Electronic Journal of Biotechnology 18 (2015) 29–34

HOSTED BY

ELSEVIER

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

A comparative proteomic study of white and black glutinous rice leaves

Wipada Phonsakhan, Kanlaya Kong-Ngern *

Department of Biology, Faculty of Science, Khon Kaen University, Khonkaen 40002, Thailand

ARTICLE INFO

Article history:
Received 22 July 2014
Accepted 5 November 2014
Available online 5 December 2014

Keywords:
2D-PAGE
Anthocyanin
LC–MS/MS
Oryza sativa L
Proteomics

ABSTRACT

Background: Black glutinous rice contains remarkable levels of anthocyanins, which possess anti-oxidative properties and thus have health benefits. The accumulation of anthocyanins in grains of thirty black glutinous rice varieties was measured, and the results revealed that the accumulated anthocyanin content ranged from 0.262 to 2.539 mg/g. Black glutinous rice Br no. 19 was selected, and its leaf protein expression profile was compared with that of white glutinous rice RD 6 using 2D-PAGE, and the protein spots were then directly analyzed after proteolysis by LC–MS/MS.
Results: The proteins from the leaves of the two rice varieties were separated using 2D-PAGE and silver stained, and the spots were analyzed using Image Master 2D Platinum version 5 software. The results showed that the protein profiles of these two rice cultivars were different, with at least six protein spots that were detected only in Br no. 19. In addition, seven protein spots accumulated at higher levels in Br no. 19 than in RD 6.
Conclusion: The protein spot S1 (AP005098.4) is homologous to the *Rc* protein. Our results suggest that some of the proteins enriched in Br no. 19 may be involved in anthocyanin synthesis in the black glutinous rice.

1. Introduction

Rice (*Oryza sativa* L.) is an important grain and a basic staple food for a large part of the world's population [1,2,3]. People in East and South-east Asian countries also consume glutinous rice in addition to the common white non-glutinous rice. Pigmented (red, brown, purple and black) glutinous rice in particular has gained a lot of attention as raw materials for production of commercial health food supplements due to its high phenolic, anthocyanin and antioxidant contents. Black rice, which includes several rice varieties with a long history of cultivation in Southeast Asian countries such as China, India and Thailand, derives its name from its black color [4]. In some varieties of black rice, anthocyanins are present in the stem and leaves as well as the kernels, in others only the grains are pigmented.

The health benefits of black glutinous rice have recently been reported by several investigators. Black glutinous rice has been shown to accumulate compounds such as anthocyanins [5,6] and gamma oryzanol [7,8,9,10]. Black rice also contains many vitamins and minerals, including iron, vitamin A and vitamin B, which are beneficial for overall health and the prevention of heart disease [11].

Anthocyanins are naturally occurring plant pigments that belong to the flavonoid family and are widely used for their antioxidant and pharmacological properties [12]. Reactive free radicals have been postulated to contribute to the development of chronic inflammatory proliferative diseases (CIPDs) [13], particularly arteriosclerosis and cancer by causing oxidative damage to essential enzymes, cells and tissues [14,15]. The anthocyanins in rice act as antioxidants, which can inhibit inflammation throughout the body [16], act as anticancer agents [17,18,19,20,21], promote blood circulation, slow damage and aging of tissues, reduce cholesterol and blood sugar levels [11,22,23, 24], affect pituitary gland function, inhibit gastric acid secretion and inhibit platelet aggregation [25].

Proteomics comprises a rapidly emerging set of key technologies that are being used to identify proteins and determine protein function. The rice proteomic studies that have been conducted to date have focused mainly on the changes in protein expression that are triggered by environmental factors [26,27]. Over 1100 proteins are expressed in rice leaves, as identified as spots on 2D-gels. The identified proteins have been classified into 10 functional categories, including cell structure, cell growth/division, energy metabolism, disease/defense, intracellular traffic, metabolism, protein destination and storage, signal transduction, hypothetical functions, and unknown proteins [28]. Most rice leaf proteomes were obtained from green leaves [29,30,31,32,33,34,35] however, proteomes from purple anthocyanin-containing leaves have never been reported. The proteomic profiling of plants that accumulate anthocyanins, such as grapes [36], and more specifically the mesocarp of vine-ripened grapes have helped to elucidate the biochemical and physiological changes that occur during anthocyanin accumulation and have been of paramount importance in advancing the understanding of berry development and the ripening process [37]. To gain information on the proteins involved in anthocyanin production in black

* Corresponding author.
E-mail address: kkanla@kku.ac.th (K. Kong-Ngern).
Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

http://dx.doi.org/10.1016/j.ejbt.2014.11.005
0717-3458/

glutinous rice, we compared the protein profiles of purple leaves of a black glutinous rice variety with those of the green leaves of a white glutinous rice using 2D-PAGE. The information obtained will be useful in further studies of the functions of proteins that are involved in the anthocyanin pathway.

2. Materials and methods

2.1. Plant materials

Thirty varieties of local black glutinous rice seeds (Table 1) collected from northeast Thailand during 2008 and 2009 were generously provided by Chumphae Rice Research Center, Khon Kaen and the Faculty of Agriculture, Khon Kaen University, Khon Kaen, Thailand.

2.2. Total anthocyanin content (TAC)

The TAC in the grain samples of all 30 rice varieties was determined using a previously described spectrophotometric method [38]. The supernatant from a crude extract was poured into a 50 mL volumetric flask and brought up to volume at 24 mL with acidified methanol. The absorbance was measured using a UV–visible spectrophotometer at 535 nm. The TAC was calculated as follows: $TAC = A \times 288.21$, where A is the absorbance reading.

2.3. Plant growing conditions

Seeds from the Br no. 19 and the white glutinous improved cultivar RD 6 were grown hydroponically in a greenhouse as described by Gregorio et al. [39], with some modifications. The experiment was conducted in a greenhouse at the Department of Biology, Faculty of Science, Khon Kaen University, Thailand. After the seedlings were grown for 35 d in nutrient solution, the leaves were collected, frozen in liquid nitrogen and stored at −70°C.

Table 1
Characteristics of thirty varieties of local black glutinous rice.

Varieties	Color			TAC (mg/g) ± SD
	Grains	Pericarp	Leaves	
Br no. 3	Black	Black brown	Green	0.954 ± 0.020
Br no. 16	Brown	Black brown	Green	0.598 ± 0.037
Br no. 18	Straw	Black brown	Green	0.437 ± 0.024
Br no. 19	Black	Black	Purple	2.539 ± 0.011
Br no. 20	Black	Black brown	Green	0.942 ± 0.022
Br no. 26	Black	Black brown	Green	0.720 ± 0.045
Br no. 27	Black	Black brown	Purple	0.41 5 ± 0.057
Br no. 28	Black	Black brown	Green	1.366 ± 0.056
Br no. 29	Black	Black	Green	1.010 ± 0.044
Br no. 30	Straw	Black brown	Green	0.919 ± 0.037
Br no. 32	Black	Black brown	Green	0.631 ± 0.039
Br no. 42	Black	Black brown	Green	0.751 ± 0.097
Br no. 44	Black	Black brown	Purple	0.663 ± 0.061
Br no. 46	Black	Black brown	Purple	0.262 ± 0.071
Br no. 50	Black	Black	Green	1.022 ± 0.071
Br no. 52	Black	Black brown	Green	1.222 ± 0.002
Br no. 53	Brown	Black brown	Green	0.752 ± 0.031
Br no. 54	Black	Black brown	Purple	0.530 ± 0.015
Br no. 55	Brown	Black	Green	0.526 ± 0.063
Br no. 56	Brown	Black brown	Purple	0.683 ± 0.056
Br no. 58	Brown	Black	Purple	0.743 ± 0.065
Br no. 59	Straw	Black brown	Green	0.405 ± 0.019
Br no. 63	Straw	Black	Green	1.243 ± 0.048
Br no. 64	Black	Black	Green	0.790 ± 0.093
Br no. 65	Black	Black	Green	0.733 ± 0.011
Br no. 68	Black	Black	Green	0.707 ± 0.037
Br no. 70	Black	Black brown	Green	0.419 ± 0.030
Br no. 71	Black	Black	Green	1.101 ± 0.037

2.4. Protein extraction

The leaves of rice cultivars RD 6 (white glutinous rice with green leaves) and Br. no. 19 (black glutinous rice with purple leaves) were ground to a fine powder in liquid nitrogen and dissolved in ice-cold double-distilled water. The homogenate was centrifuged at 13,000 × g for 15 min at 4°C. The dried protein pellets were solubilized in rehydration buffer [8 M urea, 0.5% (w/v) CHAPS, 20 mM DTT, and 0.5% (v/v) IPG buffers]. The amount of protein was determined according to the Bradford method [40].

2.5. Two-dimensional PAGE

The proteins from each sample (5 μg) were separated by 2D-PAGE as described by Berkelman and Stenstedt [41]. Isoelectric focusing gel electrophoresis (IEF) was conducted at 20°C using an IPG phor™ IEF System and a DryStrip kit (Amersham Biosciences, Uppsala, Sweden). Each of the 7-cm IPG strips (pH 3–10, non-linear) was rehydrated with 125 μL of rehydration buffer for 13 h, and the protein sample was then loaded onto the strips. The isoelectric focusing was performed in 5 steps at 150 V for 2 h, 300 V for 30 min, 1000 V for 30 min, 5000 V for 1.20 h and 5000 V for 25 min. The focused strips were equilibrated twice for 30 min in 10 mL equilibration buffer [50 mM Tris HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, and 100 mg DTT] with gentle shaking. During the second equilibration, 250 mg iodoacetamide was used instead of DTT.

The second-dimension separation was performed by SDS-PAGE (10% total monomer, with 2.6% crosslinker) using a PROTEAN II Multi Cell (Bio-Rad, Hercules, USA). The focused strips were transferred to the tops of the gels, and the two slabs were electrophoresed simultaneously with an initial current of 10 mA for 10 min and then at 25 mA until the tracking dye reached the bottom of the gel. The protein spots were visualized by staining with silver nitrate. The isoelectric point (pI) values were determined automatically using Image Master 2D Platinum version 5 software (Amersham Biosciences, Uppsala, Sweden), and the relative molecular weight (MW) of each protein spot was calculated. All 2D protein gel analyses were performed at least three times.

2.6. Chromatography coupled with tandem mass spectrometry

The protein spots were excised from the gels and analyzed after proteolysis by liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) at the National Center for Genetic engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency, Thailand. External calibration was performed, and the data were collected in linear mode. The empirical peptide mass values were matched with the theoretical digested mass and database sequence information using Mascot (http://www.matrixscience.com).

3. Results

3.1. Total anthocyanin content

Thirty varieties of local black glutinous rice seeds collected from northeast Thailand during the years 2008 and 2009 were collected and characterized for the color of grains, pericarp and leaves (Table 1). The color of grains for black glutinous rice varieties were straw color in four varieties, brown color in six varieties and black color in nineteen varieties. The colors of pericarps for black glutinous rice varieties were black color in ten varieties and black brown color in twenty varieties. The colors of leaves for black glutinous rice varieties were green in seven varieties and purple in twenty three varieties. The contents of anthocyanins in brown rice grains of thirty varieties of local black glutinous rice were measured and the results revealed that