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Synthesis of papain nanoparticles by electron beam irradiation – A pathway for controlled enzyme crosslinking

G.H.C. Varca [a,*], S. Kadlubowski [b,*], M. Wolszczak [b], A.B. Lugão [a], J.M. Rosiak [b], P. Ulanski [b]

[a] Instituto de Pesquisas Energéticas e Nucleares (IPEN/CNEN-SP) – Av. Prof. Lineu Prestes, 2242, Cidade Universitária, 05508-000 São Paulo, SP, Brazil
[b] Institute of Applied Radiation Chemistry, Lodz University of Technology, Wroblewskiego 15, 93-590, Lodz, Poland

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ABSTRACT

Crosslinked enzyme aggregates comprise more stable and highly concentrated enzymatic preparations of current biotechnological and biomedical relevance. This work reports the development of crosslinked nanosized papain aggregates using electron beam irradiation as an alternative route for controlled enzyme crosslinking. The nanoparticles were synthesized in phosphate buffer using various ethanol concentrations and electron beam irradiation doses. Particle size increase was monitored using dynamic light scattering. The crosslinking formation by means of bityrosine linkages were measured by fluorescence spectra and the enzymatic activity was monitored using Na-Benzoyl-DL-arginine *p*-nitroanilide hydrochloride as a substrate. The process led to crosslinked papain nanoparticles with controlled sizes ranging from 6 to 11 nm depending upon the dose and ethanol concentration. The irradiation atmosphere played an important role in the final bioactivity of the nanoparticles, whereas argon and nitrous oxide saturated systems were more effective than at atmospheric conditions in terms of preserving papain enzymatic activity. Highlighted advantages of the technique include the lack of monomers and crosslinking agents, quick processing with reduced bioactivity changes, and the possibility to be performed inside the final package simultaneously with sterilization.

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1. Introduction

The concept of producing protein-based nanoparticles is a theme of constant research [1,2]. Besides biopharmaceutical advantages of nanoparticulate systems [3], the main benefits of using nanostructured proteins is attributed to reduced toxicity and allergenic effects, high biocompatibility, specificity and site specific delivery [1,4,5].

Diverse techniques have been developed over the last decades in an attempt to produce stable and low-cost proteins for biotechnological and biomedical applications. Examples of commercially available protein-based drug systems include, but are not limited to, the delivery of chemotherapy agents for tumour or anticancer therapies, such as albumin bound paclitaxel [6] or crosslinked enzyme aggregates for biotechnological applications such as biocatalysts [7]. Particularly for enzymes, the former technology stands as a promising approach that was recently developed [8] and quickly upgraded.

Papain (Fig. 1) is a cysteine protease of vegetal source that has been extensively investigated with many industrial and biomedical applications [9]. Its molecule is composed of 212 amino acids divided into two domains whereas the active site is composed by a triad of amino acids – Cysteine, Histidine, and Aspartic acid [10], with a total molecular weight of 23.500 kDa [11].

Despite its broad endopeptidase, aminopeptidase, dipeptidyl peptidases and *exo*- and *endo*-peptidase activity, this enzyme is particularly stable at a broad pH range and high temperatures if compared to other enzymes [9,10]. However, in aqueous media its enzymatic activity is dramatically affected over time as a result of the oxidation of the free cysteine (Cys-25) present in its active site or/and a natural unfolding mechanism [10].

The use of a technique for the production of crosslinked enzyme aggregate by a carrier-bound free immobilization method to stabilize papain has already been demonstrated, proven effective by different researchers [12,13] and is under current commercialization. Alternatives to achieve protein particles including papain, ranging from micro [14] to nanoscale [15–17] abound in literature,

* Corresponding authors.
E-mail addresses: varca@usp.br (G.H.C. Varca), slawekka@mitr.p.lodz.pl (S. Kadlubowski).

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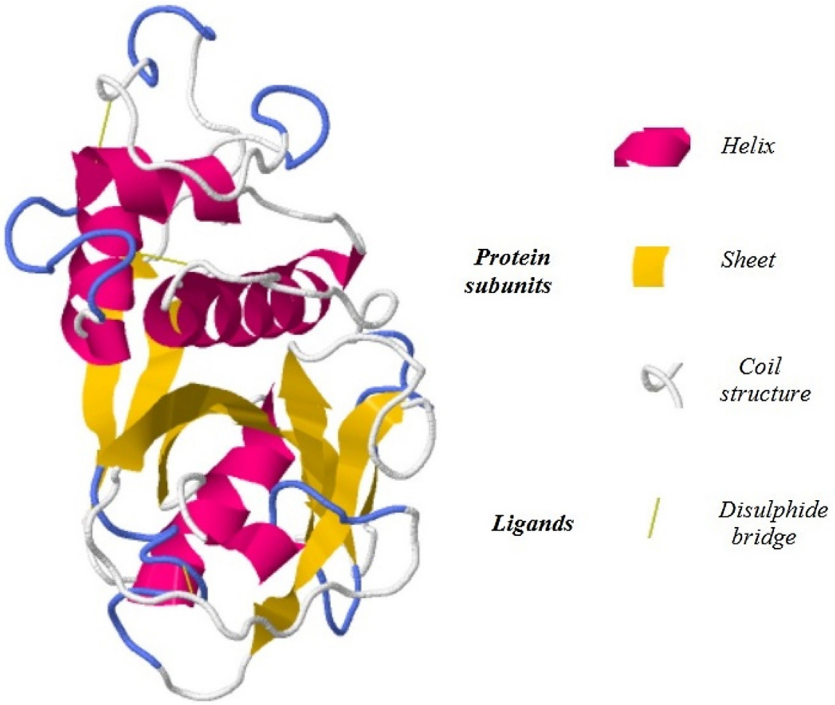

Fig. 1. Papain molecule at 1.95 Å resolution[11] (***9pap.pdb***).

whether combined with polymers [18] or by the use of bifunctional crosslinkers [19].

The synthesis of papain nanoparticles by the use of gamma irradiation has been recently described and patented [20–22]. Initially irradiation attacks the solvent molecules leading to the production of reactive species, mainly $^{\bullet}OH$ and e^{-}_{aq} as a result of water radiolysis. In subsequent steps, such cosolvent species react with protein amino acids, forming reactive centres along protein molecules, which upon certain conditions, further react to yield crosslinking [23,24].

Main advances of the technique are related to the development of size-controlled, protein-based nanoparticles with preserved bioactivity, without the need of monomers nor crosslinkers, and the possibility to allow in-situ sterilization simultaneously inside the final package. In a more specific way, under a proper microenvironment achieved by the use of cosolvents such as ethanol. Gamma-irradiation was capable of crosslinking papain nanoparticles [20], which occurred as a function of the controlled intermolecular formation of bityrosines [21]. The process itself was proved effective at a narrow papain concentration range [22].

Herein we describe the use of electron beam irradiation to achieve crosslinked nanosized papain aggregates/nanoparticles as a very quick and alternative technique for controlled enzyme crosslinking with preserved biological activity without the need of monomers or crosslinkers. In order to demonstrate this approach, we produced papain crosslinked enzyme aggregates (CLEA) using electron beam irradiation and characterized the nanoparticles by means of particle size and bityrosine formation as a function of ethanol concentration, electron beam irradiation dose and irradiation atmosphere. In addition to the absence of toxicity generated by the use of crosslinkers in conventional methods and alternatively to the use of gamma irradiation for crosslinking [23,24], electron beam irradiation does not require a radioactive source, radiation may be switched off and allows the delivery of high doses in a very short period of time inside the final package, while featuring the same properties from a mechanistic point of view.

2. Experimental

2.1. Nanoparticle synthesis

2.1.1. Sample preparation

Papain 30.000 USP-U/mg (EC 3.4.22.2) from Merck® (Germany) was solubilized in phosphate buffer (PBS) 50 mM at pH 7. Appropriate aliquots of papain solution, PBS and ethanol reagent grade from Sigma-Aldrich® (USA) were gently added to glass vials on ice bath to reach final papain concentration of 12.5 mg mL^{-1} and ethanol content ranging from 0 to 40% (v/v) to be hermetically sealed and the allowed to stabilize overnight in the refrigerator. The samples were then submitted to irradiation.

2.1.2. Electron beam irradiation

Electron beam irradiation was performed on a Linac ELU-6e irradiator (Elektronika, Russia). The following parameters were applied: pulse length 4 μs, pulse repetition 20 Hz, dose rate of 6.8 kGy min^{-1} and final doses of 2.5, 5, 7.5 and 10 kGy as determined by Alanine dosimetry [25] on a e-Scan™ (Bruker Corporation, Germany). The samples were then properly filtered using 0.45 μm cellulose acetate syringe filters and stored at 4 °C prior to analysis. Controls were prepared and handled under the same conditions.

2.1.3. Effect of gas saturation

The selected samples containing 20% (v/v) ethanol concentration were purged with argon (Ar) and nitrous oxide (N_2O) for 15 min, hermetically sealed and submitted to irradiation. Controls were prepared under atmospheric conditions.

2.2. Nanoparticle characterization

2.2.1. Particle size characterization

The samples were filtered using 0.45 μm cellulose acetate syringe and characterized according to size by Dynamic Light Scattering on a Zetasizer Nano ZS90 (Malvern Instruments GmbH,

Germany) device at 20 °C using backscatter angle (173°) in triplicates of 3 runs of 12 s each.

2.2.2. Protein crosslinking

The formation of bityrosines was monitored by fluorescence measurements on a F-4500 fluorescence spectrophotometer (Hitachi Co, Japan) according to the following parameters: λex = 325 nm and λem = 340–500 nm, scan speed of 240 nm/min, Exslit of 5 nm and Emslit of 5 nm [21,26,27]. The samples were diluted in buffer to reach equivalent protein content (λ = 280 nm) prior to analysis. The spectra were normalized by considering the maximum values registered for native papain in PBS solution as a reference value.

2.2.3. Enzymatic activity

Papain bioactivity was determined by quantifying proteolytic activity over Nα-Benzoyl-DL-arginine *p*-nitroanilide hydrochloride (Sigma-Aldrich®, USA) as synthetic substrate [28,29] at 40 °C buffer pH 7. The product was estimated by spectrophotometry analysis (λ=405 nm) on a Synergy HT microplate reader (Biotek Instruments, USA).

3. Results

3.1. Papain nanoparticles synthesized by electron beam irradiation

3.1.1. Effect of electron beam irradiation over papain particle size

The use of ethanol combined with electron beam irradiation resulted in an increase of papain size as a function of ethanol concentration (Fig. 2A), as determined by dynamic light scattering (DLS).

The combination of papain aggregation induced by ethanol followed by crosslinking by electron beam irradiation led to the formation of papain nanoparticles. Native papain irradiated at 10 kGy presented mean particle size of about 5 nm, whereas particle size increased up to approximately 11 nm as ethanol concentration increased up to 40% (w/w), when combined with irradiation. To screen out the best dose for the applied technique, a 12.5 mg mL^{-1} papain containing buffer solution (50 mM) in presence of 20% ethanol (v/v) was irradiated at doses ranging from 2.5 to 10 kGy and characterized according to particle size (Fig. 2B).

Table 1
Effect of gas saturation over particle size.

Atmosphere	Size *(d.nm)*	STD
Atmospheric O_2	8.3	0.5
Argon	8.3	0.5
N_2O	8.1	0.6

*Papain irradiated at 10 kGy in presence of 20% ethanol (v/v).

3.1.2. Nanoparticle enzymatic activity

Upon 10 kGy electron beam irradiation at the dose rate of 6.8 kGy min^{-1} the effects of ethanol over papain enzymatic activity revealed a decrease in bioactivity in the range of 10–50% approximately, as ethanol concentration increased (Fig. 3). The biological activity of the nanoparticles was determined. At a fixed ethanol concentration of 20% (v/v), by changing the irradiation dose, papain biological activity decreased proportionally to the absorbed dose (Fig. 3A). To be more specific, the use of ethanol at 20% induced a minimum biological decay of about 7% for the non-irradiated sample. Upon irradiation at 2.5 kGy bioactivity decreased approximately 14%, while at 5 kGy these values were around 22%, and 25% and 27% for 7.5 and 10 kGy respectively.

3.1.3. Effect of gas saturation

With regard to size, no significant changes were registered as a function of the gas used to saturate the solutions prior to irradiation, including Ar and N_2O as revealed in Table 1. This data indicates that the atmosphere does not influence the nanoparticle formation itself from a size perspective.

On the other hand, biological activity profile (Fig. 4), revealed that bioactivity decreased in order of $N_2O \geq Ar > ATM$ conditions. The difference between the N_2O and argon saturated atmosphere by means of bioactivity was not relevant, established as 1.75 ± 0.35%. However, if compared to the samples prepared and irradiated in presence of atmospheric oxygen, the difference reached around 18.5 ± 4.9.

3.1.3. Nanoparticle crosslinking

Protein crosslinking was evaluated by means of bityrosine formation as a function of ethanol concentration (Fig. 5A) and irradiation dose (Fig. 5B) over papain.

The effects of irradiation dose over bityrosine formation were also assayed in order to achieve the best conditions for the nanopar-

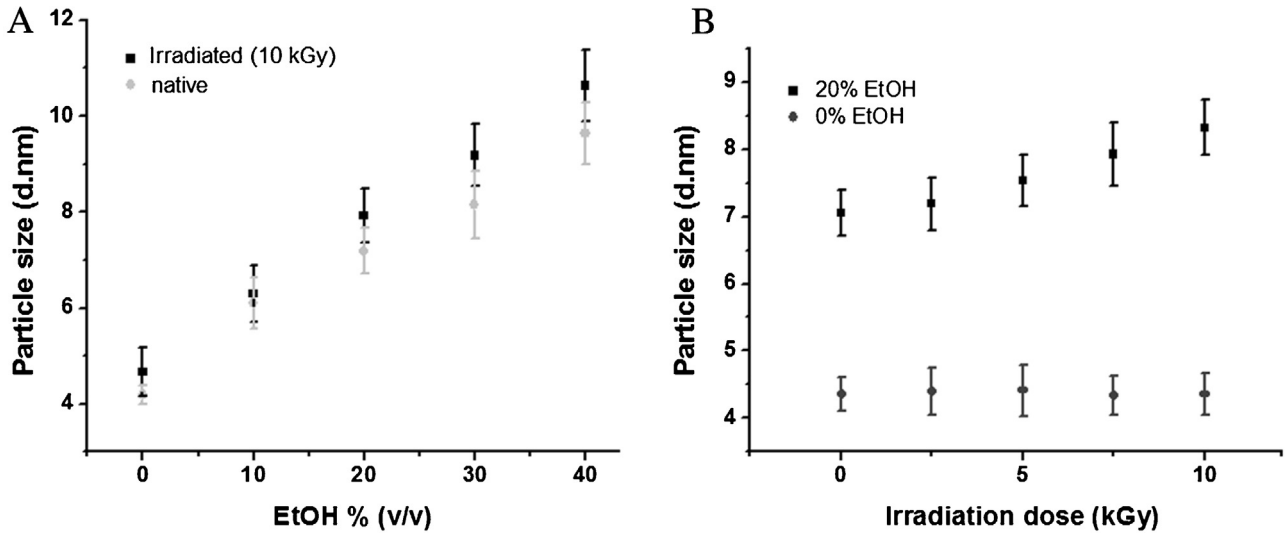

Fig. 2. Effect of ethanol concentration (0–40% v/v) over papain particle size at 10 kGy (A); Electron beam irradiation dose (2.5–10 kGy) over papain particle size in presence and absence of ethanol (20% v/v) (B).

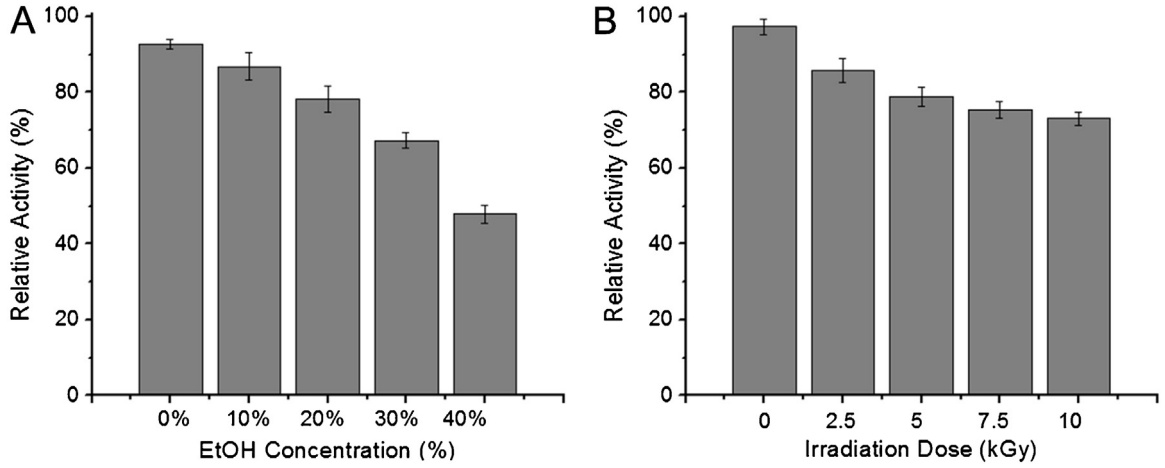

Fig. 3. Effect of ethanol concentration (0–40% v/v) upon electron beam irradiation at 10 kGy and dose rate of 6.8 kGy min^{-1} (A); Effect of electron beam irradiation dose (0–10 kGy) over papain activity at 20% EtOH (B). *Native papain activity was taken as 100%.

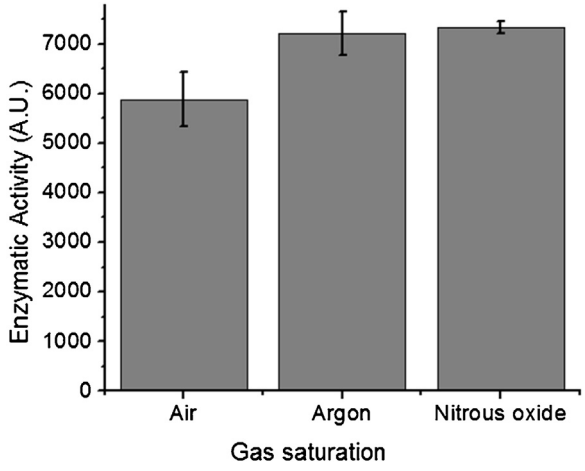

Fig. 4. Effect of gas saturation over biological activity of the papain nanoparticles. Electron beam irradiation of papain at the dose of 10 kGy in air, argon and nitrous oxide saturated aqueous solution (12.5 mg mL^{-1}) in presence of 20% ethanol (v/v). *Native papain activity was considered as 100%.

ticle formation and were presented in Fig. 5. As irradiation dose increased these linkages also increased. Particularly, higher intensity was observed at 10 kGy, even though minor changes were observed between irradiation doses of 5–10 kGy.

4. Discussion

The use of ethanol in combination with electron beam irradiation was suitable for the formation of papain nanoparticles. The technique implies on the ability of the solvent to change protein particle size, by means of protein desolvation, maintained by field or secondary forces [30,31], and the use of radiation to provide crosslinking, with some additional increase in size as a result. In a more specific way, the addition of ethanol plays an important role in the process as it is responsible for creating a milieu that induces suitable proximity of the protein particles to preferably recombine intermolecularly rather than intramolecularly. Papain particles size increase was directly related to ethanol concentration.

Ethanol has been particularly useful and effective to promote protein desolvation, as it is capable of altering the solvation layer around protein molecules, which leads to changes in particle size as a function of concentration, and crosslinking is applied to provide chemical linkages along the nanoparticle, in order to preserved the acquired rearrangements, rather than physical effects exclusively. The combination of solvation followed by high energy crosslinking has been successfully applied for the synthesis of BSA nanoparticles in recent works [23,24,32], and papain nanoparticles [21,22] exclusively by the use of gamma irradiation.

Regarding the influence of the irradiation dose over papain particle size, the results revealed that native papain did not undergo size changes as function of the applied irradiation doses. This experimental evidence allowed the conclusion that the desolvation effects promoted by ethanol were essential for the nanoparticle formation. In presence of ethanol major changes took place at 2.5 kGy, and as dose increased up to 10 kGy particle size continued to increase in a minor extent, whereas highest particle sizes were achieved at the dose of 10 kGy (Fig. 2b). Irradiation dose did not exceed 10 kGy in order to avoid excessive papain degradation as a function of the irradiation process. These results demonstrated that this technique allowed the production of enzyme nanoparticles in a less time-consuming process, capable of being performed in minutes in the final package, simply by applying electron beam irradiation, alternatively to the use of toxic monomers [33] or bifunctional crosslinkers [19].

Another relevant aspect to be considered is related to the efficiency of the process by means of biological activity. Most technological approaches for enzyme modification or immobilization are known to deeply impact enzymatic activity and as a consequence, retained enzymatic activity is usually reduced, which not rarely makes the process unviable. Taking into account a size vs. bioactivity perspective, most suitable conditions were achieved at 20% ethanol (v/v), considering a minor biological decay of approximately 25% and a particle size of about 9 nm (Fig. 3A).

The stability of papain in ethanol has already been established. Although minimum shifts in bioactivity are expected, papain is considered stable in ethanol, as identified by a minimum bioactivity loss of as ethanol concentration increases [34]. In a general perspective organic solvents, including ethanol, may impact enzymatic activity in several ways, whether as a result of the interaction of the solvent molecules with the biocatalyst, or by disrupting the secondary bonds in the native structure, as well as inducing changes over the hydration shell and the overall structure of the enzyme or an interaction with the active site of the biocatalyst, causing inactivation [35].

The effects of irradiation over papain by means of stability and sensitivity have already been studied by [36], which indicated that degradation effects occur during irradiation, with a biological loss as a function of concentration, but no compromise over stabil-

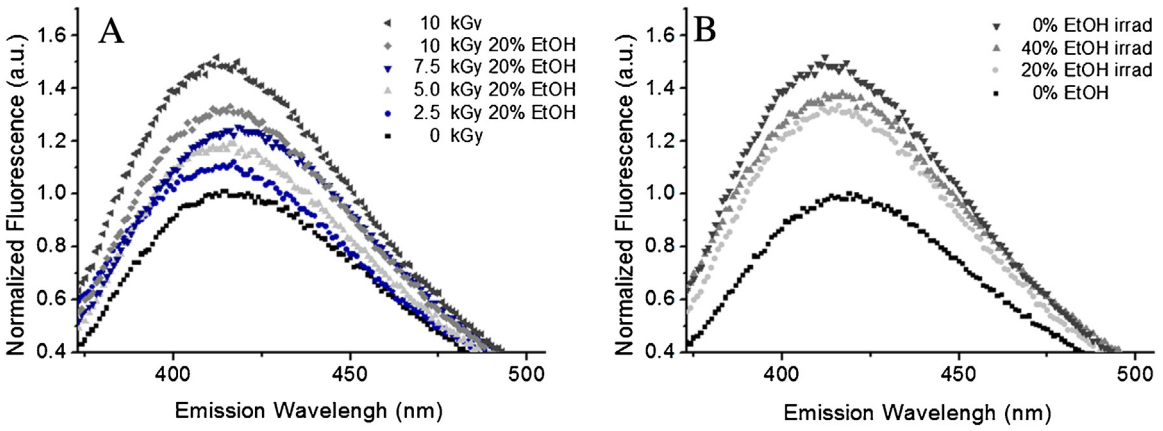

Fig. 5. Bityrosine fluorescence spectra of papain in presence and absence of solvent as a function of irradiation dose (A) and solvent concentration at 10 kGy (B).

ity. In our experiments, at a fixed ethanol concentration of 20% (v/v), by changing the irradiation dose, papain biological activity decreased proportionally to the absorbed dose (Fig. 3B). For our system, potential reasons for the bioactivity decay may result from change of solvent without irradiation as highlighted above, chemical reactions caused by irradiation taking place close to active center, changes in accessibility of active centres during crosslinking and formation of bigger nanoparticles, or even steric implications as a result of protein aggregation.

In order to identify optimum conditions for the nanoparticle synthesis induced by electron beam radiation the effect of gas saturation over particle size was also evaluated. The presence of gases in the systems was expected to play a remarkable role in the process. As an example, the negative effect of oxygen is attributed to the chain scission and oxidation of papain active site, as revealed in literature [37–39]. The motivation for saturating the solution lies on oxygen removal at a first glance, followed by inert properties featured by Ar or N_2O. In addition to that, N_2O holds solvated electron scavenging properties which in principle doubles •OH concentration [40]. Gas purge was performed for 15 min prior to irradiation.

The biological activity profile (Fig. 4) revealed that bioactivity decreased in order of $N_2O \geq Ar >$ ATM conditions. This information provided experimental evidence that the atmosphere does influence the overall bioactivity of papain rather than the particle size formation itself. On this account the process may be optimized if carried out under Argon or N_2O saturated environment. These results corroborated literature data, which indicated that the irradiation of papain in presence of oxygen leads to higher radiation damage if compared to inert atmospheres [37,38]. This information provided experimental evidence that the atmosphere does influence the overall bioactivity of papain rather than the particle size formation itself. Thus the process may be optimized if carried out under Argon or N_2O saturated environment.

In terms of protein crosslinking, the formation of dityrosines (also known as bityrosines) is attributed to one of the main mechanisms observed during enzymatic crosslinking [41] and consists of a well defined strategy to overcome stability limitations of proteins and enzymes [42]. The same bityrosine linkages were identified in protein crosslinking caused by UV irradiation [26]. As an attempt to confirm the role of such linkages in the formation of papain nanoparticles by electron beam irradiation the formation of bityrosines was monitored as a function of ethanol concentration (Fig. 5A) and irradiation dose (Fig. 5B).

Irradiation led to an increase in the signal, as described in literature [27], correspondent to bityrosine linkages, providing an experimental evidence of the mechanism involved in the nanoparticle formation. At 20% ethanol the signal increased and it was even more pronounced as ethanol concentration increased up to 40%. However, only minor differences were observed among these concentrations. This results complied with the slight particle sizes changes observed for the same samples as revealed by DLS.

The effect of ethanol concentration over bityrosine formation was irrelevant (data not shown) and reduced changes in the signal were observed if compared to irradiated papain lacking this solvent. This possibly indicated the formation of crosslinks of distinct nature – intermolecular crosslinks. A similar behavior was observed when applying gamma radiation [21].

The formation of bityrosines by free-radical modification was extensively studied by researchers in the past [43–45]. The main pathways include one-electron oxidation mediated by the formation of tyrosine •OH adduct, which leads to the formation of a bi, or polytyrosines in specific cases [44]. Regarding bityrosine formation the interaction of •OH is known to lead to the formation tyrosil radicals in which depending upon the conditions may recombined intermolecularly. In addition, an attack by reducing species, such as the e^-_{aq} and other e^-_{aq} derived radicals, under proper conditions may form bityrosines via cysteine mediated pathways [43–45].

The results supported our hypothesis that the main mechanism involved in the nanoparticle formation was attributed to the formation of crosslinks involving tyrosine residues. Although fluorescence measurements were not suitable to allow a proper distinction between the nature of such linkages, whether at intra- or intermolecular level, the levels of crosslinking were different if compared to irradiation in presence and absence of ethanol. Taking into account that papain size increase was also different as a function of such parameters, there is an evidence that there the process performed by the addition of ethanol followed by irradiation is whatsoever different than the profile observed by the effect of ethanol, involving mainly physical forces, or irradiation exclusively, experimentally supporting our claim that irradiation in presence of ethanol, lead to crosslinking of rather inter- than intramolecular nature.

It is relevant to mention that despite the extensive data to support the mechanism, this particular system still requires a detailed set of experiments in order to clarify the exact radiochemical pathway for the crosslinking, considering that its quite a complex system from a radiation chemistry point of view, e.g. one source of complexity of the system is related to the scavenging properties of OH by ethanol.

5. Conclusion

In summary, the effect of the electron beam irradiation dose over particle size in presence of ethanol indicated that major changes

took place at 2.5 kGy if compared to the other irradiated samples. Beyond this dose, such changes were mild and increased with dose, even though highest particle size was achieved at 10 kGy. From a biological perspective, a more pronounced biological decay was observed as dose increased.

Irradiation in gas saturated systems played an important role in the maintenance of biological activity during the process, where best results were achieved in presence of a non-reactive atmosphere, even though no changes in particle size were observed as a function of altering this experimental condition. Optimized conditions were established as 20% ethanol (v/v), irradiation dose of 10 kGy and N_2O or argon-saturated atmosphere. Thus, the technique was suitable for the development of engineered papain nanoparticles at the nanoscale, an alternative pathway for protein crosslinking in which the final product may be applied as a drug carrier system and bioactive nanoparticle as well.

In conclusion, the use of electron beam irradiation as an alternative to gamma ray was suitable for the synthesis of protein-based nanoparticles – papain CLEAs – with preserved bioactivity. The main advances are related to quick processing and lack of radioactive source. Ethanol was capable of inducing a proper microenvironment for bityrosine crosslinking, of intermolecular nature, which leads to a permanent increase in particle size.

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References

[1] W. Lohcharoenkal, L. Wang, Y.C. Chen, Y. Rojanasakul, Protein nanoparticles as drug delivery carriers for cancer therapy, BioMed. Res. Int. 2014 (180549) (2014).
[2] T. Suma, J. Cui, M. Müllner, Y. Ju, J. Guo, M. Hu, F. Caru, Generalizable strategy for engineering protein particles with pH-triggered disassembly and recoverable protein functionality, ACS Macro Lett. 4 (2015) 160–164.
[3] A.Z. Wilczewska, K. Niemirowicz, K.H. Markiewicz, H. Car, Nanoparticles as drug delivery systems, Pharmacol. Rep. 64 (2012) 1020–1037.
[4] S. Fuchs, C.J. Coester, Protein-based nanoparticles as a drug delivery system: chances risks, perspectives, Drug Del. Sci. Tech. 20 (2010) 331–342.
[5] G. Wang, H. Uludag, Recent developments in nanoparticle-based *drug delivery* and targeting systems with emphasis on protein-based nanoparticles, Expert Opin. Drug Deliv. 5 (2008) 499–515.
[6] W.J. Grandishar, Albumin-bound paclitaxel: a next-generation taxane, Expert Opin. Pharmacother. 7 (2006) 1041–1053.
[7] R.A. Sheldon, Characteristic features and biotechnological applications of cross-linked enzyme aggregattes (CLEAs), Microbiol. Biotechnol. 92 (2011) 467–477.
[8] R.A. Sheldon, R. Schoevaart, L.M. Van Langen, Cross-linked enzyme aggregates (CLEAs): a novel and versatile method for enzyme immobilization (a review), Biocatal. Biotransform. 23 (2005) 141–147.
[9] E. Amri, F. Mamboya, Papain, a plant enzyme of biological importance, Am. J. Biochem. Biotechnol. 8 (2) (2012) 99–104.
[10] I. Kamphuis, K. Kalk, M. Swarte, J.J. Drenth, Structure of papain refined at 1.65 A resolution, Mol. Biol. 179 (1984) 233–256.
[11] M.J. OíNeil, The Merck Index: an Encyclopedia of Chemicals, Drugs, and Biologicals, 13th ed., Merck, Whitehouse Station, NJ, 2001.
[12] M. Wang, C. Jia, W. Qi, Q. Yu, X. Peng, R. Su, Z. He, Porous-CLEAs of papain: application to enzymatic hydrolysis of macromolecules, Bioresour. Technol. 102 (2011) 3541–3545.
[13] M. Li, E. Su, P. You, X. Gong, M. Sun, D. Xu, D. Wei, Purification and in situ immobilization of papain with aqueous two-phase system, PLoS One 5 (2010) e15168, http://dx.doi.org/10.1371/journal.pone.0015168.
[14] U. Shimanovic, I. Efimo, T.O. Mason, P. Flagmeier, A.K. Buell, A. Gedanken, S. Linse, K.S. Åkerfeldt, C.M. Dobson, D.A. Weitz, T.P.J. Knowles, Protein microgels from amyloid fibril networks, ACS Nano 9 (2015) 43–51.
[15] Y. Han, D. Shchukin, J. Yang, C.R. Simon, H. Fuchs, H. Möhwald, Biocompatible protein nanocontainers for controlled drugs release, ACS Nano 4 (2010) 2838–2844.
[16] Y. Akiyama, T. Fujiwara, S. Takeda, Y. Izumi, S. Nishijima, Preparation of stimuli- responsive protein nanogel by quantum-ray irradiation, Colloid Polym. Sci. 285 (2007) 801–807.
[17] K. Furusawa, K. Terao, N. Nagasawa, F. Yoshii, K. Kubota, T. Dobashi, Nanometer-sized gelatin particles prepared by means of gamma-irradiation, Colloid Polym. Sci. 283 (2004) 229–233.
[18] A.C. Obermeyer, B.D. Olsen, Synthesis and application of protein-containing block copolymers ACS, Macro Lett. 4 (2015) 101–110.
[19] R. Fernandez-Lafuente, C.M. Rosell, V. Rodriguez, J.M. Guisan, Strategies for enzyme stabilisation by intramolecular crosslinking with bifunctional reagents, Enzyme Microb. Technol. 17 (1995) 517–523.
[20] G.H.C. Varca, C.C. Ferraz, P.S. Lopes, M.B. Mathor, M. Grasselli, A.B. Lugao, Radio-synthesized protein-based nanoparticles for biomedical purposes, Radiat. Phys. Chem. 94 (2014) 181–185.
[21] G.H.C. Varca, G.G. Perossi, M. Grasselli, A.B. Lugao, Radiation synthesized protein-based nanoparticles: a technique overview, Radiat. Phys. Chem. 105 (2014) 48–52.
[22] Varca, G.H.C., Lugão, A.B., (IPEN-CNEN/SP, Brazil). BR Patent 1020130050342, October 10, 2012.
[23] R.G. Queiroz, G.H.C. Varca, S. Kadlubowski, P. Ulanski, A.B. Lugão, Radiation-synthesized protein based drug carriers: size-controlled BSA nanoparticles, Int. J. Biol. Macromol. 85 (2016) 82–91.
[24] G.H.C. Varca, R.G. Queiroz, A.B. Lugao, Irradiation as an alternative route for protein crosslinking: cosolvent free BSA nanoparticles, Rad. Phys. Chem. 124 (2016) 111–115.
[25] ISO/ASTM516-13 Standard practice for use of the Alanine-EPR Dosimetry System.
[26] S. Garcia-Castinaras, J. Dillon, A. Spector, Detection of bityrosine in cataract us human lens, Protein Sci. 199 (1978) 897–899.
[27] C. DiMarco, C. Giulivi, Current analytical method for the detection of dityrosine, a biomarker of oxidative stress in biological systems, Mass. Spectrom. Rev. 26 (2006) 108–120.
[28] B.F. Erlanger, N. Kokowsky, W. Cohen, The preparation and properties of two new chromogenic substrates of trypsin, Arch. Biochem. Biophys. 95 (1961) 271–278.
[29] C.C. Ferraz, G.H.C. Varca, M.M.D.C. Vila, P.S. Lopes, Validation of in vitro analytical method to measure papain activity in pharmaceutical formulations, Int. J. Pharm. Pharm. 6 (2014) 658–661.
[30] C. Sabliov, H. Chen, R. Yada, Nanotechnology and Functional Foods: Effective Delivery of Bioactive Ingredients, 1st ed., John Wiley & Sons Ltd., 2015.
[31] G. Wang, H. Uludag, Recent developments in nanoparticle-based drug delivery and targeting systems with emphasis on protein-based nanoparticles, Expert Opin. Drug Deliv. 5 (2008) 499–515.
[32] E. Achilli, G. Casajus, M. Siri, C. Flores, S. Kadlubowski, S. del, V. Alonso, M. Grasselli, Preparation of protein nanoparticle by dynamic aggregation and ionizing-induced crosslinking, Colloids Surf. A Physicochem. Eng. Asp. 486 (2015) 161–171.
[33] S. Kadlubowski, Radiation-induced synthesis of nanogels based on poly(*N*-vinyl-2-pyrrolidone)—a review, Radiat. Phys. Chem. 102 (2014) 29–39.
[34] A. Szabó, M. Kotormán, I. Laczkó, L.M. Simon, Spectroscopic studies of stability of papain in aqueous organic solvents, J. Mol. Catal. B-Enzym. 41 (2006) 43–48.
[35] L.M. Simon, K. László, A. Vértesi, K. Bagi, B. Szajáni, Stability of hydrolytic enzymes in water-organic solvent systems, J. Mol. Catal. B-Enzym. 4 (1998) 14–15.
[36] M. Furuta, I. Ohashi, M. Oka, T. Hayashi, Irradiation effects on hydrases for biomedical applications, Radiat. Phys. Chem. 57 (2000) 455–457.
[37] M. Lal, Radiation induced oxidation of sulphydryl molecules in aqueous solutions. A comprehensive review, Radiat. Phys. Chem. 43 (1994) 595–611.
[38] K.J.A. Davies, Protein damage and degradation by oxygen radicals. I. General aspects, J. Biol. Chem. 262 (1987) 9895–9901.
[39] A. Saha, P.C. Mandal, S.N. Bhattacharyya, Radiation-induced inactivation of enzymes—a review, Radiat. Phys. Chem. 46 (1995) 123–145.
[40] G.V. Buxton, C.L. Greenstock, W.P. Helman, A.B. Ross, Critical review of rate constants for reactions of hydrated electrons, hydrogen atoms and hydroxyl radicals (OH/O) in aqueous solution, J. Phys. Chem. Ref. Data 17 (1988) 513–886.
[41] C.R. Thalmann, T. Lötzbeyer, Enzymatic cross-linking of proteins with tyrosinase, Eur. Food Res. Technol. 214 (2002) 276–281.
[42] J.A. Gerrard, Protein-protein crosslinking in food: methods, consequences, applications, Food. Sci. Technol. 13 (2002) 391–399.
[43] K. Bobrowski, J. Holcman, J. Poznanski, M. Ciurak, J. Wierzchowski, Long-range electron transfer between proline-bridged aromatic amino acids, J. Phys. Chem. 96 (1992) 10036–10043.
[44] C. Houée-Levin, K. Bobrowski, The use of the methods of radiolysis to explore the mechanisms of free radical modifications in proteins, J. Proteom. 92 (2013) 51–62.
[45] C. Houée-Lévin, K. Bobrowski, L. Horakova, B. Karademir, C. Schöneich, M.J. Davies, C.M. Spickett, Exploring oxidative modifications of tyrosine: an update on mechanisms of formation, advances in analysis and biological consequences, Free Radic. Res. 49 (2015) 347–373.