Amaranth-Protein Interaction in Food System and its Impact on Tryptic Digestibility.

[1]SYED MUHAMMAD GHUFRAN SAEED*, [1]SYED ASAD SAYEED, [1] SEEMA ASHRAF
[1]AFREEN QURESHI, [1,2,3]RASHIDA ALI, [4]RANA KAUSAR AND [2]ZAFAR SAIED SAIFY
[1]*Department of Food Science and Technology, University of Karachi, Karachi, 75270, Pakistan.*
[2]*HEJ Research Institute of Chemistry, University of Karachi, Karachi, 75270, Pakistan.*
[3]*English Biscuit Manufacturers (Pvt.) Limited, Plot 1-4, Sector 23, Industrial Area, Karachi-749 00, Pakistan.*
[4]*Department of Biochemistry, Federal Urdu University, Karachi, Pakistan.*
smghufransaeed@yahoo.com*

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Summary: Amaranth, a food color, is used in variety of food products to attract consumers, especially children. The purpose of the present study was to identify the component present in the food system which acts as a carrier of color and its distribution. The protein is the most possible candidate for color-conjugates and this was first explored by staining the resolved food proteins on PAGE simultaneously and separately with Amaranth as well as by Coomassie brilliant blue R250. It is the most widely used dye for protein assay. The color intensity of the Amaranth-protein complexes was slightly less than those of Coomassie brilliant blue R250, although the bands stained by Amaranth were very sharp, clearly separated and distinct. The staining procedure followed for Amaranth was quick. The impact of tryptic digestibility on amaranth–protein complexes has illustrated that dye may safely be used without any adverse effect. The possible mo0de of conjugation between amino acid and azo-bond is also discussed.

Keywords: Amaranth, nisin, BSA, nut proteins, pulse proteins, PAGE, tryptic digestibility

Introduction

The colorant exhibits a vital role in enhancing the perception and acceptability of raw and processed food in the eyes of the end consumers. The natural food, particularly plant food sources, is judge by the intensities of the colors that reflect the health and maturity of food from plant source. Most of the colorants from plant source are flavonoids mainly anthocyanin and other carotenoids compounds. All natural pigments play an important role to attract the consumer and become the quality index. Unfortunately these natural colorants are unstable, heat sensitive and pH dependent, therefore commercial food colors are being used to improve the appearance of the food and improve the perception of the raw and processed foods to the level of acceptance of the consumers.

The proper distribution of synthetic food colorants provide the evidence of strong binding affinity and stable complexation between the synthetic dye and food macronutrients during processing, package, storage and consumption. Plant-derived cyanidin, malvidin, petunidin and delphinidin enhance intensity of food colors to make it more attractive for the consumers. Conversely paprika, saffron and turmeric have been used due to their coloring, flavoring and nutraceutical importance.

Natural colorants were used until 1850. The synthetic organic dyes were produced in the 19th century as economical food colorants [1]. In the late 20th century the natural color along with the synthetic dyes were extensively used not only in raw and processed foods but also in drugs and cosmetics [2]. The first dye was synthesized in 1856 by Perkin. Since then huge number of colorants are synthesized and are being used in food, textile, cosmetics, drugs and other industries [3]. Generally most of synthetic colorants are chemically azo dyes are suspected to be carcinogenic in nature [4]. The amaranth has been used as a food colorant at the beginning of the 19th century.

The amaranth (FD&C Red No. 2, E 123) is red in color, its chemical name is Trisodium 2-hydroxy-1-(4-sulfonato-1-naphthylazo) naphthalene-3, 6-disulfonate and its molecular weight is 604.48 amu. It is used in process food like ice cream, cake mix, wine, soups, cereals, chewing gum, jams, chocolate, coffee, tinned fruits and soft drinks. The reddish shade of amaranth is also used in cosmetics and drugs [5]. It's commercially available as red powder, which are soluble in water but sparingly soluble in ethanol. Presently the amaranth, as a coloring has been used in most of the parts of EU as textile to dye wool, silk, photography and food products [6].

In current study it has been proved for the first time that amaranth shows strong interaction with protein molecules and has led to a novel idea of using amaranth as a staining agent under specific conditions of pH, solubility and concentration and for the detection of resolved proteins on PAGE (Poly acryl amide gel electrophoresis). It has been establish that amaranth is a sensitive and simplified staining methodology for the detection of proteins.

Results and Discussion

Protein Binding with Amaranth and Routine Dye

The comparative methodology applied for staining and destaining with amaranth and commassie brilliant blue R250, has been used as a routine dye, to resolve the proteins on PAGE, as given in Table-1. The solubility of amaranth in water is (50 g/L) and 0.2% of its solution is more suitable and sensitive to visualize the protein bands on PAGE. The BSA, Casein, nuts (pistachio, cashio, almond) and pulses proteins of concentration 3 mg/ml were resolved on PAGE and stained with Amaranth. They produced sharp bands as compared to those stained with commassie brilliant blue R250.

The destaining time of Amaranth was much less than the routine dyes and quick results were obtained; given in Table-1. Similar studies were conducted by Ali et al [7], Badaruddin et al [8], Abdullah et al [9] and Saeed et al [10] using Lawsone, sunset yellow, Allura red and Carmosine respectively on both natural and synthetic dyes.

Food coloring dyes such as Amaranth, Allura red and sunset yellow food colorants are commonly added in various foods to make them attractive and acceptable to consumers. These colors are also added in dairy products and in different beverages [11]. Therefore Amaranth as new staining agent is safer than coomassie brilliant blue R 250 dye.

The result have provide that method of staining and destaining of Amaranth was simple, fast and long lasting as compared to commassie brilliant blue R250 and L. Inermis [12]. The different protein bands, stained with the two dyes as shown in Figure 1(stained with coomassie Brilliant blue R250) and Figure 2 (Stained with Amaranth), have demonstrated the equivalent ability of binding with the various proteins. The Intermolecular interaction between proteins and Amaranth was intense and the bands did not fade even after storage for 4 months, whereas intermolecular binding between the coommassie brilliant blue R250 and food protein is less intense and band could not be retained above 2 months. Amaranth is believed to be electrostatically attracted to charged group on the protein like lysine, arginine and histidine residues, forming strong dye:protein complexes that are further augmented by van der Waals forces, hydrogen bonding and hydrophobic bonding [13]. Amaranth dye are classify as azo compounds containing the azo group (-N=N-) that has similar structure of commassie brilliant blue R-250. The structure reveals that commassie contains two SO_3^{-2} group in structure while Amaranth contain three SO_3^{-2} group which attract positive charges of protein molecules [8].

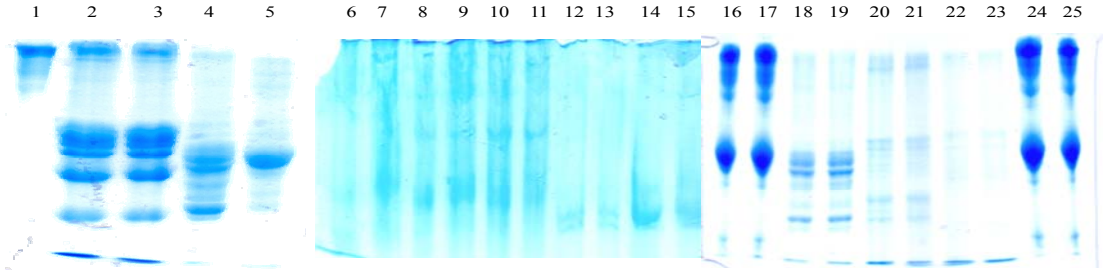


Fig. 1: The samples of BSA(lane 1, 16, 17 , 24, 25), peanut (lane 2,3, 18, 19), Pistachio(lane 4, 20, 21), cashio (lane 5), Almond(lane 22, 23), Dal chana (6,7), Sabit masoor (lane 8,9), Dal masoor(lane 10, 11), Dal mash (lane12, 13), dal moong (lane 14, 15) resolved and stained with Coomassie blue R250.

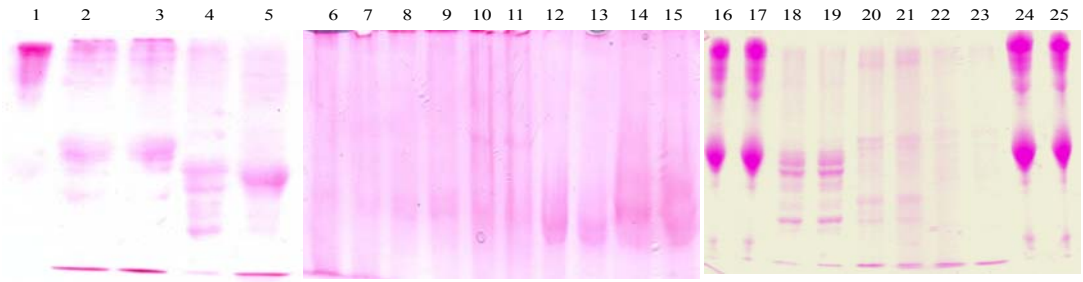
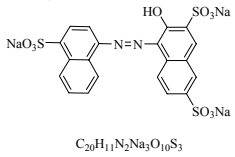
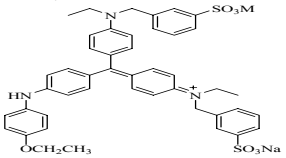


Fig. 2: The samples of BSA(lane 1, 16, 17 , 24, 25), peanut (lane 2,3, 18, 19), Pistachio(lane 4, 20, 21), cashio (lane 5), Almond(lane 22, 23), Dal chana (6,7), Sabit masoor (lane 8,9), Dal masoor(lane 10, 11), Dal mash (lane12, 13), dal moong (lane 14, 15) resolved and stained with Amaranth.

Table-1: Comparison of staining and destaining procedures used for Amaranth and Coomassie.

Methodology	Amaranth	Coomassie Brilliant Blue R-250
1. Concentration of dye	**0.2% solution of A:M:W (7.5:5:87.5)**	**0.2% solution of A:M:W (7.5:5:87.5)**
2. Staining time	**a) Heat 60 C for 10 min b) Overnight**	**Overnight**
3. Destaining time	**60 minutes**	**48 hours**
4. Intensity of bands	**Light but clear**	**Distinct, dark**
5. Nature of bands	**Sharp on edges**	**Blunt and merged on edges**
6. Stability Time	**4 months**	**2 Months**
7. Color	**Purple Red powder (stained red)**	**Blue powder(stained blue)**
8. Solubility	**50g / L H_2O**	**20g/L H_2O**
9. Cost of 10 g (Approx)	**1 US $**	**26 US $**
8. Structure	 $C_{20}H_{11}N_2Na_3O_{10}S_3$	 $C_{45}H_{44}N_3NaO_7S_2$
9. Chemical name	**Trisodium 2-hydroxy-1-(4-sulfonato-1-naphthylazo) naphthalene-3, 6-disulfonate**	**Benzenemethanaminium, N-[4-[[4-[(4-ethoxyphenyl)amino]phenyl][4-[ethyl[(3-sulfophenyl)methyl]amino]phenyl]methylene]-2,5-cyclohexadien-1- ylidene]-ethyl-3-sulfo-,**

*A= Acetic Acid, M=Methanol, W=Water

Protein Binding through Absorption Spectroscopy

Binding of protein with Amaranth dye can be measured through absorption spectrophotometry at 505 nm. Protein-dye binding can be defined as a chemical interaction of the two molecules. During the studies amount of dye kept constant (2mg/ml), the only change was in the concentration of proteins. When protein precipitated by TCA simultaneously the proteins with Amaranth interaction get minimized as demonstrated in Fig. 3.

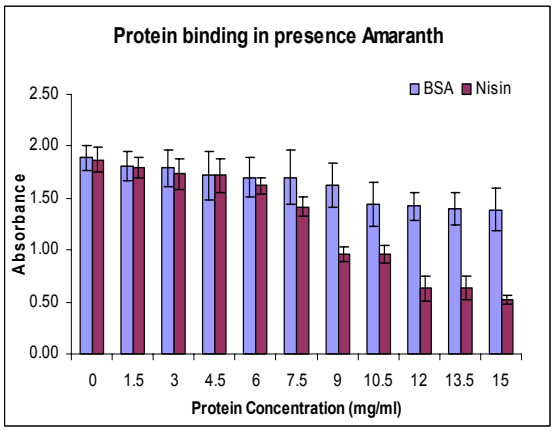


Fig. 3: *In vitro* Protein binding of BSA protein (control) and BSA-dye complexes

Protein Digestibility

Typsin is a protease enzyme acts as to cleave the peptide linkages and to digests the casein and casein-amaranth complex. In current study the agar plate method was used to observe the digestibility effects. Results in Fig. 4 have illustrated that, almost same number of the peptides bonds were cleaved. An experiment conducted with the dye incubated with enzyme for 30 min and then incorporated into the agar has same effect. However, the area digested of casein-amaranth is slightly larger indicating that extent of digestion of the peptide bond in the two cases. It did not exceed but at least is parallel to unbound casein and may easily conclude that there is no adverse effect of the amaranth binding with casein.

Fig. 4: Tryptic digestion on casein agar plate showing same area of digestion withought Amaranth (Left) and Amaranth bound protein(Right).

The protein digestibility was measured through spectrophotometery in the presence of Amaranth. In case of BSA-Amaranth the absorbance was low as compare to the BSA unbounded dye. In case of Nisin-Amaranth complex the digestibility was modified and decrease in absorbance at 280 nm, observed in comparison to the unbounded Nisin. Results presented in Fig. 5 described the digestion

quality of protein, impact of enzyme and the binding capacity of Amaranth with food proteins.

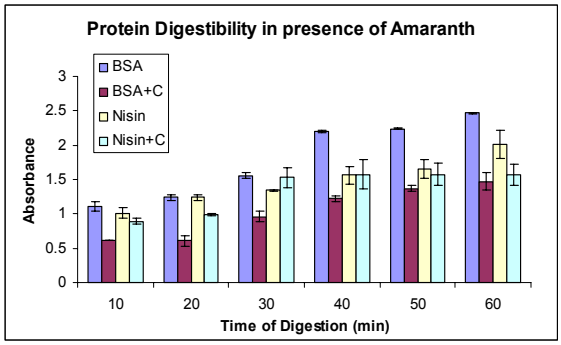


Fig. 5: *Invitro* digestibility of BSA and nisin protein (control) and dye complexes.

Experimental

The N, N'-methylene-bis-acrylamide was purchased from Scharlau (Scharlau Chemie S. A. La Jota, 86 08016 - Barcelona, Spain) while Tris (hydroxymethyl aminomethane) was obtained from Research Organics (Research Organics, Inc. 4353 E. 49th St. Cleveland, Ohio 44125 USA). Sodium dodecyl sulfate (SDS), acrylamide, ammonium peroxodisulfate (APS), glycine, Coomassie (Coomassie Brilliant Blue R 250), bovine serum albumin (BSA), TEMED, bromophenol blue and trypsin were purchased from Merck (E. Merck, 64271 Darmstadt, Germany) while Nisaplin (Nisin) was obtained from Suzhou Hengliang Import and Export (China), 2 – mercaptoethanol was supplied by Riedel-deHaen (Riedal-de Haen AG . D-30926 Seelze. Germany). Protease was obtained from Sigma (Louis, USA). The source of trypsin was pancreas and that of protease was fungal type XIII (from *Aspergillus saitoi*). Amaranth was obtained from Far Eastern Impex (Pvt) Ltd. The other chemicals used were of analytical grade. All the solutions were prepared in doubled distilled deionized (DDD) water.

Preparation of Proteins Solublzing Solution (PSS)

The PSS (40 mL) was prepared by mixing 9.6 mL of the 20% glycerol, 2.5% SDS (sodium dodecyl sulphate), 1.8 mL of mercaptoethanol and 8 mL of tris-HCl buffer of pH 6.8, few crystals of bromophenol blue were also added in the end as a marker.

Preparation of samples of proteins

The BSA protein 20 mg was dissolved separately in 1mL of DDD water. The proteins solution for nuts (peanuts, pistachios, walnuts and almonds) was prepared by taking 5g of each, crushed and defatted in 25 ml of 0.5M phosphate buffer (pH 7.6) to form a thin slurry. The mixture was constantly stirred overnight in an Orbital Shaker at 10°C and sieved through a fine cloth to recover the filtrate, consisting of proteins. The solution was centrifuged at 10000 rpm. The supernatant was used as sample for PAGE analysis.

Electrophoresis

Gel System: A 10% polyacrylamide (acrylamide/bisacrylamide in ratio of 30:0.8 w/w) was prepared according to the method of Laemmli [14] which was slightly modified by Abdullah et al. [9]. Briefly describing 20 µl of protein sample was gently placed into the wells of the gel (8 cm wide, 7.3 cm high, 0.75 mm thick). The samples were separated by using the Bio-Rad Mini-Protean 3 cell system No. 67S/ 06917, at a constant applied voltage of 120 Volt for 4 h.

Staining Solutions

A. Coomassie (0.2 g) was dissolved in 7.5 mL of glacial acetic acid and 5 mL of methanol. The volume was made up to 100 mL with double distilled deionized water (DDDW).

B. Amaranth: The solution was prepared by dissolving 0.2 g of the dye in 8.5 mL of glacial acetic acid and added 2 mL methanol. The volume was made up to 100 mL with DDD water.

Destaining Solution

The destaining solution for Commassie and Amaranth was prepared by mixing 10 mL of glacial acetic acid and 30 ml of methanol together. The volume was made up to 100 ml with DDD water.

Staining and Destaining of the Gel

The gel was heated in the Amaranth staining solution at 70°C for 10 minutes or left overnight in the staining solution at room temperature, and was washed with the destaining solution by a gap of 15 minutes to produce clear red bands on the colorless gel while the other portion of the resolved gel was stained overnight with Coomassie brilliant blue R250 and destained by the normal procedure followed by lameli [14]; washed several times with the destaining solution for 24 hour.

Protein Binding

Different concentration of BSA and Nisin protein from 0 to 15 mg/mL were mixed with Amaranth solution (2mg/mL) in separate test tubes and incubated at 37 ^{0}C for two hours. The color

bound proteins were precipitated by 40% TCA. 1 ml of the supernatant was diluted up to 10 ml with doubled distilled deionized water and protein binding capacity was determined by measuring absorbance at 505 nm. The results are given in Fig. 3.

Tryptic Digestion

The agar plate assay [15] was carried out by using commercial casein as a substrate to ensure the digestibility of proteins present in foods. For the quantification of tryptic digestibility the incubated mixtures of Amaranth bound proteins (BSA and Nisin) were digested separately by trypsin (at an enzyme concentration of 1 mg/50 mg of substrate) at various time intervals [16]. After completion the different time periods, the reactions were terminated by adding 1 ml of 10% TCA and the respective enzyme. The undigested proteins were precipitated as described above. The extent of proteolytic activity of the supernatant was measured spectrophotometrically at 280 nm.

Statistical Analysis

The statistical analysis was carried out using software Minitab version 13.1. The mean of five replicates for each analysis was calculated. The regression analysis was carried out for the digestibility of the proteins and the colour bound proteins. The analysis showed that the digestibility was linearly related to the time interval of the exposition to the enzyme since the r values calculated was in the range of 0.96–0.99. The significance of the data was also estimated by determining the p values for all the data obtained. All the p values calculated were below 0.005 by using Minitab version 13.1.

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Conclusion

During current study it was proved that Amaranth has great affinity to interact with protein molecule in food system and suitable for protein rich food as colorant. Due to its safety, economical feasibility and lifelong properties, it may be used as food proteins staining agent for routine analytical and research work in upcoming future. Its stability is pH and temperature dependent which can easily be controlled. This characteristic of Amaranth is first time elaborated to stain the resolved protein on PAGE.

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