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# Preparation of iron bound succinylated milk protein concentrate and evaluation of its stability

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ABSTRACT

Major problems associated with the fortification of soluble iron salts include chemical reactivity and incompatibility with other components. Milk protein concentrate (MPC) are able to bind significant amount of iron due to the presence of both casein and whey protein. MPC in its native state possess very poor solubility, therefore, succinylated derivatives of MPC (succ. MPC) were also used for the preparation of protein–iron complex. Preparation of the complex involved centrifugation (to remove insoluble iron), ultrafiltration (to remove unbound iron) and lyophilisation (to attain in dry form). Iron binding ability of MPC enhanced significantly ($P < 0.05$) upon succinylation. Stability of bound iron from both varieties of complexes was monitored under different conditions encountered during processing. Higher stability ($P < 0.05$) of bound iron was observed in succ. MPC–iron complex than native protein complex. This method could be adopted for the production of stable iron enriched protein, an organic iron source.



## 1. Introduction

Iron is one of the most important micronutrient being a part of many enzymes and used in many functions. Iron deficiency anemia (IDA) is the major deficiency of iron and is one of wide spread public health problems. According to a survey conducted by WHO from 1993 to 2005 about 1.62 billion people which corresponded to around 25% of world population, suffered from anaemia and more than 50% of the above suffered from IDA (Akhter et al., 2014; WHO/CDC, 2008). Iron fortification in food products is considered as an acceptable criterion to combat iron deficiency. Many different forms of iron, ranging from iron salts to iron chelates have been approved as suitable iron sources for food fortification (Hurrell, 1999). Fortification of food products with soluble iron salts poses many problems such as chemical reactivity of the fortificants, reduced stability under food processing and storage conditions, and incompatibility with other food components resulting in reduced bioavailability of the fortificants (Ellis, Mittal, & Sugiarto, 2013). Hence, many researchers are now focusing their attention on protein–iron complexes which have been demonstrated to be an efficient iron supplement for human beings without side effects.

Recent studies on mineral delivery techniques revealed that binding of iron to proteins (especially milk proteins), reduced iron reactivity and improved iron bioavailability (Sugiarto, Ye, & Singh, 2009; Sugiarto, Ye, Taylor, & Singh, 2010). Milk protein concentrate (MPC) is a functional ingredient used to enrich the nutritional properties of different products. MPC is considered as a complete protein due the presence of both casein and whey proteins similar to milk (Fang, Selomulya, Ainsworth, Palmer, & Chen, 2011). MPC shown to have better iron binding ability than whey protein isolates, mainly due to the presence of sufficient amount of caseins (Sugiarto et al., 2010). However, major drawback of MPC to be considered for food application is its low solubility, which could be improved by protein modification technique such as succinylation (Shilpashree, Arora, Chawla, & Tomar, 2015). Succinylation involved addition of carboxylic group and enhancement of negative charge on the surface; this in turn enhances the iron binding ability of proteins. Many workers have suggested the utilisation of succinyl derivatives of milk proteins for mineral binding purposes (Cremonesi & Caramazza, 1993; Cremonesi, Strada, Galimberti, & Sportoletti, 1984). However, soluble iron bound proteins in the form of lyophilised powder which would find further application in product fortification needs to be elucidated. Moreover, no work has been reported on the efficiency of succi. MPC to bind iron. Therefore, the present investigation was carried out to prepare MPC–iron complexes with following objectives: (i) comparison of the iron binding capacity of native and succi. MPC,

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(ii) standardisation of method for the production of MPC–iron complex in the form of lyophilised powder and (iii) elucidate the effect different processing condition on the stability of bound iron from native as well as succi. MPC–iron complexes.

## 2. Materials and methods

### 2.1. Materials

MPC with 85% protein was procured from Mahaan proteins Ltd. India. Folin and Ciocalteu's reagent, sodium carbonate, copper sulphate, potassium tartrate, bovine serum albumin, sodium hydroxide, succinic anhydride, lithium hydroxide, acetic acid, hydrindantin, dimethyl sulphoxide (DMSO), L-lysine monohydrochloride, ninhydrin, ferrous sulphate heptahydrate (20.07% iron), succinic acid, boron trifluoride and methane were procured from Sigma Aldrich, St. Louis, MO, USA. Triple distilled water and acid washed glassware were used throughout the experiments.

### 2.2. Methods

#### 2.2.1. Protein estimation

Protein was estimated by two methods namely Lowry, Rosebrough, Farr, and Randall (1951) for the estimation of protein solubility and Kjeldahl method as described by AOAC (1984) for the estimation of total nitrogen content.

#### 2.2.2. Succinylation of MPC

The method used for the succinylation of milk proteins and quantification of succinylation (ninhydrin method) was followed as standardised by Shilpashree et al. (2015). In the proposed method, maximum degree of succinylation was attained with 4 mol of succinic anhydride/mol of lysine content in MPC. Therefore, this concentration was used in the present work. 9.14% (w/v, 50 mmol/L of lysine content) of protein solution was adjusted to pH 8 using 2 mol/L NaOH, to this solution known quantity of succinic anhydride was added. The mixture was stirred for 1 h at 37 °C using magnetic stirrer (SPINOT MC 02, Tarsons Products Pvt. Ltd., Kolkata, India). Protein was recovered by precipitating the mixture at pH 3.5–4 with 2 mol/L HCl and followed by centrifugation at 5000×*g* for 20 min using High speed refrigerated centrifuge (KUBOTA-6500, Kubota Corporation, Tokyo, Japan). Protein precipitates were collected, washed by adding equal amount of water and stirred for another 1 h, and then centrifuged. This washing procedure was repeated for another 4 times. Finally, the washed precipitates were resolubilised at pH 7 with 2 mol/L NaOH and lyophilised (Freezone 6 – 7753030, Labconco Corp., Kansas city, MO, USA). Degree of succinylation was estimated by ninhydrin method and calculated using the following formula:

$$\text{Degree of succinylation } (\%) = \frac{A - B}{A} \times 100$$

where, $A$ = μ mol of free amino groups estimated per mg of net protein (unmodified), $B$ = μ mol of free amino groups estimated per mg of net protein (modified).

#### 2.2.3. Estimation of iron content

Iron content of the complex was estimated by dry digestion method of AOAC (2005) using Atomic absorption spectrophotometer (AAS). 100 mg of sample was weighed and ashed at 550 °C/8 h. 10 mL of triple acid (nitric acid:perchloric acid:sulphuric acid in the ratio 3:2:1) was added to ash and heated for complete dissolution. Samples were diluted suitably and iron content was estimated using AAS at $\lambda_{max}$ 248.3 nm.

#### 2.2.4. Solubility

Methods of Lawal, Adebowale, and Adebowale (2007) as modified by Shilpashree et al. (2015) and Mutilangi and Kilara (1985) with slight modification (i.e. centrifugal speed and time were standardised) were followed for the analysis of protein solubility. 1.0% (w/v) of sample solution was prepared in phosphate buffer (0.05 mol/L, pH 7). The solution was mixed for 1 h at 30 °C using magnetic stirrer followed by centrifugation at 18,000×*g* for 20 min at 4 °C. The supernatant was filtered through Whatman No. 1 filter paper and protein content was determined using the method of Lowry et al. (1951). Determinations were carried out in triplicated and solubility (percentage) was evaluated as follows.

$$\text{Solubility } (\%) = \frac{\text{Amount of protein content in supernatant}}{\text{Amount of protein in the sample}} \times 100$$

#### 2.2.5. Preparation of milk protein–iron complexes

The method of Sugiarto et al. (2009) was essentially followed for the preparation of MPC–iron complexes and the overall method used is shown in Fig. 1. Protein solution was prepared by dissolving 10 g of protein in 1000 mL of triple distilled water. To this solution, iron was added slowly from the stock solution to obtain a final mineral concentration ranging from 1 to 10 mmol/L with constant stirring using magnetic stirrer. The pH of the solution was adjusted to 6.6 and was left undisturbed for 2 h at around 20 °C. The mixture was then centrifuged at 12,000×*g* at 20 °C for 30 min. Supernatant which contained soluble mineral and protein was carefully decanted and filtered through Whatman No. 1 filter paper (supernatant iron content and protein solubility were analysed). The filtered supernatant was then passed through an Amicon ultrafiltration (UF) membrane tubes (MW cut off 10 KDa) and the iron content in permeate was analysed by AAS. Finally, the optimised iron concentration by this method was subjected for large scale production using UF membrane processing system.

*2.2.5.1. Membrane processing system.* The basic steps involved in UF membrane processing system were followed as described by Ferrer, Alexander, and Corredig (2011). Membrane with a nominal molecular weight cut-off (NMWCO) of 10 KDa hydrasart material with filtration area 0.1 $m^2$ was used for the present work. Clear filtrate was concentrated using a Sartorius ultrafiltration unit (Model No. 7578, Sartorius India Pvt. Ltd. Mumbai India) assembled with Masterflex easy load pump – 7518-00 (Thermo Fisher Scientific, Mumbai, India). Pressure gauge and flow meter were connected to the inlet of the membrane and also at exit of retentate and permeate. UF was carried out by re-circulating the supernatant from feed tank. Membrane pressure and cross-flow velocity were set to 10 psi and 300 mL/min, respectively. The experiment was conducted at room temperature (~30 °C). Free iron content was completely removed from retentate by diafiltration (i.e. constant volume washing). To ensure the presence of free mineral in permeate, 1 mL of 1 mol/L NaOH was added to 10 mL of permeate, followed by centrifugation at 2000 rpm/15 min. Diafiltration was repeated until non appearance of mineral precipitate upon addition of NaOH and pellet formation upon centrifugation. Finally, retentate with no free iron content was concentrated 4 times (4×) the original volume (i.e. 1 L to 250 mL) and freeze dried at −50 °C under 6.67 Pa pressure for 72 h.

Iron retention in protein–iron complexes was estimated as follows:

$$\text{Iron retention } (\%) = \frac{\text{Amount of added mineral}}{\text{Amount of obtained mineral in the complex}} \times 100$$