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Effect of succinylation on physicochemical and functional properties of milk protein concentrate

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ABSTRACT

Milk protein concentrate (MPC) is a complete dairy protein ingredient (containing both caseins and whey proteins) available with protein concentrations ranging from 40 to 90%. However, MPC powders are poorly soluble which thus, restricts their potential use for food application. Succinylation involves chemical derivatisation of ϵ-amino group of lysine in proteins and enhances solubility of less soluble proteins. In the present investigation, highest degree of succinylation was achieved at the level of 4 mol of succinic anhydride/mole of lysine in MPC. Findings from intrinsic tryptophan intensities stated that succinylation of milk proteins showed structural modification and increase in hydrophobicity. Further, the effects of succinylation on the functional properties (solubility, water- and oil-binding capacities, viscosity, foaming capacity and stability, emulsion activity and stability) of MPC were evaluated. Succinylation of proteins significantly ($P < 0.05$) increased solubility as a result of altered charge on protein and reduced particle size of native MPC, which in turn improved other functional properties of native MPC. The microstructure of succinylated MPC at different degrees of succinylation by scanning electron microscopy revealed that the size and number of white patch like structures present on protein increased with degree of succinylation.

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1. Introduction

Milk protein concentrate (MPC) is a newly developed functional ingredient used to enrich the nutritional properties of dairy product. However, MPC powders are poorly soluble due to their high protein content (40–90 wt.% solid content), and this restricts their potential use for food application (Fang, Selomulya, Ainsworth, Palmer, & Chen, 2011). Carr (2002), disclosed a process for making MPC with improved solubility, involving the addition of a monovalent salt to the ultrafiltered retentate prior to drying. However, this method did not solubilize the insoluble material completely. De Castro-Morel and Harper (2002) reported that the drying outlet temperatures (65–90 °C) used in making pilot-plant-produced MPC powders did not have any statistically significant effect on their solubility (at pH 7). The insolubility of proteins however sets limits for its utilization in formulated food systems and so the solubilization of MPC has been attempted to extend its usefulness in the food industry. Acylation of amino acid residues with acetic anhydride and succinic anhydride has been the most common chemical modifications used in food protein applications. Small alterations in the structure of proteins are capable of bringing about significant changes in their functional properties (Kester & Richardson, 1984). The acylation of proteins with succinic anhydride is one of the most convenient and frequently used methods for altering the functional properties of proteins (Damodaran & Alain, 1997). Succinylation involves chemical derivatization of ϵ-amino group of lysine in proteins with succinic anhydride. Succinic anhydrides result in replacement of positive charge with a negative charge at free amino (lysine) and hydroxyl groups of amino acids. Succinic anhydride (21 CFR 175.300) is generally recognized as safe (GRAS) for miscellaneous and general purpose usage in the US according to the FDA (Food and Drug Administration), with no limitation other than good manufacturing practices (Smith & Hong-Shum, 2011). Succinic anhydride can be used in the production of starch sodium succinate at the level not to exceed 4% as per Food Chemicals Codex (1981). It has been noted that succinylation of proteins causes an increase in the intermolecular electrostatic repulsion forces, resulting in an expansion of the molecule which leads to increased solubility (Johnson & Brekke, 1983). This modification had been applied to many food proteins including soy protein isolates, spray-dried egg white and whey protein isolates (Zhao, Ma, Yuen, & Phillips, 2004), soya protein (Franzen & Kensella, 1976), oat protein isolates (Mirmoghtadaie, Kadivar, & Shahedi, 2009), whey protein concentrate (Thomson & Reye, 1980),

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fish myofibrillar protein (Groninger, 1973), canola protein isolate (Paulson & Tung, 1988), cottonseed flour (Choi, Lusas, & Rhee, 1981), and egg white protein (Sato & Nakamura, 1977). However, no report is available on functionality of succinylated milk protein concentrate. The specific purposes of this study were (I) to optimize the level of reactants (succinic anhydride and protein) to achieve maximum degree of succinylation of milk proteins and (II) to determine the effect of succinylation on physicochemical and functional properties of native and succinylated milk proteins and also, to recognize the changes in microstructure of succinylated milk proteins at different degrees of succinylation.

2. Materials and methods

2.1. Materials

MPC with 85% protein was procured from Mahaan Proteins Ltd., India. Folin and Ciocalteu's, sodium carbonate, copper sulphate, potassium tartarate, bovine serum albumin, sodium hydroxide, 8-anilino-1-naphthalene-sulphonic acid (ANS), succinic anhydride, lithium hydroxide, acetic acid, hydrindantin, dimethyl sulphoxide (DMSO), L-lysine monohydrochloride and ninhydrin were procured from Sigma Aldrich, St. Louis, MO, USA. Deionized water and acid washed glasswares were used throughout the experiments.

2.2. Methods

2.2.1. Protein estimation

Protein was estimated by Kjeldahl method as described by the AOAC (1984).

2.2.2. Protein succinylation

The method used for the succinylation was followed as described by Thomson and Reye (1980) with slight modification i.e., centrifugation and washing steps in the preparation of succinylation of milk proteins were introduced. Succinylation was carried out at different levels by varying moles of succinic anhydride per mole of free modifiable amino groups i.e., lysine content. The 50 mM dispersion of lysine (9.14% of protein) solution was adjusted to pH 8.0 with 2 N NaOH using pH metre (Cyberscan pH Tutor, EUTECH Instruments, Thermo Fisher Scientific, Mumbai, India), and to this solution known weight of dry succinic anhydride was then added to obtain a final concentration ranging from 1 to 12 mol/mol of lysine in MPC. The mixture was stirred for 1 h at 37 °C using a magnetic stirrer (SPINOT MC 02, Tarsons Products Pvt. Ltd., Kolkata, India). The solution was constantly monitored for changes in pH and maintained at pH 8 with constant stirring. Milk proteins recovered by precipitating the mixture at pH 3.5–4 with 2 M 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 100 mL of deionized water and stirred for another 1 h, and this mixture was again centrifuged. The precipitate was again washed with 100 mL water and the pH was measured (if the pH was not in the range, it was adjusted to 3.5–4 with 2 M HCl). This washing procedure was repeated for another 4 times. Finally, the washed precipitates were resolubilized at pH 7 with 2 M NaOH and lyophilized (Freezone 6-7753030, Labconco Corp., Kansas City, MO, USA) at −50 °C under 6.67 Pa pressure for 72 h.

2.2.3. Quantification of succinylation

The extent of succinylation of milk proteins was measured according to the method of Friedman, Pang, and Smith (1984) with slight modification i.e., the level of standard (lysine) and protein in MPC required for estimation was optimized and formula for the estimation of degree of succinylation of milk proteins has been standardised.

2.2.3.1. Preparation of ninhydrin solution and standard curve. Lithium acetate buffer (4 M) was prepared by addition of glacial acetic acid to lithium hydroxide solution. Ninhydrin (2 g) and hydrindantin (0.3 g) were dissolved in 75 mL DMSO solution. After adding 25 mL lithium acetate buffer, the mixture was bubbled with nitrogen for at least 2 min, sealed with paraffin and stored in a refrigerator (4 °C) (fresh ninhydrin solution was prepared on each working day). L-lysine monohydrochloride was used as standard. Lysine content was quantitatively analysed and the calibration curve in the range of 50 to 500 μmol/l ($r = 0.999$) was constructed.

2.2.3.2. Testing procedure. 5 mg protein was dissolved in 5 mL of 0.1 N NaOH. From this, 1 mL sample solution was transferred to a screw-capped test tube along with 1 mL of deionized water to make final volume of 2.0 mL. To each tube (both sample and standard) 2.0 mL of freshly prepared ninhydrin solution was then added. The tubes were placed in a boiling water bath for 15 min. After heating, the tubes were immediately cooled in an ice-bath and 6.0 mL of 50% ethanol was added to each tube (total volume of 10 mL). Tubes were thoroughly mixed with a vortex mixer for 15 s (presence of any ninhydrin negative and insoluble particles was removed by centrifugation at 2000 ×g/15 min). Absorbance of the reaction mixture was measured at 570 nm using spectrophotometer (UV-2700 230 V, Shimadzu Corporation, Kyoto, Japan). Degree of succinylation was calculated using the following formula:

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

where,

A = μmol of free amino groups estimated per mg of net protein (native).

B = μmol of free amino groups estimated per mg of net protein (modified).

2.2.4. Intrinsic tryptophan intensity and hydrophobicity of succinylated MPC

2.2.4.1. Intrinsic tryptophan. Intrinsic tryptophan intensity method of Zhou et al. (2012) was followed to determine the intrinsic tryptophan intensities of native and succinylated protein samples. Protein solution (0.01%) of each sample in phosphate buffer (0.05 M at pH 7.0) was prepared and the tryptophan intensity of the samples was evaluated using fluorescence spectrophotometer (Varian SPVF, Cary Eclipse, Australia) over the emission wavelength ranging from 200 to 500 nm at a fixed excitation wave length of 280 nm with slit width of 5 nm.

2.2.4.2. Hydrophobicity. Hydrophobicity of native and succinylated protein samples was determined by the method of Yuksel, Avci, and Erdem (2010).

2.2.4.2.1. Procedure. 1.0% protein solution of each sample was prepared in phosphate buffer (0.05 M at pH 7). The fluorescent probe used was ANS and the relative fluorescence of the samples was measured by using fluorescence spectrophotometer at excitation wavelength of 390 nm, and emission wavelength of 480 nm with slit width of 5 nm. 10 mM of ANS concentration was prepared in phosphate buffer and used for ANS titration. Final concentration of ANS in the protein solution was adjusted between 0 and 140 μM during the titration. The main aim of this parameter was to reach the maximum fluorescence that shows a saturated fluorescent marker (ANS) binding. Before ANS titration, fluorescence of the samples was measured as a blank. Kinetic data were also evaluated from ANS titration curves using the method of Guo, Fox, Flynn, and Kindstedt (1996).

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