

Table 3. Showing results of protein concentrations of eight developmental stages of Nam-Dokmai mango.

Developmental stage	µg protein/µl
1	14.20
2	11.29
3	15.73
4	10.31
5	3.05
6	10.78
7	8.00
8	14.20

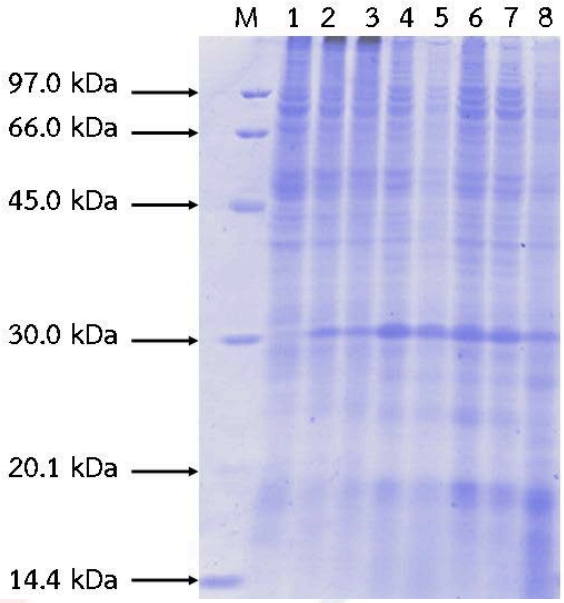
Optimization of Protein Loading for SDS-PAGE Analysis

All protein concentrations were determined by Lowry assay, where 50 µg of all protein samples were used for further experiments.

SDS-PAGE analysis

The protein samples of eight developmental stages were analyzed by 12.5% SDS-PAGE, and the gel was visualized by Coomassie Brilliant Blue R250 staining as shown in Figure 1. When SDS-PAGE gel was observed by eye, it was found that the intensity of many protein bands with in a range of 14.4-30.0 kDa had increased (up regulated) but the intensity of some protein bands had decreased (down regulated). Then these gel ranges were selected to analyze by LC-MS/MS in the next steps.

Figure 1. Showing the characteristics of 12.5% SDS-PAGE of 50 µg protein of 8 developmental stages of Nam-Dokmai mango. Lane M represents standard molecular weight marker; Lane 1-8 represent µg protein of 8 developmental stages of Nam-Dokmai mango.

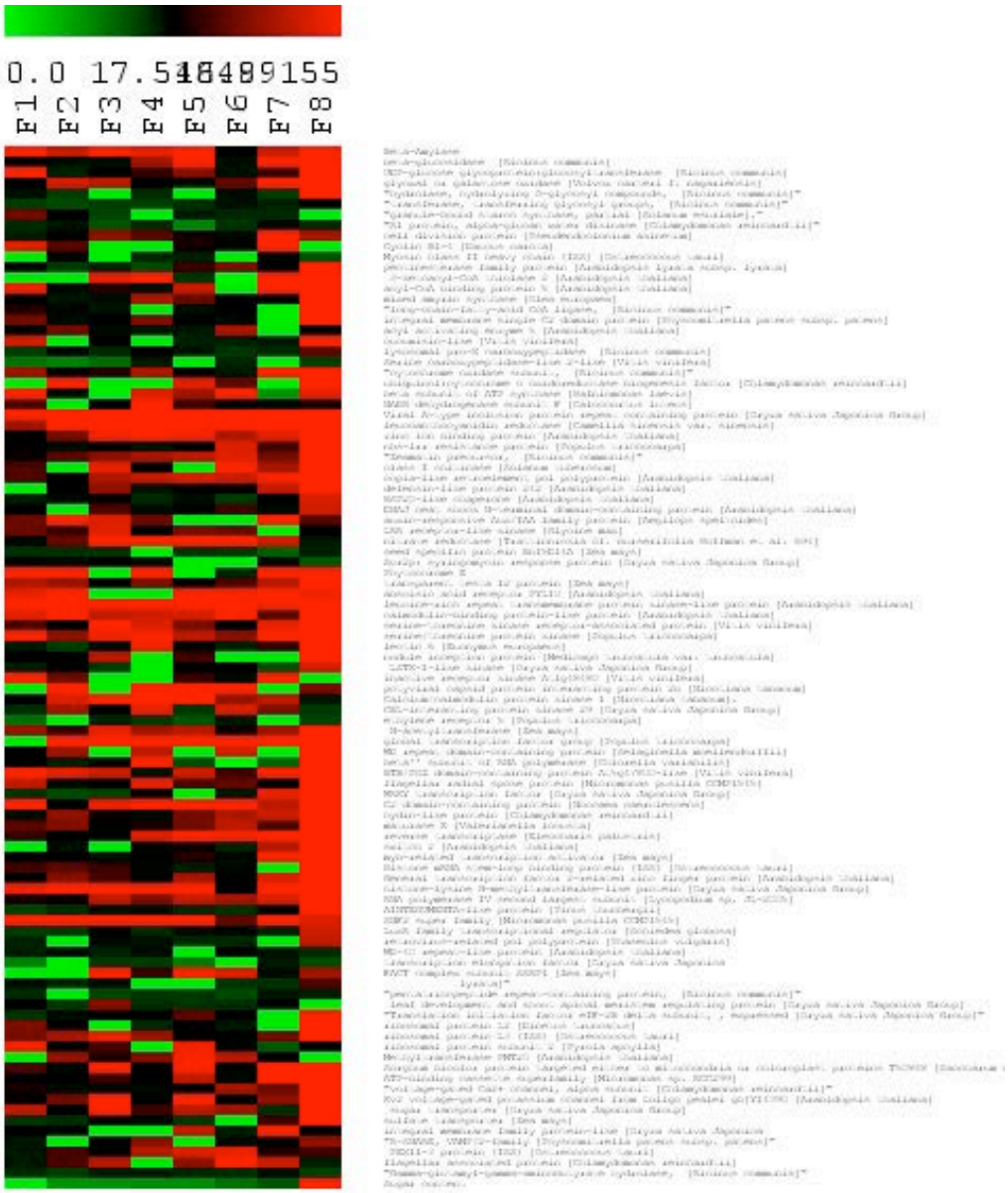


LC-MS/MS and DeCyderMS

All gel bands in the range of 14.4 to 30.0 kDa were excised to the molecular mass range of protein standard markers. The tryptic in-gel digestion was subsequently performed. The extracted peptides of each molecular mass range sample were individually injected to LC-MS/MS.

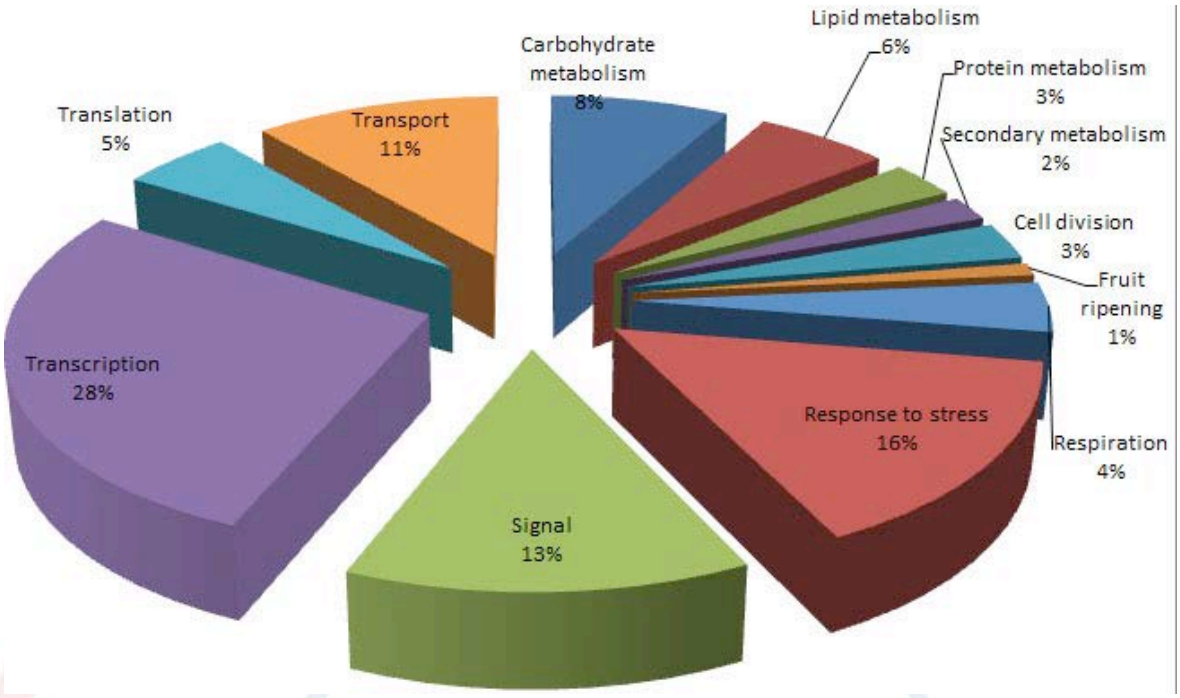
The MS/MS data of the peptides after analysis by DeCyderMS software showed differentially high expression among all eight developmental stages of Nam-Dokmai mango. They were exported and further identified by Mascot software using protein and DNA databases available in the NCBI (Johansson et al., 2006; Thorsell et al., 2007). There were 98 differentially expressed identified proteins as shown in Figure 2.

Figure 2. Showing details of 98 identified proteins that differentially express along developmental stages; different color represent different amount level of protein, green = plenty level, black = medium level, red = less level



There were several differentially expressed proteins identified in this study, which were related to carbohydrate metabolism, lipid metabolism, protein metabolism, secondary metabolism, cell division, fruit ripening, respiration, signal, transcription, translation and transport. Results are summarized in Figure 3.

Figure 3. Schematic diagrams showing functional classification percentages of 98 identified proteins.



There were 11 proteins that showed a similar trend in expression to sugar production as shown in table 4, i.e., glycosyl transferase, beta-glucosidase, lysosomal pro-X carboxypeptidase, defensin-like protein 242, lectin 6, global transcription factor group, FACT complex subunit SSRP1, AINTEGUMENTA-like protein, N-acetyltransferase, LuxR family transcriptional regulator and hydin-like protein. These proteins were related to carbohydrate metabolism, protein metabolism, responded products to stress, signal, and transcription.

Table 4. Showing proteins related to Namdokmai-mango and their relative expression levels along developmental stages.

Protein name	Accession number	1	2	3	4	5	6	7	8
transferase, transferring glycosyl groups	gi|255584380	14.01	13.16	13.98	14.08	14.93	16.38	15.70	17.44
beta-glucosidase	gi|255584818	17.53	16.62	16.51	18.35	19.61	16.57	19.84	20.83
lysosomal pro-X carboxypeptidase	gi|255565027	14.14	16.76	15.34	17.40	16.23	14.61	15.24	17.97
defensin-like protein 242	gi|42572919	0.00	17.39	18.21	16.34	17.19	17.58	18.50	18.97
lectin 6	gi|158562101	16.06	14.12	16.63	18.82	16.85	15.65	17.80	19.88
global transcription factor group	gi|224136059	0.00	20.68	21.16	19.44	22.45	18.95	22.42	21.60
FACT complex subunit SSRP1	gi|162462425	0.00	0.00	18.87	17.79	20.51	18.70	16.73	18.33
AINTEGUMENTA-like protein	gi|46395275	13.68	15.03	16.57	16.51	15.17	14.64	16.97	18.87
N-acetyltransferase	gi|226529942	18.30	17.78	18.73	18.31	18.43	17.64	18.28	22.96
LuxR family transcriptional regulator	gi|302172131	14.11	14.36	17.19	15.01	16.71	16.95	15.59	18.57
hydin-like protein	gi|159463534	16.42	18.22	17.62	16.58	18.07	18.39	18.60	20.39

DISCUSSIONS AND CONCLUSIONS

Here, proteomic approaches were selected for identified protein that related to sweetness during developmental stages of mango fruits. All fruit samples of mango were collected from an orchard and were segregated into eight stages of the development according to the time intervals or days after flowering and the morphological appearance of the fruits. This collection step was differ from the former work that fruits were collected in unripe stage and were allowed to ripen naturally at 25ºC (Andrade et al., 2012). However, the sugar result in our study was similar to the previous work.

According to Table 1, sugar production in mango flesh started during stage 2 (30 DAF) and remained stable to stage 5 (75 DAF). Then sweetness gradually increased from stage 5 to stage 8, reaching a maximum at 25.97 degree Brix. The trend of soluble sugar accumulation is similar to the observations of previous works about mango (Saluuke and Wu, 1973; Andrade et al., 2012) and also in another fruit (Wanitchang et al., 2010; Hubert and Mbéguié, 2012).

After SDS-PAGE, it was found that the intensity of many protein bands in 14.4-30.0 kDa were increased or up regulated then were selected and individually cut for identified by LC-MS/MS and Mascot. The 98 differentially expressed protein during eight developmental stages of Nam-Dokmai mango flesh were identified and found to be involved in several cellular processes including carbohydrate metabolism, lipid metabolism, protein metabolism, secondary metabolism, cell division, fruit ripening, respiration, signal, transcription, translation and transport. This protein result was similar to previous proteomic study on mango using 2-DE gel and LC-MS/MS (Andrade et al., 2012). Besides, in these amounts, there were 11 identified proteins that showed a similar trend in expression to sugar production through developmental stages. They may play a major role in sugar production of Nam-Dokmai mango.

Furthermore, these results may be helpful for mango harvesting in different purposes such as readiness for consumption, dehydration, pickling, juicing, etc. Our next study will use HPLC technique to study the quantity and quality of each sugar type and hope these will be valuable for other industrial crops in Thailand and Asian countries.

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