Food Protein: Food Colour Interactions and its Application in Rapid Protein Assay

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Abstract

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The uniform distribution of colours as additives in a majority of the food systems is a reliable indication that one or more components of foods are able to bind with colour molecules and act as their carriers. However, the food components acting as the colour carriers have not been identified. The present paper describes the binding capacity of Carmoisine with a variety of food proteins, our results have shown that the intensity, staining, and sharpness of the stained protein bands were excellent as compared to Coomassie Brilliant Blue R 250, which is an established staining agent for visualising electrophoretically resolved proteins. The data illustrates that Carmoisine is a fast reacting dye forming colour-complexes with all types of food proteins including curry leaves proteins. The protein bands are visualised within an hour which is useful for the initial immediate protein identifications. The experiments related to the staining of the resolved proteins with Carmoisine have shown that the dye is highly sensitive, rapid, and lasting. The food-dye can provide a quick protein assay as often desired in research works, the results may be later confirmed by using Coomassie if so required. In view of its strong binding with almost all proteins, it was thought that human proteases present in the digestive tract may not hydrolyse the bound proteins completely and may restrict the proteolytic digestion. However, the experiments based on the tryptic digestibility *in vitro* revealed that colour binding has no adverse effect on hydrolysis of peptide bonds by the intestinal proteases.

Keywords: Carmoisine; food proteins; staining agent; PAGE; tryptic digestibility

In spite of the controversial findings about the safe use of synthetic food colours, these are constantly added for enhancing the appearance of the food products f to be more attractive and appealing from the consumer's point of view. Carmoisine Red-3 has been used as a food colour for decades. However, the chemical nature of food components and their interactions with Carmoisine have not been fully investigated. The uniform distribution of the colour in a variety food systems is an authentic indications that a strong affinity and stable complexations between the dye and ligands exist throughout the food processing and storage. It shows that the colour complexes are heat resistant and insoluble in biological fluids. Carmoisine belongs to monoazo class of food colours and is identified as C1 Food Red 3, C1 (1975) No. 14720, INS No. 122. Chemically it is 2-(4-sulfo-1-napthol-4-sulfonic acid) or {disodium 4-hydroxy-3-(4-sulfonate-1-napthylazo) naphtalene-1-sulfonate} with a molecular weight of 502.44. It is commercially available as red to

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maroon powder or granules, which are soluble in water but poorly soluble in ethanol.

The purpose of the present investigation was to identify the component of the food that binds with Carmoisine and is responsible for the uniform colour distribution, secondly to clarify if the colour-bound-food-proteins are equally digestible by trypsin as the native protein, trypsin being a major intestinal protease. Carmoisine was used for staining a variety of proteins such as proteins from curry leaves and rice bran, and presently we are isolating and identifying several proteins from these food sources. Well known proteins such as BSA, nisin, casein, and wheat glutens were also included in the experiment to show the wide range of proteins capable of binding with Carmoisine. Carmoisine is a nontoxic food colour as it is rapidly distributed into tissues and efficiently excreted through the gastrointestinal tract (Galli *et al.* 1981). The dye is reduced in the gut by azoreductase of bacterial origin (Walker 1970). The bioavailability of Carmoisine calculated from the blood radioactivity curves after oral and intravenous administration of (^{14}C) Carmoisine was found to be below 10% of the total intake (Galli *et al.* 1982a). This further indicates that the colour in food systems may not be harmful. Carmoisine is reported to inhibit succinic oxidase activity by 40% as demonstrated in rats (Sikorska & Krauze 1962), and is not carcinogenic (Anonymous 1982), nor leaving any negative effects on placenta or during pregnancy (Galli *et al.* 1982b). It has been observed that no mortality or tumour incidences were reported after its consumption as colour additive (Hecht 1966), its adverse activity being also nil (Bar & Griepentrog 1960).

The embryotoxic or teratogenic effects were absent in the animals on Carmoisine administration (Smith *et al.* 1972). In conclusion, it is clear that Carmoisine is neither carcinogenic nor shows any adverse effect if consumed at the level of 400 mg/kg/day.

The detection and estimation of Carmoisine in foods and other cosmetic products has been well studied. It has been determined by capillary electrophoresis (Suzuki *et al.* 1994) and UV visible spectrometry combined with the mathematical treatments of the spectra (Saguy *et al.* 1978; Hofer & Jenewein 1997). Other methods of the dye identification involve electrochemistry (Thomas & Boto 1975), differential pulse polarography (Fogg & Yoo 1979), and their degradation (Fogg & Summan 1983). The stripping voltammetry at the mercury drops (Fogg *et al.* 1986) is also a reliable method to identify the presence of colours in food samples. Furthermore, the dye analysis by HPLC as compared with differential pulse polarography was found to be equally applicable for analysis (Chanlon *et al.* 2005). Reversed phase HPLC was also found to be a sensitive tool for the determination of synthetic food colours including Carmoisine (Minioti *et al.* 2007). Recently, synthetic food colorants including Carmoisine were accurately detected by capillary electrophorsis with laser induced fluorescence detection (Ryvolova *et al.* 2007). The advantages of using Carmoisine in medical practice have been demonstrated by the fact that it removes cutaneous neoplasia by intraoperative visual enhancement (Murphy & Hall 2003).

We have been working on food colour interactions with a variety of known and unknown proteins resolved on PAGE and have earlier reported that both natural and synthetic food colours bind with all types of plant and animal proteins. Sunset Yellow and Allura Red proved to be good staining agents for SDS-PAGE assay (Badaruddin *et al.* 2007; Abdullah *et al.* 2008). The natural colours including lawsone from Hina leaves used as protein staining agent produced better results than produced by Commasie (Ali & Sayeed 1990; Ali *et al.* 1995). The purpose of this study was to demonstrate the protein complexation with Carmoisine and its possible use as a rapid staining agent for electrophoretically resolved proteins to get immediate results that are often required in the research. Moreover, we also wanted to make it clear that Carmoisine-colour complexes are easily digestable by human gut proteases such as trypsin.

MATERIALS AND METHODS

Materials

N, N'-methylene-bis-acrylamide was purchased from Scharlau (Scharlau Chemie, Barcelona, Spain). Tris (hydroxymethyl aminomethane) was obtained from Research Organics (Reserach Organics, Inc., St. Cleveland, USA). Sodium dodecyl sulfate (SDS), acrylamide, ammonium peroxodisulfate (APS), glycine, Coomassie (Coomassie Brilliant Blue R-250), bovine serum albumin (BSA), TEMED (Tetramethylethylenediamine), bromophenol blue, and trypsin

were supplied by Merck (E. Merck, Darmstadt, Germany). Nisaplin (nisin) was purchased from Suzhou Hengliang Imp. & Exp. Co., Ltd. (Suzhou, China), 2-mercaptoethanol was supplied by Riedel-deHaen (Riedal-de Haen AG, Seelze. Germany). Protease was obtained from Sigma (St. Louis, USA). The source of trypsin was pancreas and that of protease was fungal type XIII (from aspergillus saitoi). Carmoisine (Carmoisine-Red-3) 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 double distilled deionised (DDD) water.

Preparation of proteins solubilising solution (PSS). PSS (40 ml) was prepared by mixing 9.6 ml each of 20% glycerol, 2.5% SDS (sodium dodecyl sulphate), 1.8 ml of mercaptoethanol, and 8 ml of tris-HCl buffer of pH 6.8. A few crystals of bromophenol blue were added at the end.

Preparation of proteins samples and their hydrolysates. Twenty milligrams of each of the used proteins (BSA, casein, nisin, wheat gluten) were dissolved separately in 1 ml of DDD water.

The rice bran proteins (RBPs) solution was prepared by mixing 100 g of fresh, defatted rice bran with 1 l of 0.5M phosphate buffer (pH 7.6) to form thin slurry; the mixture was stirred overnight in an orbital shaker at 10°C, then it was sieved through fine cloth to recover the filtrate containing proteins. The mixture was concentrated on a rotary evaporator to the total volume of 10 ml. Protein solutions were prepared by following the same method as above using PSS for dilution and as the solvent used in upper chamber during electrophoresis.

The hydrolysates of all the proteins, i.e. BSA, casein, nisin, wheat gluten, and rice bran protein were prepared by adding 20 µl of each protein to 20 µl of PSS. 20 µl of trypsin (1 mg/ml) was then added to each protein solution. The mixture was incubated for 10 min, then boiled for 10 min to deactivate the enzyme, and 20 µl of 40% trichloroacetic acid was then added to precipitate the non-hydrolysed proteins. The mixture was allowed to cool and was centrifuged. The protein hydrolysates (20 µl) were used for PAGE.

Electrophoresis

Gel system. A 12.5% polyacrylamide gel (acrylamide/bisacrylamide in the ratio of 30:0.8 (wt/wt)) was prepared according to the method of Laemmli (1970) which was slightly modified (Abdullah *et al*. 2008). Briefly, 20 µl samples of protein were carefully 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 (Alfred Nobel Drive, Hercules, USA), at constant current of 120 V for 4 hours.

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 DDD water.

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

Destaining solution. The destaining solution for Commassie was prepared by mixing 10 ml of glacial acetic acid and 30 ml of methanol. The volume was made up to 100 ml with DDD water, while only 10 ml of methanol in 90 ml of DDD water was used for the preparation of the destaining solution for Carmoisine. The rest of the procedure was the same.

Staining and destaining of the gel

The gel was heated in the Carmoisine staining solution at 70°C for 10 min or left overnight in the staining solution and was subsequently

Table 1. Comparison of staining and destaining procedures used for Carmoisine and Coomassie

Methodology	Carmoisine-Red-3	Coomassie Brilliant Blue R-250
1. Concentration of dye used	0.2% solution	0.2% solution
2. Staining time	overnight	overnight
3. Destaining time	30 min	48 h
4. Intensity of bands	light but clear	distinct, dark
5. Stability of bands	more than 6 months	more than 3 months

washed twice with the destaining solution with 15 min intervals to produce clear red bands on the colourless gel background while the other half of the gel was stained overnight with Coomassie and destained by washing several times with the destaining solution which took almost 24 hours. The procedures of staining and destaining using the two dyes are compared in Table 1.

Digestibility of Carmoisine-Proteins-Conjugates (CPC)

Prepration of CPC. Standard solutions of various proteins were prepared using the amount of 15 mg/ml of 0.1M phosphate buffer, pH 7. By dilution, different concentrations were prepared, ranging from 1.5 mg/ml to 15 mg/ml. The Carmoisine solution was prepared by dissolving 10 mg in 1 ml water and diluted to 2 mg/ml.

Equal volumes of each protein and Carmoisine solution were mixed in separate test tubes and incubated at 37°C for two hours. The colour bound proteins were precipitated with 40% TCA. 1 ml of each supernatant was diluted up to 10 ml with DDD water and protein binding capacity was determined by measuring the absorbance at 516 nm according to the method of Abdullah *et al.* (2008).

Tryptic digestion. The incubated mixtures of Carmoisine bound proteins as given above were digested separately by trypsin and pepsin (at the enzyme concentration of 1 mg/50 mg of substrate) for various periods of time (Pfleinderer & Krauss 1965). After completion the different time periods, the reactions were terminated by adding 1 ml of 10% TCA the respective enzyme and to precipitate the undigested proteins were precipitated as described above. The extent of proteolytic activity of the supernatant was measured by Bradford Assay (Bradford 1976).

Statistical analysis

The statistical analysis was carried out using software Minitab version 13.1. The mean of five replicates for each analysis was calculated and is shown in Figures 3 and 4.

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 were 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.

RESULTS AND DISCUSSION

Protein binding with Carmoisine and routine dye

The strong affinity of Carmoisine to food proteins is shown by staining proteins BSA, casein, wheat proteins, curry leaves proteins, and rice bran proteins resolved by PAGE and comparing the results with the protein bands stained by the standard dye Coomassie (Figures 1 and 2). The

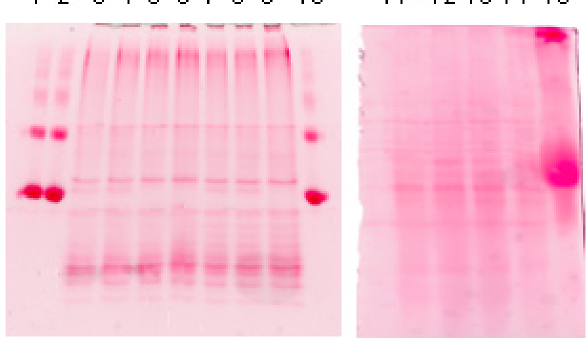

Figure 1a. The samples of curry leave proteins (lane 3–9). BSA (lane l, 2, 10), rice bran protein (lane 11–14) and mixture of BSA, nisin, casein and wheat gluten (lane 15) resolved and stained with Cammoisine

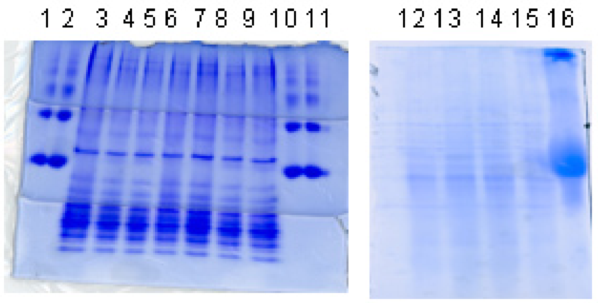

Figure 1b. The samples of curry leave proteins (lane 3–9). BSA (lane l, 2, 10, 11). rice bran protein (lane 12–15) and mixture of BSA, nisin, casein and wheat gluten (lane 16) resolved and stained with Coomassie

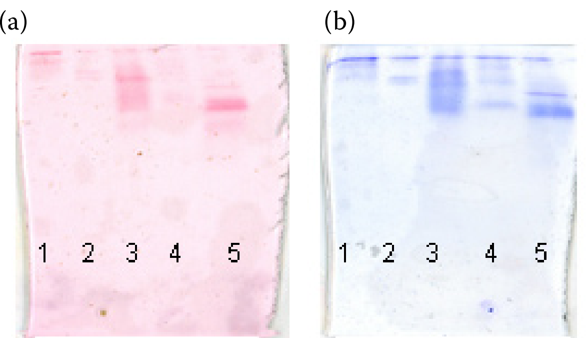

Figure 2. Trypsin hydrolysate in lane 1, 2, 3, 4, 5 correspondingly as BSA, casien, nisin, wheat gluten and rice bran protein resolved and stained with Carmoisine (a) and Coomassie (b)

results indicated that the destaining time in the case of Carmoisine-Red-3 is shorter in comparison with the routine dyes. The intensity of the bands is lower in Carmoisine staining but is more clear with sharp edges as compared to those of the bands stained with Coomassie Brilliant Blue-R-250, where the bands are slightly dispersed. The stability of the bands was also observed and it was found that the colour was retained for a longer period of time in the case of Carmoisine-Red-3 (six months) while the bands stained with Coomassie Brilliant Blue-R-250 were stable only for three months.

Carmoisine may be introduced here as a new staining agent for proteins; the positive results obtained with others than food proteins are not reported here. Other advantage over the Coomassie is that it is safe, being the food colour. The bands stained with Carmoisine were stable for six months when stored in water: acetic acid: methanol (6:3:1) while the bands disappeared/dimmed in three months in the gel stained with Coomassie and stored in the same solution. The results are summarised in Table 1. Carmoisine-Red-3 is believed to be electrostatically attracted towards the charged groups in the protein chains as amino acids like lysine, arginine and histidine residues, forming strong dye:protein complexes that are further augmented by van der Waals forces, hydrogen and hydrophobic bondings (Silberstein *et al.* 1985).

The method of Carmoisine staining is economical, rapid, and safe and it demonstrates that Carmoisine-Red-3 may also be used as a staining agent for routine analysis in PAGE in the near future apart from its utilisation as a food colour.

Digestibility effect on dye-protein-complex

The protein binding capacity of Carmoisine is illustrated by spectrophotometric measurements as shown in Figure 3. The decrease in optical density with the increase of the protein addition was the indication of the dye-protein complex formation. A correlation existed as for every 1 mg protein addition the absorbance at 516 nm decreased in the same order by 0.04165 in the case of BSA. The slope on the bar diagram shows that in the course of its linking to protein, the amount of free molecules of Carmoisine decreases and only a fixed amount of dye is bound to the protein,

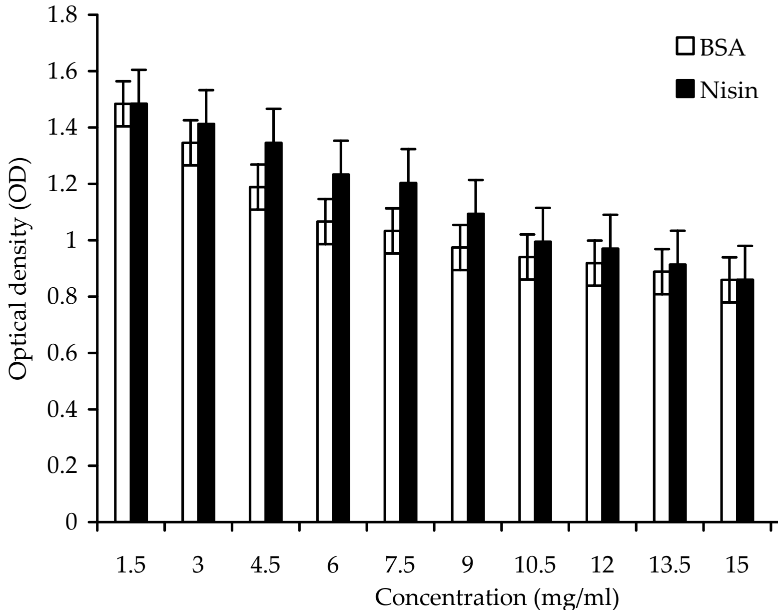

Figure 3. Decline in absorbance due to Carmoisine-protein complex formation with increase in protein concentration

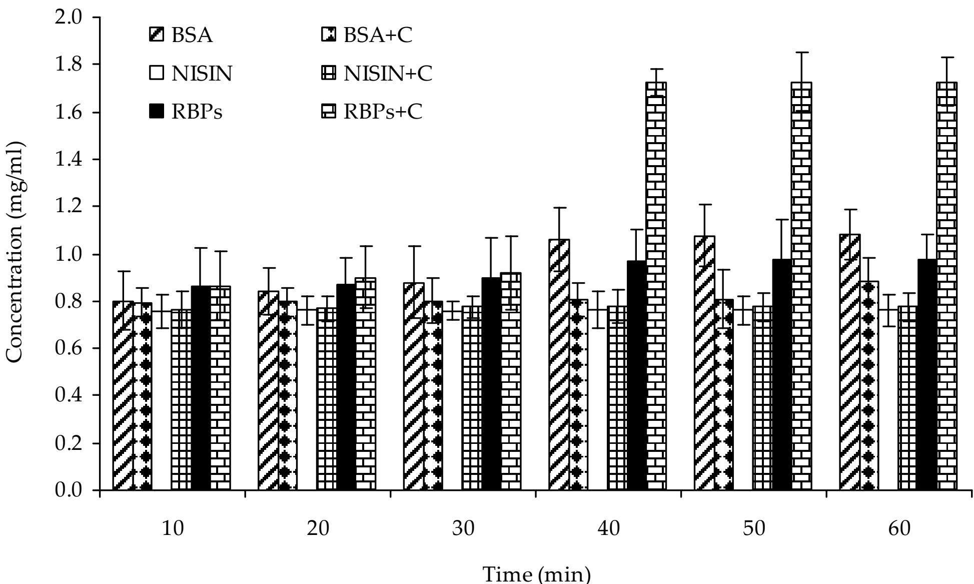

Figure 4. Tryptic digiestibility of BSA and color bound BSA (BSA-C), nisin and color bound nisin (NISIN-C), RBPs and Color Bound RBPs (RBPs-C)

i.e. Carmoisine binds on molar basis. This shows that carmoisine may be used for the estimation of soluble proteins.

The tryptic digestibility of Carmoisine linked to BSA, nisin, and RBPs (crude rice bran protein solution) complexes as compared to unbound proteins (Figure 4) has clearly illustrated that Carmoisine bound nisin was almost equally digestible. However, Carmoisine slightly restricted the proteolytic process in the case of BSA, perhaps the active sites for the trypsin action were found equally available in the BSA-Carmoisine complex and BSA alone. However, in the case of RBPs, the process was totally reversed and the increased digestibility of the protein may have been due to the action of Carmoisine on the substrate which, was responsible for uncoiling of RBPs molecules thus enhancing the trypsin action.

In conclusion, the results of the present study have shown for the first time that Carmoisine-Red-3

Table 2. Comparison of Carmoisine and Coomassie

Carmoisine-Red-3	Coomassie Brilliant Blue-R-250
HO, SO_3Na, N=N, SO_3Na	H_3C, N, SO_3M, CH_3, HN, $\overset{+}{N}$, CH_3, OCH_2CH_3, SO_3^-
Red powder(stained red)	Blue powder(stained blue)
Carmoisine is not carcinogenic and that the no-untoward-effect level in this study was 400 mg carmoisine/kg body weight/day	Slightly hazardous in case of skin contact (irritant), of ingestion, of inhalation
Usually use as Food Color in confectionary, beverage, ice cream and in cosmetics etc.	Not suitable for edible use

binds to all the proteins, and that the dye is suitable in food processing at high or low temperatures. The fast staining capacity of the dye to produce sharp colour bands in the eletrophoretic gel with a persistent stability has proved that it may be used as protein staining agent where the results are quickly desired. The easy tryptic digestibility of the colour-bound proteins has shown that no adverse effect is by this food colour to restrict the protein digestion.

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