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Safety evaluation of formulations containing carboxymethyl derivatives of starch and chitosan in albino rats

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Abstract

Two composite formulations, based on carboxymethyl derivatives of starch (formulation I) and chitosan (formulation II), used in the preparation of coating formulations to enhance post harvest shelf-life of fruits and vegetables, were evaluated for safety by single dose dietary (formulation I, coating on feed pellet-1.3% w/w and formulation II, coating on feed pellet-1% w/w) and oral (1 ml, 2% aqueous solution) administration to albino rats. Experiment was carried out for 4 weeks. No significant changes were observed in gain in weekly body weight, weight of vital organs and in parameters of haematology and histopathology among experimental groups, thus indicating safety (and non-toxicity) of the coating formulations.

Keywords: Composite coating formulations; Carboxymethyl starch; Carboxymethyl chitosan; Oral/dietary administration; Albino rats; Safety

1. Introduction

Safety and security of fruits and vegetables are important issues concerning with their protection and preservation from oxidative and microbial spoilage. As the demand for freshness of fruits and vegetables is increasing, the methods of their storage are also getting improved. The existing methods in this direction are controlled atmosphere and modified atmosphere packaging (MAP) techniques in conjunction with refrigerated storage systems. Due to interactions between the product and the packaging materials, as well as cost ineffectiveness and huge infrastructural requirements, MAP techniques are expensive. Though, plastic based materials are fulfilling the needs for packaging of fruits and vegetables, it is of great concern to environmentally conscious individuals as these are petroleum based products and therefore non-biodegradable, and persist in nature for a long period. Nearly, 1.4×10^6 ton of plastic materials are being used for a variety of packaging purposes (Roper & Koch, 1990) and over 25 million tons of non-biodegradable plastics are being accumulated in the environment annually (Lee, Pometto, Fratzke, & Banaras, 1991). Alternatively, biodegradable polymers are getting more importance, especially for short term usage such as agricultural mulches, beverage and fast food packages

Of late, edible coatings are getting increased attention as alternative means to preserve the freshness of fruits, vegetables, nuts, etc. Though, protein (such as corn zein, soy protein, peanut protein, keratin, gelatin, casein, milk whey proteins, etc.) based films and coatings are available, some individuals exhibit allergic responses to specific protein sources used in the film (Skerritt, Devery, & Hill, 1990). Lipid-based coating solutions (mainly of triglycerides and waxes) show poor flexibility and high degree of cohesiveness (Kester & Fennema, 1986, 1989). On the other hand, polysaccharides, such as starch, cellulose, pectin, etc. are non-toxic, available in abundance and are getting importance as promising biopolymers, which both in their native and derivatised forms can modify the micro-atmosphere inside the packaging. Examples for such materials include coating mixture composed of sucrose fatty acid ester (SFE), sodium carboxymethyl cellulose

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(TAL-Prolong), Nutri-Save (*N,O*-carboxymethyl chitosan), chitosan, etc. (Banks, 1983, 1984; Dhalla & Hanson, 1988; Ghaouth, Arul, Ponnampalam, & Boulet, 1991). Some of the above coatings however, are not effective in extending the shelf-life of bell pepper or long green pepper stored at 21 °C (Banaras, Lownds, & Bosland, 1989). Further, TAL-Prolong coatings resulted in inferior colour and texture in banana (Krishnamurthy & Kushalappa, 1985). Recently, composite coating formulations, based on starch and chitosan {*N*-deacetylated chitin, a poly-β-(1-4)-*N*-acetyl-D-glucosamine} derivatives have been prepared and evaluated for their effectiveness in extending the shelf-life of banana and mango (Kittur, Saroja, Habibunnisa, & Tharanathan, 2001). These coatings improved the characteristics of banana and mango by reducing the water loss and maintaining the colour. The above coatings showed beneficial effects on firmness, titrable acidity and reducing sugar content of fruits. Chitosan-based coatings, in addition to delaying ripening, had antifungal property as the added advantage. Sensory evaluation of fruits coated with these polysaccharide-based formulations showed acceptable colour, flavour and taste. In food industry, consumer has to be assured about the safety of new materials that are used for preservation. Safety evaluation of the above two polysaccharide-based composite coating formulations, used in preservation study, is the focus of the present investigation.

2. Materials and methods

2.1. Materials

NaOH, toluene, isopropanol, methanol, glacial acetic acid were of analytical reagent grade from Qualigens, Bombay. Glycerol monostearate and Tween-80 were from Sigma, USA.

Potato was purchased from the local market. Laboratory rat feed pellets were from Kamadhenu Agencies, Bangalore. Shrimp chitin was procured from CFTRI Regional Centre, Mangalore.

2.2. Isolation of potato starch

Peeled potato (500 g) was kept in cold water for 30 min, crushed (wet grinder) into a fine paste and suspended in water. The slurry was centrifuged at 4000 rpm for 30 min. the residue was slurried with 0.1 N NaOH to pH 9.0; stirred continuously for ∼10 min and centrifuged. The starch was washed thoroughly with water to remove residual alkali. The sedimented starch was treated with 0.1 N NaCl–toluene (10:1, v/v) for ∼20 min, centrifuged and the purified starch was thoroughly washed with water. Finally it was washed in alcohol and dried at 60 °C in an oven (Madhusudhan, Susheelamma, & Tharanathan, 1993).

2.3. Carboxymethylation (CM) of starch

Starch (4 g), NaOH (3.2 g) and monochloroacetic acid (4 g) were taken in a beaker, with 10 ml of water and the contents were subjected to continuous stirring to homogeneity. Subsequent reaction was allowed to proceed at 60 °C for 2 h. The reaction products were precipitated with ethanol and washed alkali free and dried in an oven at 80 °C for 3 h (Khalil, Hashem, & Hebeish, 1990).

2.4. Chitosan preparation

Sieved shrimp chitin (30 g) was placed in a 1 l round-bottom flask. NaOH (40%, w/v solution, 300 ml) was added to this flask. The reaction was carried out for different intervals of time (4–10 h) at ∼100 °C. The mixture was continuously stirred by a glass rod. At the end of the specific time period, the reaction mixture was poured into cold water. The precipitate was filtered using a Buchner funnel and Whatman No. 1 filter paper. The precipitate was washed several times with distilled water to neutrality (pH 7). The product was then dried in an oven at 60 °C. Chitosan samples were purified by dissolving in 1% aqueous acetic acid to remove insolubles. The soluble portion was precipitated with 2 N NaOH, washed thoroughly with double distilled water and lyophilized.

2.5. N,O-carboxymethylation of chitosan

To chitosan (5 g) suspension in 50 ml isopropanol, 13 ml of 10 M NaOH was added in six equal portions over a period of 20 min under agitation. The alkaline slurry was stirred for an additional 45 min. Different amounts of monochloroacetic acid were added to achieve different degrees of substitution. The reaction mixture was heated at 60 °C for 2 h. Cold distilled water (5 ml) was added and the pH was adjusted to 7.0 with glacial acetic acid. The reaction mixture was filtered and the solid *N,O*-carboxymethyl derivative was washed with 70% methanol and dried in an oven at 60 °C. The derivatives were purified by dissolving in water to remove insolubles (scarcely substituted products), filtered, dialysed against running water for 2 days and against double distilled water for one day and lyophilized.

2.6. Composite coating formulations

To prepare 100 ml of coating solution, 1–2 g polysaccharide derivatives were dissolved in distilled water and blended with glycerol monostearate (0.8%). Tween-80 (0.2 ml) was added to the solution to emulsify and to improve wettability. The above solution was stirred for 30 min and the insolubles were removed by filtration (Tharanathan, Kittur, Krishnaprakash, & Habibunnisa, 1998; Tharanathan, Saroja, Habibunnisa, & Krishnaprakash, 1998). Formulations based on CM starch and CM chitosan were marked as Formulations I and II, respectively.

2.7. Coating of feed pellets

Rat feed pellets were coated with formulations I and II by giving a dip (∼5 min) in respective formulations followed by draining out excess solution and drying in sun light for 1.5 h. Weight of pellets, before and after coating, were noted. The difference in weight was used to calculate percentage of coating.

2.8. Animal experiments

Groups of six male and six female albino rats (*Rattus norvegicus*, OUT B, Wistar albino, IND-Cft), with average body weight of 85 g, were used in this study, with the consent of Animal Ethics Committee, constituted in our Institute. Animals were kept individually in experimental cages. In one set of experiment, I group and II group of animals were fed once with pellets coated with formulation I (1.3% w/w) and formulation II (1% w/w), respectively. III group served as control. In the second set of experiment, I group and II group of animals were intubated once with formulations I and II (1 ml, 2% aqueous solution), respectively. III group served as control. All animals were fed with control pellet diet and water ad libitum for 4 weeks. Weekly body weight was recorded. At the end of the experiment, animals were sacrificed and weight of heart, liver, kidneys, brain, spleen, testes, adrenals and lungs were noted.

2.9. Histopathology and haematology

Respective tissues were fixed in 10% formaldehyde and processed for paraffin blocks. 6 μm thick sections were stained with haematoxylin and eosin. Observations were made under the microscope for any change in histology. Collected blood was subjected to haematological parameters (Hb, RBC, WBC, PCV and differential counts).

3. Results and discussion

Carboxymethylation is one of the important etherifying derivatization processes of polysaccharides resulting in products of potential applications in both food and non-food industries. In food industry, assurance of consumers health is important before any new applications. In the present investigation, safety of solution of composite coating formulations based on carboxymethyl starch and chitosan was evaluated in experimental rats. The purpose of designing coated pellet feeding experiment was to be more nearer to the mode of product application. Dip method of coating is the common practice for fruits, vegetables and meat products where commodity is dipped in coating formulations, removed and allowed to air dry (Tharanathan, 2003). This results in the formation of a thin membranous film over the surface of commodity, and thus facilitating

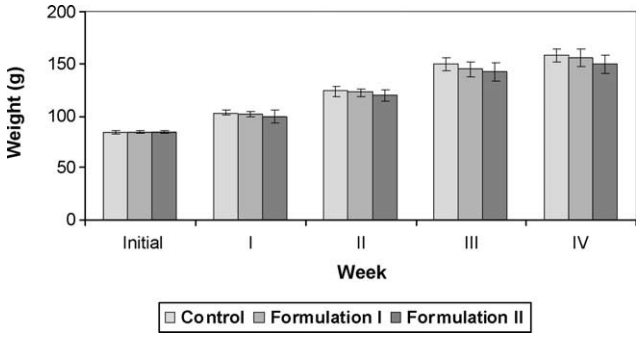

Fig. 1. Gain in weekly body weight of male rats fed with coated pellet diet.

MAP conditions inside the package. Second experiment of oral administration was aimed at exposing experimental animals to an acute oral dosage of 1 ml of 2% aqueous solution of these formulations (>2%, the solution was found to be too viscous).

In the experiment with coated pellet diet, all animals expressed no discomforts. Gain in weekly body weight of male and female rats during the experimental period is shown in Figs. 1 and 2, respectively. Accordingly, there was no significant change in the body weight gain among the groups. Similarly, no significant changes among male and female rats were discernable in the weights of their vital organs (Figs. 3 and 4). All the haematological parameters were found within the normal range in both male and female rats among different groups (Table 1). In histopathology, all tissues were found to be normal in all groups.

In oral administration experiment also, all animals were found to be normal. Gain in weekly body weight of male and female rats showed no significant changes among the groups (Figs. 5 and 6). In the organs weight of both male and female rats there was no significant change among the groups (Figs. 7 and 8). All the haematological parameters were found within the normal range in both male and female rats among different groups (Table 2). In histopathology, all tissues were found to be normal in all groups.

The observation of no ill effects on health of experimental rats clearly indicates the safety and the non-toxicity of the composite coating formulations. The non-toxicity of CM starch has already been documented. In *Salmonella*

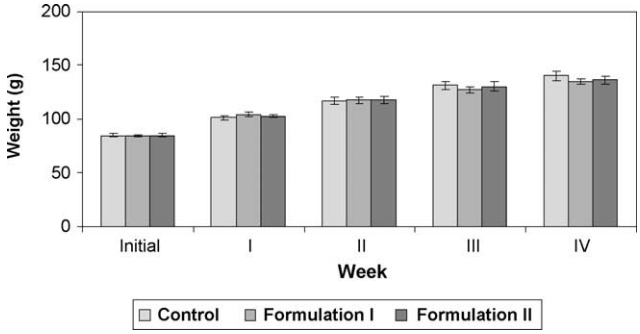

Fig. 2. Gain in weekly body weight of female rats fed with coated pellet diet.

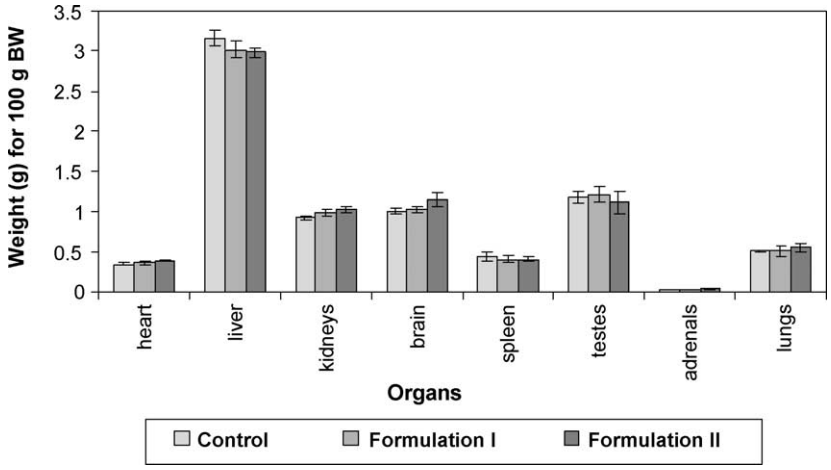

Fig. 3. Organ weights in male rats fed with coated pellet diet.

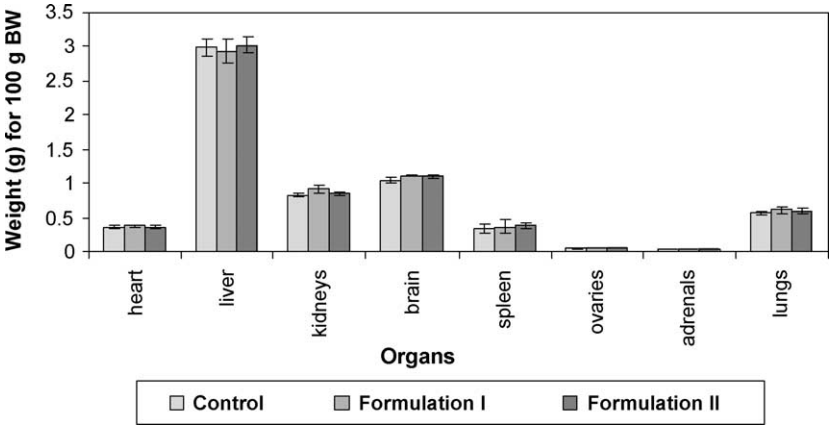

Fig. 4. Organ weights in female rats fed with coated pellet diet.

typhimurium (TA 98 and TA 100) it was found to be non-mutagenic (Strizhel'chik & Kul'shin, 1994). Tsuji, Tsuji, and Suzuki (1977) observed no effect of CM starch on cholesterol metabolism in rats upon dietary administration at 5% level. Chitosan also has been found to be safe in mice and rats when used as food additive (Mita, 1987). The documented oral LD_{50} of chotosan in rats is over 1.5 g/kg. No haematological and physiological effects have been observed in cats, mice and cows upon subcutaneous administration of chitosan at 200 mg/kg (Minami et al., 1996). However, repeated subcutaneous administration of chitosan at 150 and 200 mg/kg, in dogs resulted in hemorrhagic pneumonia.

Multiple actions of chitin and chitosan in food systems relate to their effect as dietary fibre and as a functional food ingredient. The US Food and Drug Administration has approved chitosan as a feed additive (Knorr, 1986). The nutritional significance of chitinous polymers in animals as a feed additive has been demonstrated (Hirano, 1989). Accordingly, lower cholesterol and triacylglycerol values

Table 1
Profile of haematological parameters in rats fed with coated pellet diet

	Hb (g/dl)	RBC (10^6/μl)	WBC (counts/μl)	PCV (%)	L (%)	P (%)	M (%)	E (%)	B (%)
Male rats									
Formula I	13.66±0.16	11.67±0.40	15040±1960	39.00±0.84	84.40±1.86	14.40±2.38	2.00	0	0
Formula II	13.50±0.10	9.78±0.28	14010±2981	36.60±1.12	87.80±3.80	11.40±3.59	1.60	0	0
Formula III	13.66±0.31	10.23±0.54	17660±3794	37.20±0.80	89.20±2.92	8.80±2.90	2.00	0	0
Female rats									
Formula I	13.40±0.48	10.69±0.77	11890±2574	38.60±1.21	88.40±1.96	9.4±2.06	2.20	0	0
Formula II	13.92±0.35	9.83±0.52	10730±2155	34.80±0.58	89.00±1.45	9.80±1.20	1.50	0	0
Formula III	13.86±0.32	10.82±0.69	13870±2030	37.00±1.38	87.60±1.72	10.80±1.74	2.00	0	0

L, leukocyte; P, polymorph; M, monocyte; E, eosinophil; B, basophil.

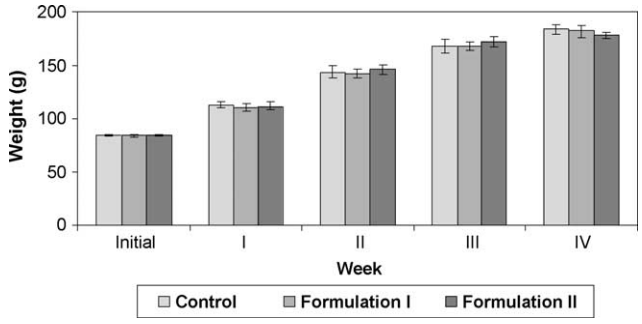

Fig. 5. Gain in weekly body weight of male rats administered orally with formulations.

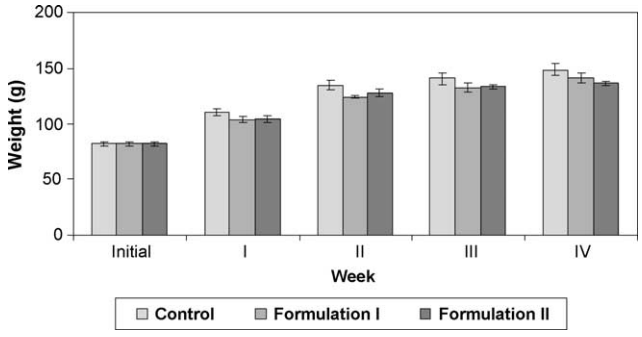

Fig. 6. Gain in weekly body weight of female rats administered orally with formulations.

were obtained in rabbits, hens and broilers upon feeding on chitin and chitosan at $<1.4\ g\ kg^{-1}$ b.w. without any adverse effect on normal growth pattern. Austin, Brine, Castle, and Zikakis (1981) reported the effect of chitin as a feed additive on the enhanced growth of bifidobacteria in guts of chickens.

Further, versatile physiological activities of oligomers of chitin and chitosan have been observed and these include antitumour activity, immunoenhancing effects, protective effects against some infectious pathogens in mice, antifungal and antimicrobial activities (Hirano & Nagao, 1989; Kendra, Christian, & Hadwiger, 1989; Muzzarelli, 1996; Tokoro et al, 1988; Yamada, Shibuya, Kodama, & Akatsuka, 1993; Zacour, Silva, Cecon, Bambirra, & Vieira, 1992). With regard to metabolism of chitin oligomers, reports are available on the presence of enzymes capable of cleaving chitin oligomers in human plasma, articular chondriocytes and in synovial cells (Renkema, Boot, Muisers, Donker-Koopman, & Aerts, 1995). Recently, chitosan has also been found to be susceptible to the hydrolytic activities of several other enzymes namely, cellulose, pectinase, lipase and protease such as pepsin, pepain and bromelin (Pantaleone, Yalpani, & Scollar, 1992).

As a source of dietary fibre, the chemically modified derivatives may undergo colonic degradation by

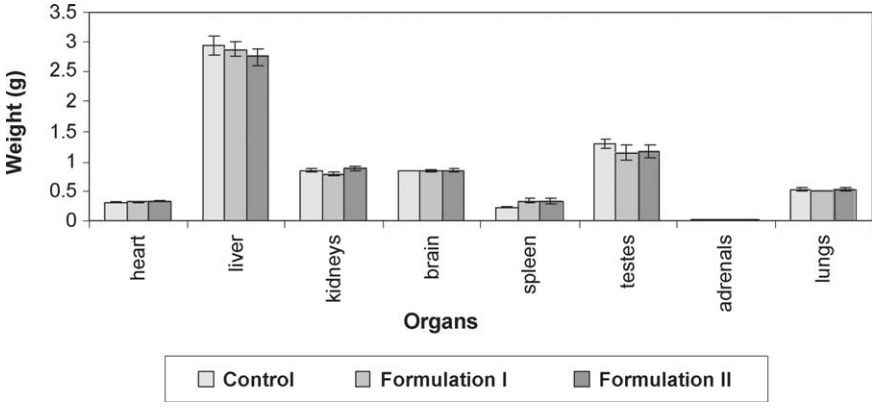

Fig. 7. Organ weights in male rats administered orally with formulations.

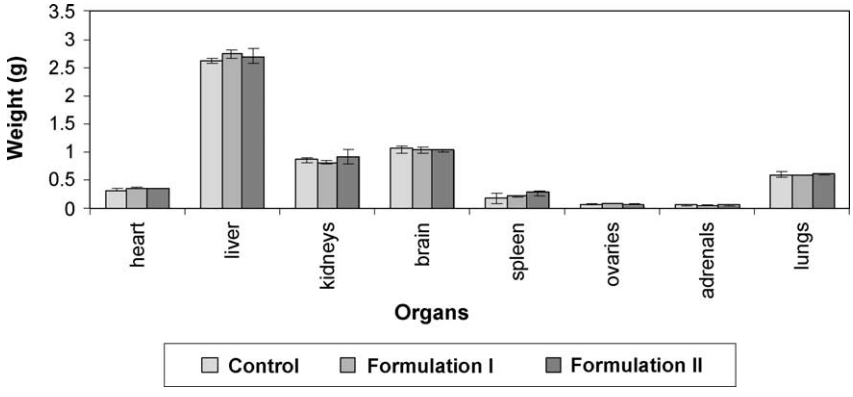

Fig. 8. Organ weights in female rats administered orally with formulations.

Table 2
Profile of haematological parameters in rats administered orally with formulations

	Hb (g/dl)	RBC (10^6/μl)	WBC (counts/μl)	PCV (%)	L (%)	P (%)	M (%)	E (%)	B (%)
Male rats									
Formula I	14.27±0.30	10.19±0.61	10568±2817	40.50±1.19	77.50±1.32	21.75±1.11	1.00	0	0
Formula II	14.05±0.26	11.10±0.34	12000±1668	38.75±1.11	70.76±1.08	22.00±0.71	2.30	1.00	0
Formula III	13.80±0.30	11.65±0.22	12762±2033	41.00±1.29	68.75±0.85	27.50±0.96	2.75	1.00	0
Female rats									
Formula I	14.50±0.35	11.24±0.61	9587±1742	37.00±1.47	74.50±1.71	22.75±2.10	2.50	1.00	0
Formula II	13.80±0.29	10.14±0.67	8612±1754	37.25±0.48	71.75±1.11	27.00±1.29	1.25	0	0
Formula III	14.27±0.50	10.51±0.28	11112±1816	40.00±1.15	70.25±1.31	27.25±1.11	2.00	1.30	0

L, leukocyte; P, polymorph; M, monocyte; E, eosinophil; B, basophil.

the anaerobic microbial fermentation forming gases such as H_2, CH_4 and CO_2, and short chain fatty acids (SCFA) such as acetate, propionate and butyrate (Muir et al., 1993). Butyrate metabolism is important for it provides energy, which is utilized by the colon epithelial to inhibit or avoid transformation of these cells into malignant cells (Mathers, 1992).

4. Conclusions

Application of coating formulations is an emerging and promising area for preservation of freshness of fruits and vegetables. Carboxymethyl derivatised starch and chitosan based composite coating formulations, found to be useful in enhancing the shelf-life of some fruits/vegetables, were found to be generally safe. Biodegradability of these derivatised polysaccharides is the added advantage. These formulations get value addition upon further observations of their safety in long term/generation studies.

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