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Swapnil S Thakare
Division of Biochemistry, ICAR-Indian Agricultural Research Institute (IARI), New Delhi, India

Monika Awanaa
Division of Biochemistry, ICAR-Indian Agricultural Research Institute (IARI), New Delhi, India

Shilpi Aggarwal
Division of Plant Pathology, ICAR-Indian Agricultural Research Institute, New Delhi, India

Veda Krishnan
Division of Biochemistry, ICAR-Indian Agricultural Research Institute (IARI), New Delhi, India

Archana Singh
Division of Biochemistry, ICAR-Indian Agricultural Research Institute (IARI), New Delhi, India

Corresponding Author:
Archana Singh
Division of Biochemistry, ICAR-Indian Agricultural Research Institute (IARI), New Delhi, India

Fluorescence intensity-based analysis of binding interaction of biomolecules during ageing of rice (*Oryza sativa* L.)

Swapnil S Thakare, Monika Awanaa, Shilpi Aggarwal, Veda Krishnan and Archana Singh

Abstract
Rice (*Oryza sativa* L.), is a crucial cereal crop that provides the bulk of nutrients upon consumption. Due to storage, there is an alteration in the physico-chemical properties, digestibility and matrix component interactions of rice but the exact mechanism is not known till date. Present study was performed to elucidate the role of binding interaction between different biomolecules during ageing in mostly consumed and grown varieties in India- 2 basmati varieties (PB-1121 and PB-1509) and 2 non-basmati varieties (BPT-5204 and Swarna) by analyzing intensities of different fluorescent dyes. Starch in rice showed high fluorescence intensity (35 Grey value) compared to protein (30 Grey value) and for lipid (25 Grey value). Fluoresceine isothiocyanate (FITC) showed greater increase in fluorescence intensity during ageing period. Findings also indicated that PB-1509 and BPT-5204 showed a strong increase in fluorescence intensity after 12 months of storage. Obtained results also suggest that there must be alignment and regular arrangement of these biomolecules that happened during ageing could be responsible for the improved eating and cooking quality of aged rice.

Keywords: Rice ageing, fluorescence, binding interactions, starch granules

1. Introduction
Half of the world's population depends on rice, a crucial cereal food crop as their primary source of food (Saikrishna *et al*., 2018) [1]. The amount of amylose and amylopectin in rice starch granules varied from around 18% to 35% and 70% to 90%, while crystallinity ranged from 32% to 46% (Kong *et al*., 2015) [2]. With granule sizes ranging from 2 to 10 m, rice starch is one of the grains with the smallest granule size. Due to its unique features and functions, rice starch is frequently employed in industry (Lindeboom and Tyler, 2004) [3].
Since starch is the primary source of carbohydrates for many animal species, including humans, it is also one of the key components of diet (Zhang *et al*., 2014) [4]. The total production of rice had remained high during the previous ten years. The majority of the rice was directly ground and processed for consumption, with a small quantity being utilized as raw materials for food processing and animal feed. As a result, rice must be kept in storage for the time interval before consumption. Rice storage is also called as ageing which is a complicated process where starch, protein, lipids and even endogenous amylases all play important roles to affect the rice grain properties during storage. Studies suggested that the interactions among macro- and micro-compositions in rice grains during storage would play key roles on the changes contributing towards overall physico-chemical, sensory, texture and cooking properties of rice (Pal *et al*., 2019; Zhou *et al*., 2015) [5, 6]. Because of the increased breakdown of -1,6-glycosidic linkages caused by rice storage, the amount of amylose increased whereas the amount of long branch-chains of amylopectin decreased (Wang *et al*., 2022) [7]. Increasing AC content and interaction of starch with other non-starch components could be the reason for increasing RS content during ageing. According to research by Chakraborty *et al*. (2023) [8], the resistant starch (RS %) content rose as storage duration and temperature increase. Recent study by Tiano *et al*. (2022) [9] also endorsed that starch granule disaggregation after cooking gets reduced which could be the reason for the alteration in pasting properties (high peak viscosity and final viscosity) of rice grain during ageing.
It has been observed that a number of variables, including starch structural parameters such as amylose/amylopectin ratio, molecular/supra-molecular structure, and amylose-lipid, regulate the pace and amount of enzymatic hydrolysis of starches.

Barrier factors, such as protein matrices, fibers, and the integrity of cell walls, include complexes, chemical modification, processing, granule/ particle size, and porosity (Annor *et al*., 2017; Dhital and Gidley, 2017) [10, 11]. The interactions between important macronutrients like proteins, lipids, and starches can be one of these elements, and they can have a significant impact on the glycemic response brought on by starchy diets (Bhattarai *et al*., 2016; Parada and Santos, 2016) [12, 13].

According to Bhattarai *et al*. (2016) [12], research on the relationships between proteins, lipids, and starches and how they affect enzymatic susceptibility in various grains is still in its early stages. According to Yu *et al*. (2018) [14], barley proteins, amylase may bind to starch granules and to water-insoluble protein, resulting in a reduction in starch hydrolysis. Proteins, in particular water-soluble proteins, can impede starch digestion. The types and amounts of fatty acids, according to Annor *et al*. (2013) [15], are crucial factors in the hypoglycemic qualities of millets. There has been a lot of research on how amylose chains can form inclusion complexes when the right guest molecule is present (Putseyset *et al*. 2010) [16]. Polar visitors are strongly drawn to the hydrophobic surface of the amylose helix cavity (Rutschmann *et al*., 1990) [17]. Previous study examined the existence of complexes between amylose and lipids (fatty acids and phospholipids) (Biliaderis *et al*., 1991) [18]. Amylose-lipid inclusion complexes have been demonstrated to affect the food's rheological properties and digestion (Putseyset *et al*. 2010) [16]. It is consequently essential to comprehend the nature and dynamics of the process. One of biophysical technique that can be used to study this interaction between different biomolecules is confocal laser scanning microscopy (CLSM).

CLSM has already revolutionized the field of structural biology and it continues to find more and more uses in a variety of scientific fields. Development of CLSM leads to fluorescence imaging in life science and it has great advantages over conventional microscopy. Its high axial resolution, sharp image quality and associated quantitative picture analysis provide vital structural information about micro-structure (Tata and Raj, 1998) [19]. According to the widely recognized model, the polar group, which includes the carboxylic acid, stays outside the helix as a result of electrostatic interactions, whereas the aliphatic chain of the fatty acid molecule is housed inside the hydrophobic cavity of the amylose helix (Godet *et al*., 1993) [20]. Recent research has demonstrated the development of distinct inclusion complexes between lipophilic molecules and waxy and normal starch granules from various plant sources using CLSM (Manca *et al*., 2015) [21]. Study of binding of non-starch components to starch is the most recent area of research. This field of study is not much explored due to unavailability of literature about direct determination of interaction between various biomolecules through the use of different dyes. Most of the studies have mostly focused on the identification of particular isolated biomolecules only. In this study, we have tried to observe the binding between different grain components specially starch, protein, and lipids in rice flour using different fluorescent dyes.

2. Methodology

2.1 Storage

Four widely consumed rice varieties (Basmati-PB-1121 and PB-1509 and non-Basmati-BPT- 5204 and Swarna) having largest area under cultivation were used for the current study. Grains of selected varieties were stored at a control temperature (28 °C) and were removed after every 6 months for further analysis.

2.2 Sample preparation

100 mg of rice grain flour was weighed and taken in 2 ml eppendorf tube and 1 ml of distilled water was added. Vortexed for 1 min and then cooked for 20 min at 90 °C in the water bath (shaken after every 5 min). Rhodamine B (10 ul, 0.025%) was first added, and vortexed for 1 min. Then Nile Blue (NB) (10 ul, 1%) was added, and vortexed for 1 min. FITC 10 ul, 0.25%) was added at last (and vortexed for 1 min. All the procedures were carried out at room temperature. After preparation of rice slurry, suspension with dye immediately covered with aluminium foil or kept at dark till the observation under CLSM were not taken in order to avoid light absorption by different dyes that may affect the fluorescence intensity of dyes during microscopic observation.

2.3 Microscopic Visualization

During microscopic observation, the slides were prepared using a thin cover slip and a single drop of rice slurry suspension was sucked by using a suction pressure tube and added to the glass slide. Excitation wavelength were set according to different dyes such 488 nm, 588 nm and 633 nm for FITC, Rhodamine B and NB respectively. Intensity of fluorescence were calculated by selecting the prominent area in the captured images and values represented in unit Grey value, which give idea about the brightness of pixel.

3. Results and Discussion

According to previous study (Martisek and Prochazkova, 2017) [22] introducing specific fluorophore probes throughout the materials, the distributions, shapes and sizes of internal structures of cell can be directly visualized by the CLSM technique. FITC, Rhodamine B and NB dyes were used to visualize the rice slurry sample using CLSM. Fig 1 shows a representative micrograph network of starch granules components observed in the images. Starch binds with FITC and emits green light upon excitation, proteins bind with Rhodamine B and emits yellow light whereas lipids bind with Nile blue and emits red light. Since there is high percentage of starch in rice it showed high fluorescence intensity (35 Grey value) as compared to protein (30 Grey value) and lipid (25 Grey value). Although FITC showed high fluorescence intensity but it was not as high as the according to starch concentration (60 to 70%), this indicates that FITC binds less efficiently compared to Rhodamine B and Nile blue to the respective biomolecules. Rhodamine B showed highest binding efficiency among all the used dye. Two distinct CLSM variants reflectance-mode and fluorescence-mode CLSM have so far use in characterizing material structures. The surface texture or roughness of material surfaces is characterized using CLSM in the reflectance mode (Webb, 1996). In our study we have exploit the fluorescence-mode CLSM approach. It is the most common type of CLSM, which produces image contrast via excitation of fluorophores (Ille *et al*., 2019 and Liu *et al*., 2016) [29, 24].

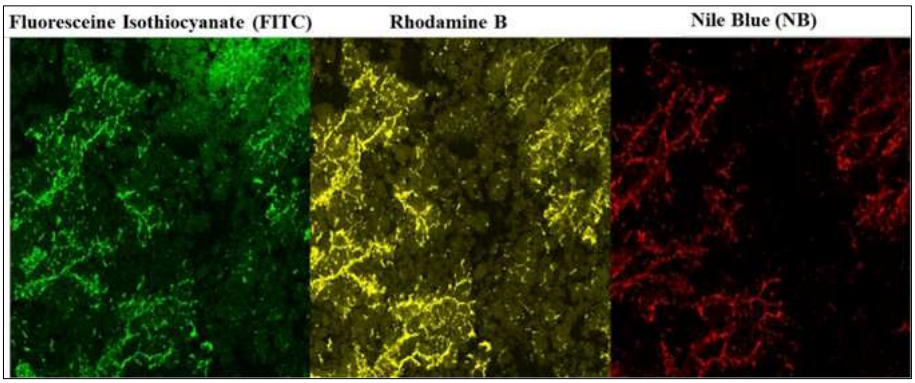

Fig 1: Representative confocal micrograph of rice slurry visualized using Fluoresceine Isothiocyanate (FITC), Rhodamine B and Nile Blue (NB)

When the intensity of individual dye in samples of different ageing period was evaluated, it was found that there is increase in the intensity in significant manner (Fig 2). In PB-1121 for FITC dye, freshly harvested sample showed 7% fluorescence intensity while 12%, 17%, 29% and 35% intensity were observed in 6 months, 12 months, 18 months and 24 months sample respectively. In case of Rhodamine B dye, freshly harvested sample of PB-1121 showed 12% fluorescence intensity and for 6 months, 12 months, 18 months and 24 months sample it was 14%, 19%, 26% and 29% respectively. Whereas, NB showed 11%, 14%, 18%, 26% and 31% intensity in freshly harvested, 6 months, 12 months, 18 months and 24 months samples respectively. Likewise, fluorescence intensity of individual dye was checked for other basmati (PB-1509) and non-basmati rice varieties (BPT-5204 and Swarna) for different ageing duration as shown in Fig 2 & 3. In PB-1121 FITC showed ~2 fold, ~2.5 fold, ~4 fold and ~5 fold increase in the fluorescence intensity of 6 months, 12 months, 18 months and 24 months stored sample respectively as compared to freshly harvested sample. For Rhodamine B in comparison to freshly harvested sample it was ~1.25 fold, ~1.5 fold, ~2 fold and ~2.5 fold increase in the fluorescence intensity of 6 months, 12 months, 18 months and 24 months stored sample respectively. Whereas Nile blue showed ~1.25 fold, ~1.75 fold, ~2.5 fold and ~3 fold increase in the fluorescence intensity of 6 months, 12 months, 18 months and 24 months stored sample respectively as compared to freshly harvested samples. Hence among 3 dyes, FITC showed greater increase in fluorescence intensity during ageing period.

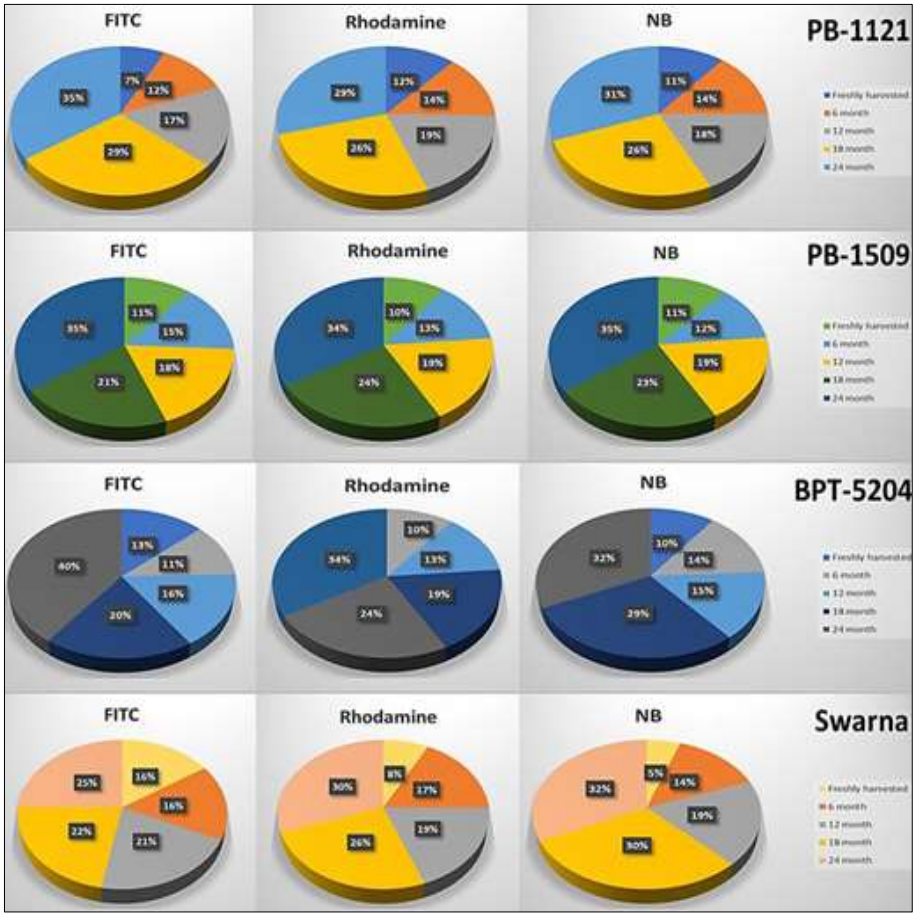

Fig 2: Fluorescence intensity of individual dye [Fluoresceine Isothiocyanate (FITC), Rhodamine B and Nile Blue (NB)] during the different ageing periods for selected Basmati (PB-1121 and PB-1509) and non-basmati rice varieties (BPT-5204 and Swarna)

Study by Dai *et al*., (2019) [25], showed that binding interaction of rice glutelin protein with the phenolic compound are getting alter due to their varying content and analysed it based on fluorescence intensity in CLSM. Various components of grain, inside and outside of the starch granule before ageing are randomly distributed and present in diffused form. During ageing and cooking of such aged rice it undergoes numerous physico-chemical changes that results in increase in retrogradation, gelatinization kinetics, regular arrangement of alternate lamellae and amorphous structure of starch. Also allow alignment of different functional groups of amino acids in the proteins that help in proper arrangements of proteins surrounding to starch those results in increase in compactness of starch and other non-starch components. Hence due to the combined effect of ageing and cooking, this diffused form of biomolecules comes together and form their condensed state, this could be the possible reason for increase in the fluorescence intensity of individual dye during ageing of rice. The structural breakdown and phase transfer of starch molecules with enhanced structure and lower the amount of hot water dissolving during the cooking of aged rice may be responsible for better eating cooking quality (ECQs) traits of rice (Peng *et al*., 2019) [26].

Analysis of fluorescence intensity of merged images of all 3 dyes showed that freshly harvested samples of basmati varieties namely PB-1121 and PB-1509 showed lower fluorescence (15 and 18 Grey value respectively) compared to non-basmati varieties namely BPT-5204 and Swarna (19 and 25 Grey value respectively) as showed in Fig.3A and 3B. During storage of 24 months, it has been found that PB-1509 and BPT-5204 showed highest increased in the fluorescence intensities ~6.5 fold in each compared to freshly harvested sample. Finding indicates that in freshly harvested sample of basmati varieties, starch, lipids and proteins are present in more diffused form than non-basmati varieties. Further analysis of aged sample was carried out and it was found that there is great increase in the fluorescence intensity after 12 months of storage. In the aged sample, PB-1509 and Swarna showed the highest increase in fluorescence intensity than other varieties. According to earlier studies (Xu *et al*., 2015) [27] Increase in the hydrophobic interaction of rice glutelin protein with amylose also could be the reason for more firmer structure of matrix and increasing fluorescent intensity of selected sample.

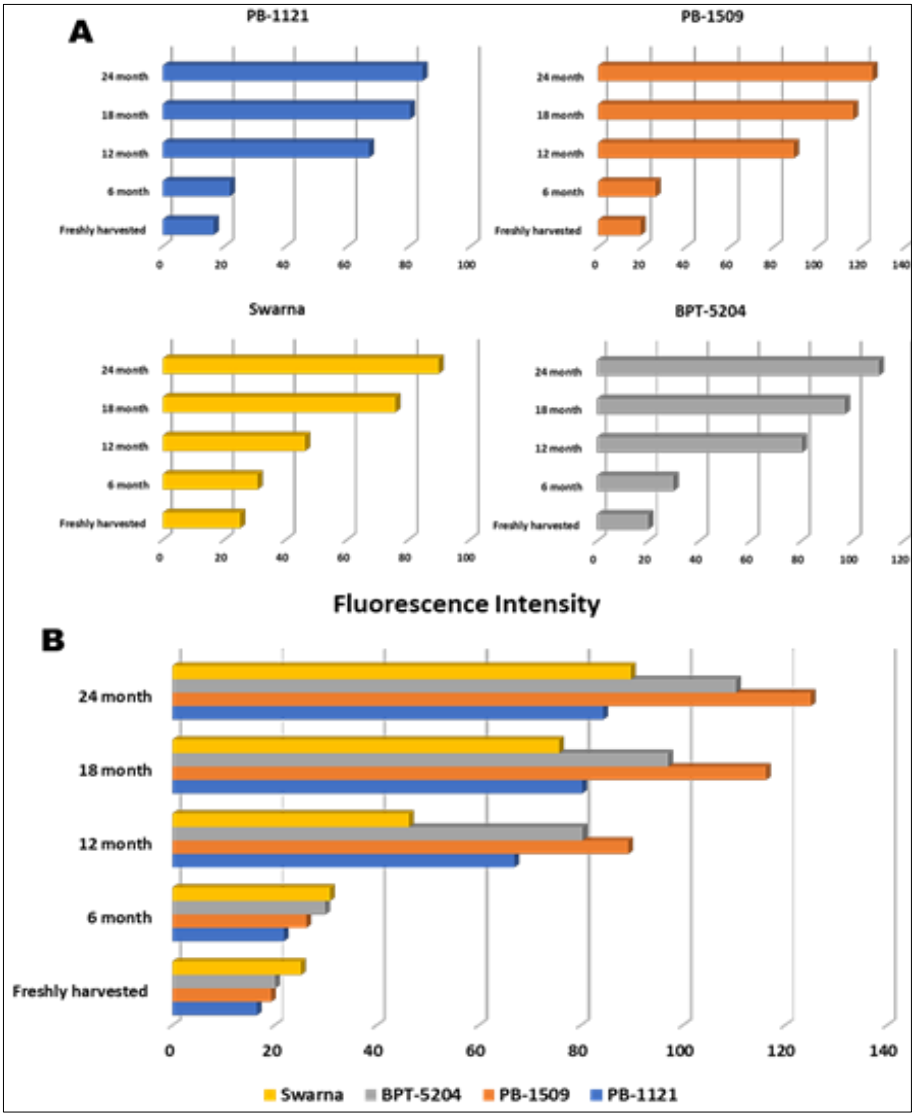

Fig 3: A) Combined fluorescence intensity of Fluoresceine Isothiocyanate (FITC), Rhodamine B and Nile Blue (NB) during the different ageing periods for selected basmati (PB-1121 and PB-1509) and non-basmati (BPT-5204 and Swarna) rice varieties B) Comparative analysis of fluorescence intensity of different aged sample of selected basmati (PB-1121 and PB-1509) and non-basmati (BPT-5204 and Swarna) rice varieties

4. Conclusion

Fluorescence intensity-based analysis of binding interaction of biomolecules is the novel method to study the interaction among the biomolecules. We can use this method to trace the various physico-chemical changes occurring during ageing of rice (*Oryza Sativa* L.) Our finding suggests that there must be increased interaction among starch and non-starch components (proteins, lipids and others) during ageing. This leads to better eating and cooking properties in the aged rice samples. We also found that aged sample of PB-1509 and BPT-5204 showed greater increase in the fluorescence intensity compared to other selected varieties. Based on fluorescence intensity 12 months stored samples of rice could be best for cooking and eating point of view. Further investigation is needed for the study of the binding efficiency of different dyes to respective biomolecules. Also there is a need to focus on how matrix components interaction play important role in determining the digestibility of starch and ultimately GI of food specially rice.

5. Conflict of interest

There is no conflict of interest associated with this work

6. Acknowledgments

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7. Author contributions

Swapnil S. Thakare: Methodology, Investigation, Formal analysis, Validation, Visualization, Writing - original draft & drafting of manuscript. Monika Awana: Writing - review & editing. Shilpi Aggarwal: Methodology and Visualization. Veda Krishnan: Conceptualization, Writing - review & editing. Archana Singh: Conceptualization & supervision, Writing - review & editing, Funding acquisition.

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