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Radio-synthesized protein-based nanoparticles for biomedical purposes

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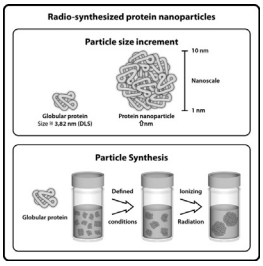
HIGHLIGHTS

- Novel technique for the development of protein nanoparticles using γ-irradiation.
- Size control of papain particles with preserved conformation and bioactivity.
- Alternative method for controlled protein crosslinking.
- Bioactive protein nanoparticles of biotechnological and clinical interest.
- Protein-based drug carrier potential of biotechnological and clinical interest.

GRAPHICAL ABSTRACT



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ABSTRACT

Protein-crosslinking whether done by enzymatic or chemically induced pathways increases the overall stability of proteins. In the continuous search for alternative routes for protein stabilization we report a novel technique – radio-induced synthesis of protein nanoparticles – to achieve size controlled particles with preserved bioactivity. Papain was used as model enzyme and the samples were irradiated at 10 kGy in a gammacell irradiator in phosphate buffer (pH=7.0) and additives such as ethanol (0–40%) and sodium chloride (0–25%). The structural rearrangement caused by irradiation under defined conditions led to an increase in papain particle size as a function of the additive and its concentration. These changes occur due to intermolecular bindings, of covalent nature, possibly involving the aromatic amino acids. Ethanol held major effects over papain particle size and particle size distribution if compared to sodium chloride. The particles presented relative retained bioactivity and the physic-chemical characterization revealed similar fluorescence spectra indicating preserved conformation. Differences in fluorescence units were observed according to the additive and its concentration, as a result of protein content changes. Therefore, under optimized conditions, the developed technique may be applied for enzyme nanoparticles formation of controllable size and preserved bioactivity.

1. Introduction

Globular proteins are complex structures that occur in specific microenvironments which makes these molecules sensitive to physico-chemical changes and unstable when exposed to

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non-natural millieu, triggering degradation mechanisms like denaturation and unfolding. As a consequence, protein formulation in pharmaceutics remains a challenging task.

A few classical techniques may be applied in order to provide a controllable biological decay during formulation stages and storage. Immobilization is a common tecnique (Sheldon, 2007) although imposed mobility restrictions combined with chemicals sensitivity often lead to low residual activity and possible toxic residual content. The use of additives, such as sugars (Arakawa and Timasheff, 1982) stabilizes the structures by weak interactions or indirect effects, that combined or not, increase protein stability to a time limited extent. In this work we report the use of radiation-induced protein crosslinking, as a novel tool to achieve biologically-active enzyme nanoparticles with controllable particle size.

Papain, a proteolytic enzyme (EC 3.4.22.2) of biotechnological and biomedical relevance was selected for this study. Stability issues in non-natural environments make common available pharmaceutical forms – solid, semi-solid and liquid – inadequate for papain formulation. In solution, natural unfolding, oxidation and hydrolysis correspond to major degradation mechanisms (Kamphuis et al., 1984; Govardhan and Abeles, 1996).

Protein crosslinking offers advantages over many immobilization processes since it can be done using chemical agents, enzymes (Fernández-Lafuente et al., 1995; Govardhan, 1999), or the molecule itself, often preserving flexibility and conformation. The use of chemical agents may lead to toxic or undesirable reactions if the compound is not properly removed. Crosslinking reached by enzymatic route, on the other hand, is not associated to the production of toxic compounds, although it is time consuming and highly especific, being limited to enzyme specifity for a determined subtrate or amino acid sequence (Matheis and Whitaker, 1987).

Seeking for alternative routes for development of stable papain particles, we evaluated the use of ionizing radiation to achieve nanostructured enzyme particles, by means of radiation-induced protein crosslinking. The irradiation effects over protein solution are mainly attributed to indirect effects, particularly, concerning the generation of radicals as a result of water radiolysis, commonly related to chain scission and conformational changes which can alter the protein structure and induce degradation as a consequence (Davies, 1987; Saha et al., 1995). Such effects rely upon radiation source, dose, dose rate and the irradiation atmosphere.

The use of radiation to control the nanostructure of peptides and proteins was demonstrated by Furusawa et al. (2004), where nanometer-sized gelatin particles were produced and by Akiyama et al. (2007) that synthesized protein nanogels. Recently, the synthesis of radio-induced nanoparticles using more complex protein structures such as globular proteins, like bovine serum albumin was demonstrated by Soto Espinoza et al. (2012).

This article reports the development of radio-induced papain nanoparticles for biomedical and biotechnological applications, demonstrating the use of ionizing radiation for particle size control of protein structures with preserved bioactivity.

2. Experimental

2.1. Materials

Papain (30,000 USP mL^{-1}) and ethylenediaminetetraacetic acid were purchased from Merck (Germany); L-cysteine hydrochloride monohydrate, Dimetilsulfoxide, sodium chloride, ethanol, Sodium hydroxide, Chloridric acid, Acetic acid and Heptahydrate disodium phosphate from Synth (Brazil), and Nα-Benzoyl-DL-arginine p-nitroanilide hydrochloride from Sigma-Aldrich® (Brazil). All reagents were of analytical grade.

2.2. Methods

2.2.1. Particle synthesis

Phosphate buffer, papain solution and cosolvent were added dropwise to glass vials on ice bath and allowed to stabilize overnight prior to the beginning of experiments at 4 °C. Samples were exposed to gamma radiation at 8 °C and dose rate 1.2 kGy h^{-1} using ^{60}Co as radioactive source in a gamma cell 220 Irradiator. The samples were properly filtered using 0.22 μm filters and stored at 4 °C prior to analysis. Controls were prepared under the same conditions.

2.2.2. Effect of co-solvents/additives

Ethanol was used as cosolvent in the concentration range of 0–40%(v/v). Sodium Chloride was added in the concentration range of 0–25%(w/v).

2.2.3. Particle characterization

Tryptophan fluorescence was determined using λ_{Ex}=280 nm/λ_{Em}=300–400 nm on a Hitachi F-4500 fluorescence spectrophotometer. Particle size measurements were performed by Dynamic Light Scattering analysis (DLS) using a Zetasizer Nano SZ90 device at 25 °C using dual scattering angles – 13° and 90°, in triplicates of 3 runs of 60 s each. Enzymatic activity was determined by spectrophotometry analysis (λ=405)using Nα-Benzoyl-DL-arginine p-nitroanilide hydrochloride as substrate at 40 °C, pH 7.0.

2.2.4. Computational studies

Solvent acessible surface area was calculated using the program Volume, Area, Dihedral Angle Reporter –VADAR (Willard et al., 2003) considering Van der Waals radii from Shrake and Rupley (1973). Graphical representation was performed using Jmol ***v. 12.0.4.1.***. All calculations were performed with files available at the Protein Data Base (PDB) – (**9pap – 1.65 Å**).

3. Results and discussion

3.1. Effect of ethanol

The use of ethanol as a co-solvent led to shifts in papain particle size and particle size distribution after irradiation at 10 kGy. Distinct sizes were achieved with particle size ranging from 5.3 ± 0.5 (control) to 11.0 ± 0.3 nm (20% ethanol), and larger structures from 117.9 ± 9.0 (10% ethanol) to 910.8 ± 10.4 nm (40% ethanol) as a function of ethanol concentration (Table 1). At 10% ethanol minor changes were observed, while above 30% ethanol particle distribution shifted to large structures with monomodal distribution ranging from 703.5 ± 51.8 to 910.8 ± 10.4 nm, for 30–40% ethanol respectively, indicating that the induced agglomeration was too intense and therefore inadequate for the purpose. Regarding small particles, the system achived highest particle size at 20% ethanol.

The changes in particle size are a result of the irradiation under specific microenvironment that induces a structural disposition of the protein molecules in the system, suitable for the nanostructuring. The effects of such modifications were also evaluated in terms of bioactivity, since conformational changes and the irradiation process itself may induce protein degradation and decreased bioativity. The bioactivity profile of the samples is showed in Fig. 1.

Nanoparticles synthesis using ethanol showed relative activity ranging from 97 to 34% compared to papain irradiated in buffer. Particularly at higher ethanol concentrations, up to 30%, bioactivity