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Radio-synthesized protein-based nanoparticles for biomedical purposes

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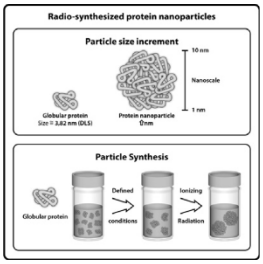
HIGHLIGHTS

- 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

decay is more pronounced making the process inviable under such conditions, considering the purpose of developing bioactive nanoparticles. As observed in the DLS measurements, at 10% ethanol minimum changes were observed and at 20% the remaining bioactivity was established around 74%, thus being suitable for our purpose.

The use of ethanol combines the protein aggregation, essential for the involved process and radioprotection due to its intrinsic scavenger property, capturing specific radicals generated by water radiolysis, known to cause a deep impact on protein integrity.

In terms of preserving protein structure from radio-induced denaturation, the results (Fig. 2) indicated a preservative effect using ethanol concentrations ranging from 10–35%. At 40% ethanol concentration, fluorescence units decreased significantly with values below positive control and highlighted a destabilizing effect rather than a protective one. No formation of new peaks nor conformational changes were observed for the samples, only changes in terms of fluorescence intensity were identified, highlighting that protein structure is preserved after the process.

Table 1
Mean papain particle size and size distribution (DLS) as a function of ethanol concentrations.

Ethanol (%)	Particle size					
	Peak 1		Peak 2		Peak 3	
	Diameter (nm)	*SD*	Diameter (nm)	*SD*	Diameter (nm)	*SD*
0	5.08	0.03	69.68	10.16	401.5	9.12
0 irradiated	5.30	0.46	15.94	9.77	172.06	9.01
10	5.55	0.19	–	–	117.86	21.22
20	2.42	0.59	11.05	0.29	202.1	10.06
30	–	–	–	–	703.53	51.79
35	–	–	–	–	730.3	66.80
40	–	–	–	–	910.83	10.36

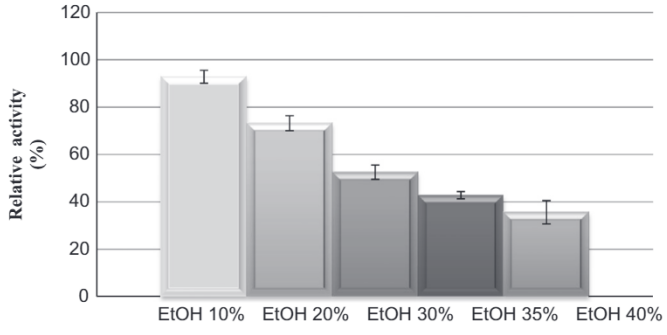

Fig. 1. Relative bioactivity of the particles as a function of ethanol concentration.

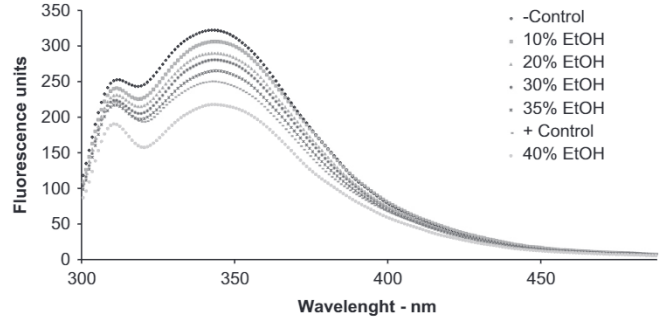

Fig. 2. Fluorescence spectra of papain samples as a function of ethanol concentration 0–40%; (Negative control -Non irradiated without ethanol; Positive control – Irradiated without ethanol).

3.2. *Effect of sodium chloride*

In presence of sodium chloride smaller particles of aproximately 5.3 ± 0.05 nm shifted to 7.5 ± 0.4 nm and larger particles from 164.7 ± 9.0 up to 638.0 ± 31.7 nm were achieved. Similar particle size distribution was observed for the samples indicating that this additive was not capable of altering this parameter (Table 2).

The effects of sodium chloride concentration over particle size was limited if compared to ethanol, since the results revealed less effects over particle size. Specifically, at 5% sodium chloride minor changes were observed. As concentration increased up to 15% particles size increased. At 20% sodium chloride small nanoparticles diminish their size and large structures became bigger. The activity changes observed for sodium chloride as a function of concentration indicated no protective effects of the use of such additive for irradiation, since the bioactivity profiles were the same for the samples containig distinct concentrations of this compound (Fig. 3).

The fluorescence spectra (Fig. 4) indicated similar profiles, however, the microenvironment around the aromatic residues changed resulting in higher quantum yield instead of a decrease in fluorescence units as observed for ethanol. No new peaks nor relevant shifts in the spectra were identified.

The concept of using different co-solvents concentrations did influence nanoparticle formation and was effective in terms of allowing changes in size of the developed systems. Due to intrinsic limitations of the DLS, the analysis were performed using two different angles, 90°and 13°, in order to assure precise measurements with low deviation and error.

Similar changes in terms of particle size were observed if compared to the non-irradited samples in presence of the additive or cosolvent. In both cases the so-called aglomeration process takes place and by definition the nature of the binding forces involved that promote intermolecular association involved are mainly physical (Pappu et al., 2008) and under usual conditions, solvent changes, such as dilution tends to revert the process (Chi et al., 2003). Corroborating the literature, after dilution the irradiated samples did not suffer any changes in particle size, indicating that the forces involved in this case are more stable, possibly of covalent nature, and highlighting the formation of intermolecular crosslinking.

Table 2
Mean papain particle size and size distribution (DLS) as a function of sodium chloride concentrations.

Sodium Chloride (%)	Particle size					
	Peak 1		Peak 2		Peak 3	
	Diameter (nm)	*SD*	Diameter (nm)	*SD*	Diameter (nm)	*SD*
0	5.29	0.030	61.81	10.16	401.5	9.12
0 irradiated	5.30	0.46	15.94	9.77	164.66	9.01
5	5.50	0.57	1.37	0.34	584.43	103.39
10	6.77	0.25	65.11	29.11	212.9	51.137
15	7.47	0.40	69.72	13.04	540.53	105.66
20	7.27	0.31	25.63	12.40	638.9	31.68

3.3. Mecanism approach

The exact mechanism by which crosslinking takes places by the use of radiation is still unclear, however size variation (Tables 1 and 2) and the formation of insoluble particles provided an evidence of the occurrence of radiation-induced crosslinking.

The irradiation of samples in presence of cysteine led to no detectable changes by DLS as achieved in absence of this amino acid (data not shown). This demonstrated the essential role of radicals generated, specifically oxidizing species in the involved crosslinking formation. Further experiments must be done to clarify the involved mechanism.

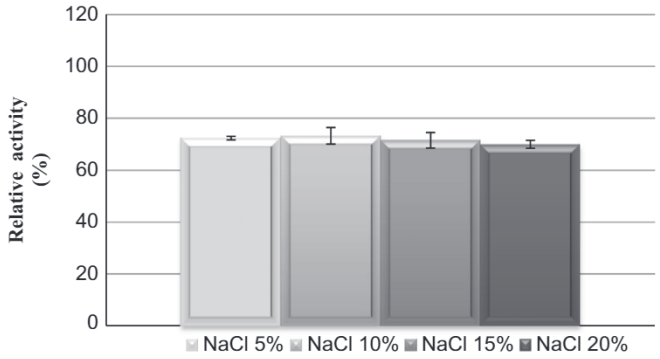

Fig. 3. Relative bioactivity of the particles as a function of sodium chloride concentration.

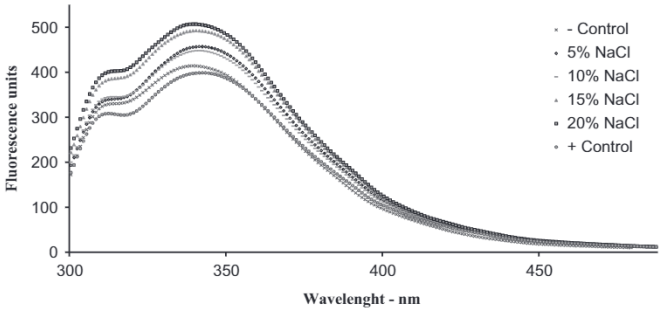

Fig. 4. Fluorescence spectra of papain samples as a function of sodium chloride concentration 0–20%; (Negative control – Non irradiated without additive; Positive control – Irradiated without additive).

According to literature, enzymatic-induced crosslinking is known to lead to tyrosine residues attachment, such as di and tri-tyrosines formation, depending upon possibility of contact between tyrosine residues (Bailey, 1991). Previous work describes tyrosine cross-links due to the action of free radical formed by UV irradiation (Garcia-Castinaras et al., 1978).

In order to provide a theoretical background to this hypothesis, we evaluated the solvent acessibility of tyrosine residues located in papain 3D structure. Out of 19 tyrosine residues present in papain amino acid sequence, 10 of them were solvent acessible with side-surface values ranging from 58.1 to 150.9 $Å^2$, considering that such values increase as accessibility increases. This information strongly supports the possibility of crosslinking involving these specific residues of distinct papain molecules. Tyrosine solvent accessibility profile is described in Fig. 5.

From a technological point of view the technique seems to be promising for the development of protein based nanoparticles for biomedical applications using globular proteins. In this particular work, enzymes with retained activity. Possible drawbacks of the technique could be attributed to the low yield of the process, established as around 30–40% even though the enzymatic activity of the produced particles was managed to be kept around 69–70%, if compared to the control samples. Such points can be overcome by possible balanced overall performance and high operational stability conferred due to biochemical and biological changes as a result of the process. Further studies should be performed in order to provide detailed data of such modifications and the new stability profile conferred.

At last, regarding the application of large structures, the idea of developing more stable structures with high bioactivity seemed remote due to high additive content required and the low residual activity observed as a consequence. However, the use of such nanomaterials as protein-based drug carrier remains a possible application to be explored, considering the biocompatibility of the system by its nature.

4. Conclusions

The technique was suitable for the synthesis of papain nanoparticles with controllable size distribution and retained biological activity. Particle size and activity varied as a function of precipitant agent used in the process, even though both agents were effective in terms of providing a microenvironment for particle-size increase. Ethanol was able to lead to larger particle formation,

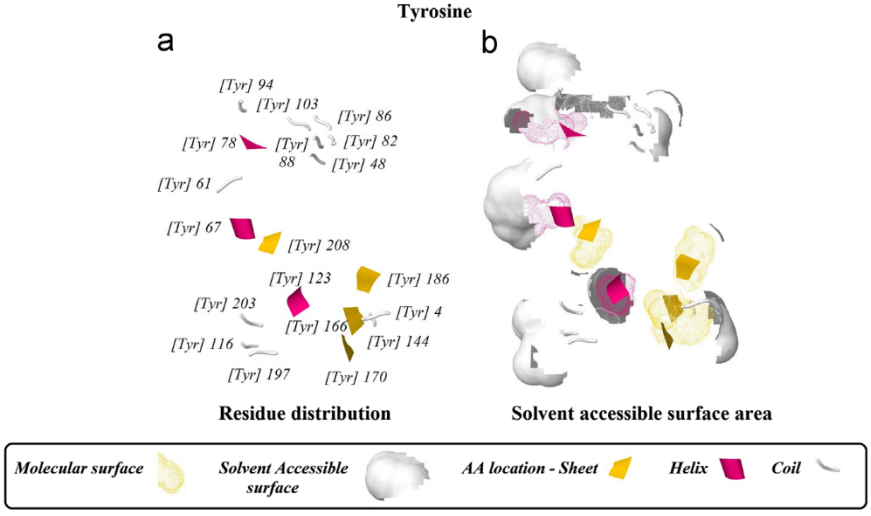

Fig. 5. (a) Tyrosine distribution in papain structure (frontal view); (b). Solvent accessibility of tyrosine amino acids present in papain structure.

while sodium chloride led to minor changes. Ethanol was also capable of changing papain particle size distribution. This event was not observed in presence of sodium chloride.

Computational studies confirmed that many tyrosines present in papain structure are solvent accessible, providing in-silico evidence of the possibility of such residues to be involved in the proposed mechanism, on a structural based approach. The enzymatic activity results indicated relative biological decay, altough bioactivity was less affected under optimized conditions. Physicochemical characterization revealed preserved protein conformation, thus highlighting the potential of the produced particle as bioactive nanoparticles for biomedical or biotechnological purpose.

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