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Binding ability of Allura Red with food proteins and its impact on protein digestibility

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Abstract

Allura Red-40 is a safe colour additive (permissible by the FDA and Health Canada) that is used in a variety of foods to make them more attractive and appealing for consumers. However, limited information is available about its binding to macronutrients that are responsible for its uniform distribution in food products. In the present study, the binding capacity of Allura with food proteins is compared with Coomassie Brillant Blue R 250, which is an established staining agent for visualizing electrophoretically resolved proteins. The data illustrate that Allura is a fast reacting dye and binds with a variety of food proteins including peanut, rice bran, garlic and mixture of proteins [(Takadiastase, nisin, a microbial protein and bovine serum albumin (BSA)]. The Allura bound proteins retained their colour at high and low temperatures and in a wide range of pH. The experiments on the resolution of proteins and staining with Allura have shown that the dye is highly sensitive, rapid, lasting and is easily linked with a variety of proteins. The binding of Allura to various proteins had almost no adverse effect on protein digestibility, as predicted by in vitro digestibility determinations.

Keywords: Allura; Protein binding; PAGE; Protein digestibility

1. Introduction

Colour plays a very important role in choice and quality perception of food by appealing to the consumers. The colour of a food is a primary food quality characteristic, set naturally by a consumer of any age or group. That is why food processing industries are always anxious about the right quality and standard of food colourant to be used for decoration of food for consumer's attraction. Even the natural unprocessed foods are judged by their colour intensity that reflects the maturity and health of fruits and other commodities.

Most of the natural colours belong to the either anthocyanin or carotene families in general, which are heat labile and are faded during processing. One of the reasons to add synthetic colours, which are heat resistent like Allura Red, is to overcome such losses. Commercial colourants are added to give natural appealing look to foods. Allura has been used as a food colourant for decades (Anonymous., 2003) however, the nature of its binding to food components has not been fully investigated. The uniform distribution of a colourant in food systems is an authentic indication that a strong affinity and stable complexation between the dye and a ligand exists throughout the food processing and storage.

Allura belongs to the monoazo class of food colourants and is identified as C1 Food Red 17, Food Drug & Cosmatic (FD&C) Red No. 40, C1 (1975) No. 16035, INS No. 129. Chemically it is disodium 6-hydroxy-5-(2 methoxy-5-methyl-4-sulphophenylazo)-2-naphthalenesulphonate with a molecular weight of 496. It is commercially

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available as dark red or maroon powder or granules, which are soluble in water but insoluble in ethanol. The joint FAO/WHO Expert Committee on Food Additives has suggested acceptable daily intake (ADI) for Allura as 7 mg/kg/day (Ito, 2000). Allura is reduced by human intestinal anaerobes (Chung, Fulk, & Egan, 1978) and is approved by FDA and Health Canada as a nontoxic food colourant.

The dye can easily be isolated and estimated in food products by spectrophotometric determination at 504 nm and reverse phase ion pair high-performance liquid chromatography (HPLC) (Lancaster & Lawrence, 1991; Richfield-Fratz, Baczynskyj, Miller, & Bailey, 1989). The food colours are also estimated by capillary electrophoresis in nano quantities (Huang, Shih, & Chen, 2002), and recently three new techniques including adsorptive stripping voltammetry (Alghamdi, 2005), microemulsion electrokinetic chromatography (Huang, Chuang, Chiu, & Chung, 2005) and complex spectral-pH three-way array (Marsili, Lista, Band, Goicoechea, & Olivieri, 2005) have been described for quantitative analysis of food colours including Allura.

The purpose of this investigation was to study the binding capacity of Allura with food proteins. Allura was used for staining proteins such as peanut, garlic, and rice bran proteins (RBP), and a mixture of proteins (Takadiastase, BSA, and nisin, a microbial protein often used as a preservative in variety of foods), and was then separated on sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) just to prove that it exclusively binds with protein molecules in the complex system of different foods. The garlic extract being acidic in nature has better keeping quality and was separated well on acrylamide gel, garlic extract contains garlic proteins, especially lectins (Ahmad, Pischetsrieder, & Ahmed, 2007; Ghananfari, Hassan, & Khamesipour, 2006; Smeets et al., 1997; Wang & Ng, 2001). Furthermore, the Allura–protein complexes were studied to assess the effect of colour binding on protein digestibility.

2. Materials and methods

2.1. Materials

The *N*,*N*′-methylene-bis-acrylamide was purchased from Scharlau (Scharlau Chemie, Barcelona, SPAIN) while Tris (hydroxymethyl aminomethane) was obtained from Research Organics (Research Organics, Inc. Cleveland, OH, USA). Sodium dodecyl sulphate (SDS), acrylamide, ammonium peroxodisulphate (APS), glycine, coomassie (coomassie brilliant blue R 250), BSA, *N*-tetramethylethylenediamine (TEMED), bromophenol blue, trypsin and protease were supplied from Merck (Darmstadt, Germany), while Nisaplin (nisin) was purchased from Suzhou Hengliang Imp and Exp, Jiangsu, China, and 2-mercaptoethanol was supplied from Riedel-deHaen (Riedal-de Haen AG Seelze, Germany). The ficin and bromelain were given as a gift from the Department of Food Science, University of California, Davis, while milk powder, liquid milk and eggs were purchased from Top ten super market, Karachi, Pakistan, and protease was obtained from sigma (Louis, USA). Allura (Allura Red – 40) was obtained from National Foods (Pvt.) Ltd. Karachi, Pakistan as a gift. The other chemicals used were of analytical grade. All the solutions were prepared in doubled distilled deionized (DDD) water.

2.2. Electrophoresis

2.2.1. Preparation of slab gel

The following solutions were first prepared to prepare a Gel for SDS.

Acrylamide (30 g) and bis-acrylamide (0.80 g) were dissolved in 100 mL water and filtered through Whatman filter paper no. 1 to prepare a solution A. Tris–HCl buffer (1.5 M) was prepared by mixing 18.02 g of Tris dissolved in 80 mL of water and pH was adjusted to 8.8 by using 1 M HCl, and the volume was finally made up to 100 mL. Tris (12.114 g) was dissolved in 80 mL water, the pH was adjusted to 6.8 with 1 M HCl and the volume was increased to 100 mL with water to prepare its solution. SDS (10 g) was dissolved in water and the volume was made up to 100 mL to make SDS solution. APS (1 g) was dissolved in water and the volume was increased to 10 mL, while TEMED was used as supplied.

The 12.5% slab gel of thickness 0.75 mm was prepared by using 2.083 mL of solution A, 0.666 mL of solution B, 0.500 mL of solution D and 0.250 mL of solution E. The mixture was degassed and 1.7 μL of TEMED was added before use. The gel was left for 15 min for settling.

2.2.2. Staking gel

Solutions were added in the ratio of 0.416:0.830:0.333:0.166, respectively. The mixture was degassed immediately, and finally 1.7 μL of TEMED were added before the pouring of the stacking gel. The wells were prewashed with a reservoir buffer after the gel was settled.

Reservoir buffer: Tris (0.9 g), glycine (3.6 g) and SDS (1 g) were dissolved in 50 mL water and the volume was made up to 1 L.

2.2.3. 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 increased to 100 mL with water and filtered to avoid any contamination and stored at room temperature (R).

B. Allura: The solution was prepared by dissolving 0.2 g of the dye in 7.5 mL of glacial acetic acid and 5 mL of methanol. The volume was made up to 100 mL with water (R).

C. Allura (acidic): The staining solution B (25 mL) was adjusted at pH 2.0 by using 2 N HCl (R).

D. Allura (basic). The staining solution B (25 mL) was adjusted at pH 10.0 by using 5 N NaOH (R).

2.2.4. Destaining solution

Destaining solution was prepared by mixing 10 mL of glacial acetic acid and 30 mL of methanol together. The volume was increased up to 100 ml with water (R). All the solutions were stored at 4 °C in the dark to prevent auto-oxidation, except where (R) is given at the end of the preparation method, which represents storage at room temperature.

2.3. Preparation of samples

2.3.1. Peanut proteins

The protein solubilizing solution (PSS) (5 mL) was specially prepared by mixing 1.2 mL each of aqueous solutions of 2.5% SDS, 0.0025% bromophenol blue and 20% glycerol to which 1 mL Tris–HCl buffer and 0.2 mL 2-mercaptoethanol were added. A dehulled mass of peanuts (*Arachis hypogaea*) was ground to a fine powder of mesh size 60, and 50 mg of the above sample were separately mixed with 1.5 mL of PSS. The mixture, after having been left overnight at room temperature, was continuously shaken in a boiling water bath for 30 min. It was centrifuged at 2000 rpm (500*g*) for 15 min. The clear supernatant was separated by using a pipette and used as peanut protein sample.

2.3.2. Mixture of proteins

The equal quantities of takadiastase, nisin and BSA were dissolved in water to a final concentration of 20 mg/mL. Twenty microliters of this sample were mixed with 20 μL of PSS. The sample was boiled for 3 min and then centrifuged at 2000 rpm (500*g*) for 15 min and the clear supernatant was collected as the mixed sample.

2.3.3. Rice bran proteins (RBP)

The RBPs were prepared in the same way as the peanut proteins. Fifty milligrams (50 mg) of fresh, defatted and dried rice bran of mesh size 60 was suspended in 1.5 mL of PSS.

2.3.4. Garlic proteins

Fresh cloves of the garlic bulbs obtained from top ten super market, Karachi, Pakistan, were peeled, blended and suspended in 20 mM phosphate buffer saline (PBS) (100 g:100 mL)) overnight with continuous orbital shaking in a refrigerated incubator (LabTech, LabTech, Namyangju-city, Kyungki-Do Korea) at 10 °C. The extract was concentrated up to a protein content of 10 mg/ml in a rotary evaporator at a temperature of 40 °C.

2.4. Electrophoretic resolution

A small quantity of 50 μL of the supernatant of each sample was resolved on SDS–PAGE gel using reservoir buffer for upper and lower chambers at a constant voltage of 100 V for 4 h.

2.5. Staining and destaining of the gel

2.5.1. Procedure A

In the case of Allura, the gel was left overnight in the staining solution and was washed twice with the destaining solution with a gap of 15 min to produce clear red bands on colourless gel, while the other half portion of the gel was stained overnight with Coomassie and destained by washing several times with the same solution. The procedures of staining and destaining with the two dyes are compared in Table 1.

2.5.2. Procedure B

The same procedure for resolution of proteins was followed and half of the gel was stained by heating the gel in the Allura solution for 15 min at 60 °C. The other half was stained in Coomassie; both were destained in the same way.

2.5.3. Procedure C

In order to explore the effect of the acidic or the basic pH on the extent of binding of the dye with the protein bands, the samples were resolved in the same way as described above. The gel was cut into two halves; both of them were stained separately with the Allura staining solutions C and D overnight and destained accordingly.

2.5.4. Procedure D

In order to see the impact of heating on the method of staining at acidic and basic pH, the samples were resolved in the same way. The gel was also stained with solutions C and D, with the only difference being that they were heated at 60 °C for 15 min. All gels were destained with the same destaining solution.

2.6. Digestibility of Allura bound proteins

2.6.1. Protein standard solution

The standard solutions of 15 mg/ml of nisin and BSA were separately prepared and diluted to different concentrations ranging from 1.5 mg/ml to 15 mg/ml.

2.6.2. Allura solution

Allura Red solution was prepared by dissolving 10 mg of Allura in 1 ml water and diluted to 2 mg/ml.

Table 1
Comparison of staining and destaining procedures used for Allura and Coomassie

Methodology	Allura Red-40	Coomassie Brilliant Blue R 250
1. Concentration of dye used	0.2% Solution	0.2% Solution
2. Staining time	15 min	Overnight
3. Destaining time	30 min	48 h
4. Intensity of bands	Light but clear	Distinct, dark
5. Stability of bands	More than six months	More than three months

2.6.3. Protein binding

Equal volumes of proteins and Allura solutions were mixed in separate test tubes and incubated at 37 °C for two hours. The colour bound proteins were precipitated with 40% trichloroacetic acid (TCA). One (1) milliliter of the supernatant was diluted up to 10 ml with DDD water and protein binding capacity was determined by measuring the absorbance at 504 nm.

2.6.4. Tryptic digestion

The agar plate assay (Schumacher & Schill, 1972) was carried out by using commercial casein as substrate to ensure the digestibility of proteins present in foods. The incubated mixtures of proteins (BSA and nisin) and Allura bound proteins as mentioned above were digested separately with trypsin and protease (at an enzyme concentration of 1 mg/ml of substrate). The source of trypsin was pancreas and that of protease was fungal type XIII (from *Aspergillus saitoi*) at various intervals of times (Pfleinderer & Krauss, 1965). The extent of proteolytic activity was measured spectrophotometrically by observing the optical densities at 280 nm.

2.7. Statistical analysis

The colour binding assay was performed at least four times while the determination of digestibility by spectrophotometer and agar plate method was performed five times. All the other experiments were repeated at least five times. To represent the data, the bar diagrams were developed using Microsoft Excel (MS Office). To analyze the data statistical analysis software Minitab version 13.1 was used.

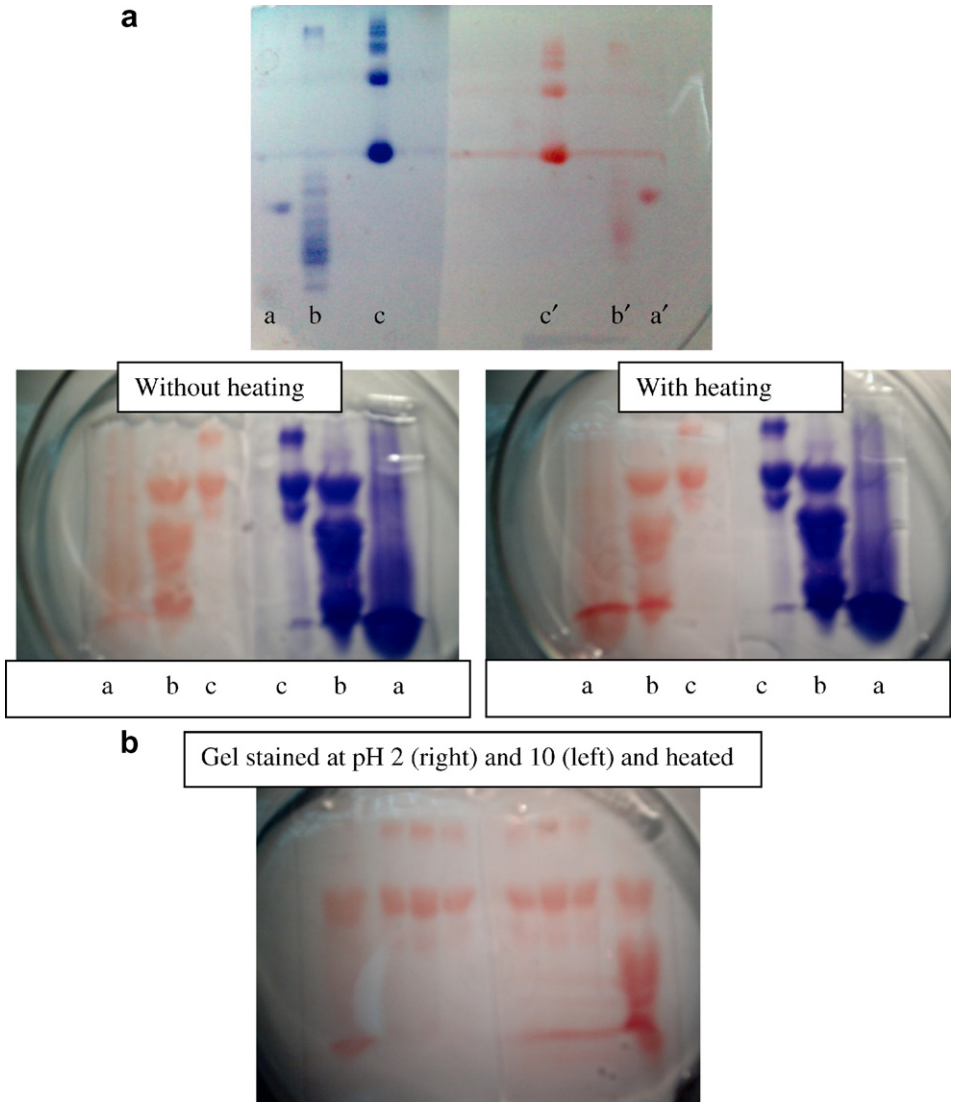

Fig. 1. In this figure, electrophoretically resolved proteins stained with Allura (right) and Coomassie (left). The samples a:a, b:b and c:c represent the [takadiastase and casein mixture (extremes)], garlic proteins and BSA respectively. While in figure 1a electrophoretically resolved proteins with and without heating the gel separately in presence of Allura and Coomassie (a:a), (b:b) and (c:c) represents nisin, peanut protein and BSA respectively. Figure 1b is showing gel stained at pH 2 (right) and 10 (left) and is heated.

2.7.1. Regression analysis

The regression analysis was carried out for the digestibility of the protein and the colour bound proteins. The analysis showed that the digestibility was linearly related to the time interval exposed to the enzyme, since the r values calculated were in the range of 0.96–0.99.

2.7.2. p value

The significance of the data was also ascertained by determining the *p* values for all the data obtained. All *p* values calculated using Minitab version 13.1 were less than 0.005, except for the binding of Allura, which was 0.117.

3. Results and discussion

The strong affinity of Allura with food proteins is shown by staining the resolved proteins on PAGE with Allura and comparing it with the standard dye Coomassie (Fig. 1). The destaining of Allura was much quicker than Coomassie, and immediate results required in research may be obtained by using this food colourant as the staining agent (Table 1). Similar results were obtained by Badaruddin, Abdullah, Sayeed, Ali, and Riaz (2007) by using the sunset yellow food colour. According to them sunset yellow is fast reacting dye for food protein and may be used as a fast staining for all types of resolved proteins with PAGE, which is important because Coomassie Brillinat Blue R 250 and Amido Black 10 B take at least 24 h for visualization of protein bands with PAGE.

Colourants are commonly added to a variety of foods to make them attractive and acceptable to consumers and even being thoroughly mixed, there are specified methods to detect them (Alghamdi, 2005). Food colours are also added to different beverages including milk and milk-based drinks (Huang et al., 2002) and there the colours bind proteins as it has been demonstrated in the present study that Allura is linked to various fractions of casein resolved on polyacrylamide gel electrophoresis. The impact of heating showed better and persistent dye binding, as the protein-stained bands were preserved for a period of at least six months (Fig. 1a). It is possible that some other binding sites are created in view of the unfolding of protein molecule due to the heat denaturation process. Allura was found suitable in food processing at both elevated and low temperatures. The results are evidence-based as in common practice of food processing; Allura is used in preparation of custard, gelatins, puddings, dairy products, confectionary, and beverages and the colour is stable in both types of products, i.e. processed at low and at elevated temperatures (Anonymous, 2008) The colour intensity was slightly affected at very low or very high pH. The staining procedure of the proteins at pH 2, 4.5 and 10 has shown (Fig. 1b) that the colour binding is pH dependent and extreme ends in pH are not suitable for binding with proteins. The solubility of Allura decrease with increases in pH, and the optimal pH was found to be from 4 to 7.5, which are normally found in the food systems. The heating

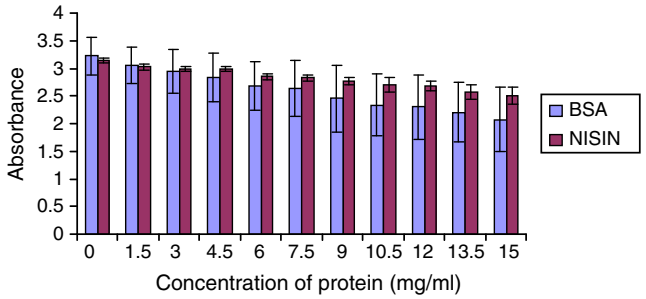

Fig. 2. The decline in absorbance due to Allura–protein complex formation with increase in protein concentration.

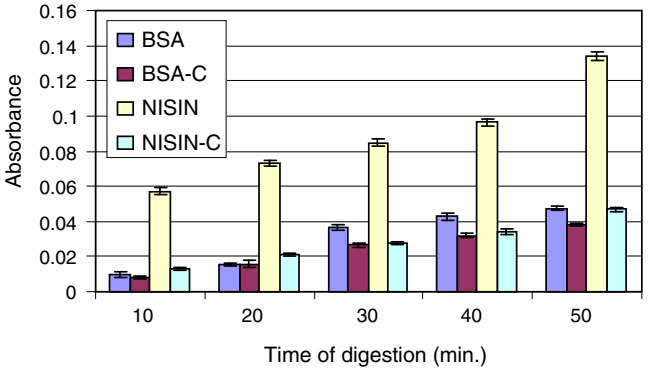

Fig. 3. Tryptic digestibility of BSA and colour bound BSA (BSA-C), nisin and colour bound nisin (NISIN-C).

up to 60 °C made the bands visible even at pH 2 and 10, which suggested that heating facilitates the dye binding irrespective of the pH.

The protein binding capacity of Allura is illustrated by spectrophotometrically as shown in Fig. 2. The decrease in optical density with increasing on the protein addition was an indication of dye-protein complex formation. A correlation existed, and for every 1 mg addition of protein the absorbance at 504 nm decreased by 0.103 in the case of BSA. The slope on the bar diagram shows that as it links to protein, the free molecules of Allura decreased and a fixed amount of dye is bound to the protein.

The tryptic digestibility of Allura linked to nisin and BSA complexes as compared to unbound proteins (Fig. 3) has clearly illustrated that Allura bound BSA were almost equally digestible. However, Allura restricts the proteolysis process in the case of nisin, for perhaps the active sites for trypsin action are blocked. However, a correlation between the time of exposure to enzyme and digestibility is always established significantly in either Allura bound or unbound proteins, and in both the cases the *r* values calculated were 0.95–0.99. Moreover, digestion of Allura bound casein by trypsin revealed that Allura binding had no adverse effect on digestibility of other protein sources from foods (Fig. 4). [1]Fig. 4 has two plates, the

[1] (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

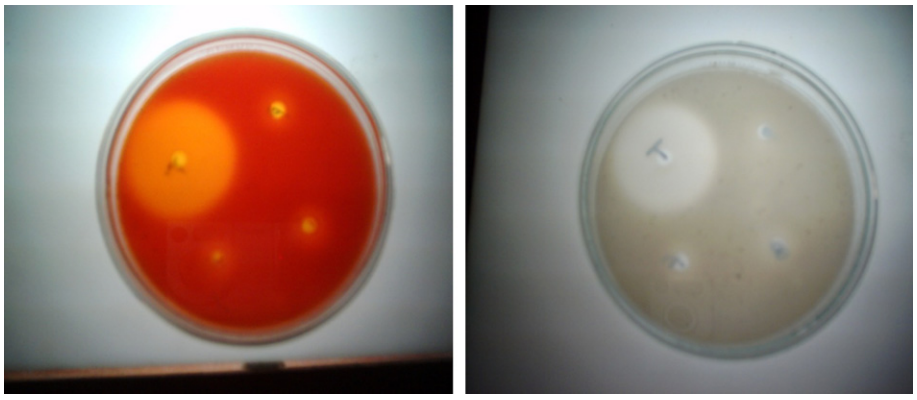

Fig. 4. Shows the zones of tryptic digestion in plate (right) consisting casein and (left) consisting Allura bound casein with the same area hydrolysed.

red one consisting of casein bound with Allura Red and left one without Allura, the big circle shows the digestibility with trypsin in two cases and supports finding that Allura bound proteins are almost equally digestible with proteolitic enzymes.

In summary, the results of the present study have shown for the first time that Allura Red-40 binds with all proteins and the dye is suitable in food processing at high or low temperatures. The colour intensity is not much affected at mildly acidic or basic pH, and the dye may be used in various processed foods, as most of the food systems are normally in the range of pH 4–7.5 where dye binding is optimal.

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