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PEGylation of black kidney bean (*Phaseolus vulgaris* L.) protein isolate with potential functironal properties

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ABSTRACT

In this work, we investigated PEGylated black kidney bean protein isolates (BKBPI) by PEG succinimidyl carbonate (PEG-SC), PEG succinimidyl succinate (PEG-SS) and PEG succinimidyl propionate (PEG-SPA) conjugation. The functional properties, thermodynamic stability, *in vitro* digestion stability, and hemagglutination activity of the modified products were evaluated. The degree of PEGylation was measured, and FTIR analysis revealed that protein-PEG conjugations were formed, and that no obvious changes in water- and fat-holding capacities were observed. The solubility, emulsifying property, and foaming property were all improved through the modification, while, higher thermodynamic stability was achieved with the increase in Td values and reduction of ΔH. The PEGylated proteins were found to be more resistant to *in vitro* digestion, and the hemagglutination activity was significantly ($P < 0.05$) decreased, indicating the higher safety of the protein isolate. The results showed that the functional properties, thermodynamic stability, and biological safety of BKBPI were improved by PEGylation, which could serve to increase the applications for this protein.

1. Introduction

The utilization of food proteins as an important ingredient in food processing has expanded in recent years, due to their unique properties, such as solubility, water- and fat-holding capacity, foaming properties, emulsifying properties, gelling capacity, and basic nutritional values [1–3]. Although many legume protein isolates have been explored, many of these have critical problems, including low special functional properties, susceptibility to enzyme hydrolysis, and strong allergenicity [2,4]. Thus several physical, chemical and enzymic strategies have emerged to improve the physicochemical properties of protein isolates. To date, it has been widely documented that high pressure processing, alcohol washing, acid or alkali treatment, acetylation, glycation, phosphorylation and enzymatic modification could offer interesting possibilities for proteins in practical applications [1,5,6]. Nevertheless, the nutrition and safety of the modified proteins should be further evaluated. The isomerization and amino acids degradation, introduction of toxic chemicals and increasing complexity of production could accompany the application of the modification methods [3]. In addition, there is growing interest in seeking novel approaches to enhance the functional properties of proteins with easy operation and high safety.

PEG (Polyethylene glycol) and its derivatives are neutral, hydrophilic polyethers. The PEG oligomers with a molecular weight above 1000 Da have been approved for pharmaceutical formulation designs for human use by Food and Drug Administration (FDA) because of their non-toxic, hydrophilic and highly flexible properties [7,8]. Over the past decades, various PEGylation of therapeutic proteins have been described, and the reactions between activated forms of monomethoxypoly(ethylene glycol) (mPEG) and the N-terminal amine and surface lysine residues of the proteins in neutral or mild alkali solutions system were widely applied with no other chemicals introduced [9]. Due to the PEG modification, the structures of proteins in solution have been altered, resulting in ameliorative effects on the physical and functional properties, such as the increased solubility, improved mechanical and ther-

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mal stability, enhanced proteolytic resistance and half-life, and the reduced antigenicity and immunogenicity [10]. Hence, PEG oligomers are receiving attentions as they open new directions in bio-catalysis and bio-modification technology where many proteins can be more easily administered.

There are, however, some concerns regarding PEGylation that need to be addressed. When the conjugated protein possesses more than one reactive sites, the conjugation products might be not unique and well defined because of the nonselective attachment of PEG chains to the protein and could result in a significant loss of biological function [11–13]. Recently, several activated PEG derivatives have therefore been developed based on the activation of esters. mPEG succinimidyl carbonate (mPEG-SC) and succinimidyl succinate (mPEG-SS) have been widely applied to improve the selectivity of the PEG-conjugation sites depending on the availability of amino groups. In addition, the introduction of carboxymethylated PEGs, such as mPEG succinimidyl propionate (mPEG-SPA), has reduced the frequency of diol PEG contaminants. The clinical trials for these conjugates have been successfully completed; these trials provide clear proof of their safety. However, the knowledge of the PEGylated legume protein isolates is still scanty.

The black kidney bean (*Phaseolus vulgaris* L.) is a common bean. It is an important industrial crop in subtropical and tropical countries and is especially popular in Asia, Africa, and Latin American [14]. It contains a good balance of amino acids and is rich in nutrient such as isoflavones, vitamin E, saponins, carotenoids, anthocyanins, polyphenols, and especially proteins (more than 25%, wet weight) [15]. Because of the potential functional properties of bean proteins, black kidney bean flour has become an ingredient of choice in many diverse food applications, such as dairy products, meat extension, baked products, flour products and nutritional supplements [15,16]. With improved standard of living and customer demand, proteins with better functional properties are strongly desired in the food industry. However, it is worth noting that the high content of lectin is retained after the protein extraction process, which could induce allergic reactions and reduce food safety [17]. Therefore, to promote a wider application of black kidney bean protein, proper technology is needed to enhance functionality and safety.

In this study, black kidney bean protein isolates (BKBPI) were extracted, and a novel PEGylation method was applied using PEG-SC, PEG-SPA, and PEG-SS conjugation. The PEGylated proteins were investigated by PEGylation degree test, ^{1}H nuclear magnetic resonance (NMR) spectroscopy, and Fourier transform infrared spectroscopy (FTIR). The effects of PEGylation on solubility, water and fat-holding capacities, emulsifying and foaming activities, thermodynamic stability, *in vitro* digestibility, and hemagglutination activity were also investigated.

2. Materials and methods

2.1. Materials

Dried black kidney beans (*Phaseolus vulgaris* L.) were purchased from a local supermarket in Hefei, Anhui Province, China. mPEG-SC (5 kDa), mPEG-SPA (5 kDa) and mPEG-SS (5 kDa) were purchased from YareBio Biotech, Inc. (Shanghai, China). Pepsin (1:10000) and pancreatin (1:250) were purchased from Sigma Aldrich (St. Louis, Missouri, USA). A 2% (v/v) erythrocyte suspension was purchased from Hong Quan Biotech, Inc. (Guangzhou, China). Precast gels (4–15%) used for the SDS-PAGE analysis were obtained from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). All of the other reagents and chemicals were of analytical grade.

2.2. Preparation of black kidney bean protein isolate

The beans were ground to flour and degreased by petroleum ether, followed by dispersion in de-ionized water (1:10, w/v), and the pH adjusted to 7.0 with 2 M NaOH. After stirring for 6 h, the suspension was centrifuged at 8000 tr/min for 30 min and the precipitates were obtained by adjusting pH to 4.5 with 2 M HCl and centrifugation at 5000 r/min for 30 min. Then, the protein was re-suspended in water and adjusted to pH 7.0 with 2 M NaOH. And the protein solution was subsequently lyophilized for the protein isolate analysis.

2.3. Conjugation of black kidney bean protein isolate with mPEG

Black kidney bean protein isolate was dissolved in phosphate buffer (10 mM, pH 7.0) at a concentration of 10 mg/mL using a magnetic stirrer to avoid the formation of air bubbles or foam. mPEG-SC, mPEG-SPA, or mPEG-SS was slowly added to the protein isolate solution to a final molar ratio of 1:10. The conjugation reaction was conducted for 8 h at room temperature and was then dialyzed using a dialysis bag (MWCO 8000) against deionized water for 2 days to clear the free PEG derivatives. The dialysate was lyophilized, and the obtained PEGylated protein isolate powders were stored in sealed vials at −20 °C.

2.4. PEGylation degree determination

The PEGylation degree was determined according to the amino modification rate test as described with slight modifications [18]. Sodium 2,4,6-trinitrobenzene-sulfonate (TNBS, 25 μL) was added to 1 mL of the sample solution (1 mg/mL), and the mixture was incubated for 2 h at room temperature. The absorbance at 420 nm was recorded with native BKBPI as the negative control. The degree of PEGylation (PD) was expressed as:

$$PD\,(\%) = \left(1 - \frac{A_1}{A_0}\right) \times 100\% \quad (1)$$

where A_1 is the absorbance of PEGylated BKBPI, A_0 is the absorbance of native BKBPI.

2.5. ^{1}H MNR spectroscopy measurement

The conjugations of PEG modifiers and BKBPI were confirmed by the ^{1}H MNR analysis (AVANCE II 400 MHz NMR Spectrometer, Bruker Co., Switzerland). Fifteen milligrams of each dry sample was dissolved in D_2O (99.9%, 1.0 mL), and 500 μL was injected into the NMR tube for the a full spectrum (0–10 ppm) determination.

2.6. FTIR spectroscopy analysis

FTIR measurements were performed using a BioRad FTS-60A FTIR spectrometer (Bio-Rad Laboratories, Richmond, CA, USA). The samples were measured in solid form at 25 °C. To enhance the signal to noise ratio, 32 scans were taken with a resolution of 4 cm^{-1} over a wavelength range from 500 to 4000 cm^{-1}.

2.7. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis

SDS-PAGE was conducted using 4–15% precast gradient gels (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Samples were prepared by mixing proteins with loading dye (2.0 mL glycerol, 1.6 mL 10% SDS, 0.8 mL β-mercap-toethanol and 0.4 mL 1% bromophenol blue) at 1:1 (v/v) ratio. After heating at 100 °C for 5 min, the samples were centrifuged for 1 min. Then, 20 μL of each sample was loaded in each well of the gel, and run for 75 min at a constant