Effect of Glutaraldehyde or Tranglutaminase as Crosslinking Agent in the Release of Coacervated Microparticles

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Introduction

Coacervation is an efficient technique used for encapsulation due to the high load of the core inside the particles and mild conditions used to produce the particles, like absence of organic solvents, low temperatures and the use of natural biopolymers. The porosity of these particles can be modified to obtain sustained release by the incorporation of crosslinks, usually between protein chains. Chemical agents used for covalent crosslinking of proteins have included glutaraldehyde, glyceraldehyde, formaldehyde, glyoxal and others (Hernández-Muñoz et al., 2004). On the other side they show possible toxicity, which make its use questionable for foods (Galietta et al., 1998). In this way, enzymatic crosslinking could be a better alternative. An enzyme that has received extensive recent attention for its ability to crosslink proteins is transglutaminase (De Jong & Koppelman, 2002). The introduction of crosslinks constricts the protein network and can reduce the porosity of the particles. The effects of two crosslinkers, glutaraldehyde and transglutaminase were the aim of this work. The morphology of the microparticles and the release behaviour of the core material were used to evaluate the reticulations process.

Materials and Methods

Materials. Gelatin (type A, from bovine skin, gel strength 244 bloom, batch LF 21502/04, viscosity 6.66 mps, from Leiner Davis, SP, Brazil) and gum arabic (ref. IRX49345, Colloides Naturels Brasil Comercial Ltda, SP, Brazil) were used as the wall materials to make the microcapsules. Commercial vetiver oil (batch 0210222, Dierberger Óleos Essenciais, Barra Bonita, SP, Brazil). Dansyl chloride (Dansyl-Cl) 5-dimethylamine-1-naphthalenesulphonylchloride 99%, Avocado Research Chemicals Ltd. (Heysham, LA, England) and ethanol (99%, batch 78133, Synth, SP, Brazil). All the solvents and other reagents were of analytical grade and deionized water was used in all the experiments.

Fluorescent compound preparation. Zizanoic acid was extracted from the vetiver oil by liquid-liquid extraction (Martinez et al., 2004), purified by flash chromatography and reduced to khusimol using $LiAlH_4$ (Perez et al. 2003). Khusimol was esterified to khusimyl dansylate (khusimyl 5-dimethylamino-1-naphthalenesulphonate [KD]) by addition of the dansyl chloride in pyridine (1 mmol/mL) in excess to the alcohol (2:1) in an ice bath 6 hours. The pyridine was extracted by washing with diethyl ether (2x/50mL), HCl (10%) (2 x 50mL) plus a saturated solution of copper sulphate according Prata (2006).

Preparation of the fluorescent mixture (FM). The derivatized compound KD was added to the vetiver essential oil before making the capsules. The KD:oil proportion used was approximately 1:100.

Microparticles preparation. The microparticles were produced by complex coacervation with gelatin and arabic gum (1:1, 2.5%wt./v) according to the methodology described by Lamprecht et al. (2001).

Chemical and enzymatic crosslinking. Glutaraldehyde*:* 1 mmolar of glutaraldehyde/ g of protein was used.10 mL of the solution (0,7% wt (25% vol) /v deionized water) was added to 1g of moisty microparticles, 25°C during 15 hours. The crosslinked microparticles were washed 3 times with destilated water. **Transglutaminase***:* The enzyme (Ajinomoto, 100U/g de atividade) was added to

1g of moisty microparticles in 1:4 enzyme:microparticles to produce a relation of the 20U/g of the protein.

Microparticles Characterization. **Size:** image analyses, software Scion (www.scioncorp.com) counting minimum 150 particles. **Swelling*:*** moist microparticles in water and SDS solution (5%wt./v) at 25ºC after 5 hours using image analysis (average diameter). **Encapsulation efficiency*:*** determined by fluorimetric assay (330/520nm; analytical curve [$R^2 = 0,9999$, $y = 3,0.10^8 x + 117,11$, where x = concentration of KD (g/mL) and y = fluorescence intensity], spectrofluorimeter Hitachi 7500 (Tokyo, Japan)). The microparticle wall was broken by the action of the enzyme alcalase [Novozymes 2.4L, PLN 05209, 04/09/2003, 0.1% (10 mL/0.3 g microparticle), pH 8 with NaOH 1N, 37 ºC, 3h). The KD was extracted with dichloromethane. The effect of the enzyme was followed using optical microscopy.

Release of KD. Moisty microparticles (1.0000g) in 300mL of SDS medium (5% wt/v) was assayed at 25°C, following the KD release from the microparticles, fluorimetrically. Sampling (5mL) after 15, 30, 45, 60, 120, 180, 240, 300 minutes, with solvent reposition were used.

Results e Discussion

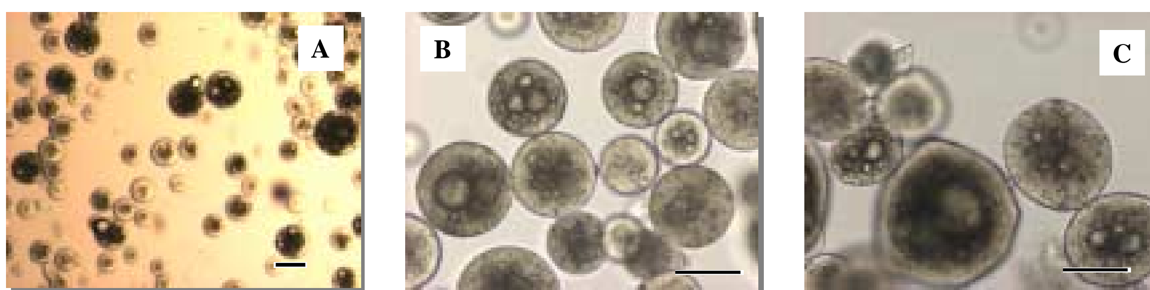

Figure 1. Microparticles in water: A. Without reticulation treatment; Crosslinked with B. Glutaraldehyde; C. Transglutaminase. Bars size:40µm.

The encapsulation efficiency of the fluorescent compound KD was high, reaching 95% considering the moist particles without reticulation. Multinucleate (monolithic distribution) particles were obtained. The morphology of the particles crosslinked with glutaraldehyde did not shown differences compared to the particles without reticulation, both presenting spherical geometry after being produced. However, those crosslinked by transglutaminase showed irregularities in the wall where the spherical form was slightly lost (Fig. 1). The average sizes showed no differences between particles reticulated using glutaraldehyde and without reticulation, whereas reticulation using transglutaminase increase the average size of the particles (Table 1).

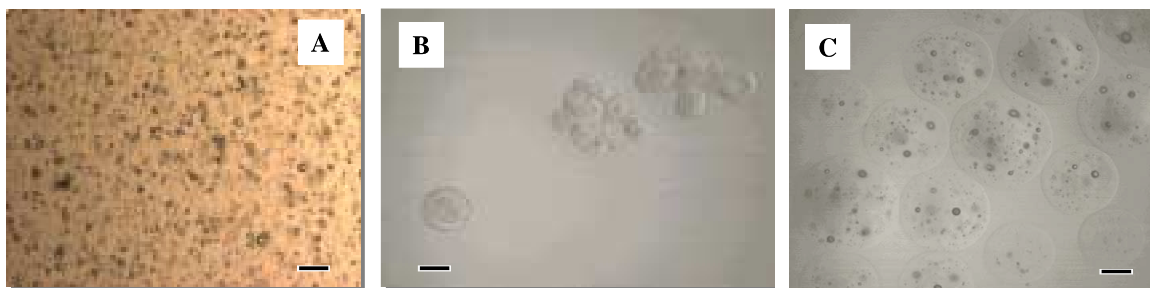

Figure 2. Microparticles in SDS solution (5%wt./v): A. Without reticulation treatment; Crosslinked with B. Glutaraldehyde; C. Transglutaminase. Bars size:40µm.

	Without reticulation	*Glutaraldeído*	*Transglutaminase*
Water	44 ± 9	39 ± 9	52 ± 7
SDS 5%wt./v	-	73 ± 33	121 ± 40

Table 1. Average size and SD (µm) of the microparticles in water and SD S(5% wt/v) after 5 hours

When SDS (5%wt./v) was used as the release medium only reticulated microparticles could be used considering that the particles without reticulation (Fig. 2A) were dissolved (Fig. 2). Also in SDS (5%wt./v) both reticulated particles showed swelling, increasing the average size of the microparticles compared to the size of the microparticles measured in water. The swelling effect was much bigger when trasglutaminase was used to reticulate the microparticles (Table 1).
The effect of reticulation in the polymeric matrix was assayed using a hydrophobic fluorescent marker (KD) to follow the release in the surfactant solution (5%wt/v). The microparticles crosslinked with transglutaminase were less effective in the retention of KD compared to particles reticulated with glutaraldehyde.

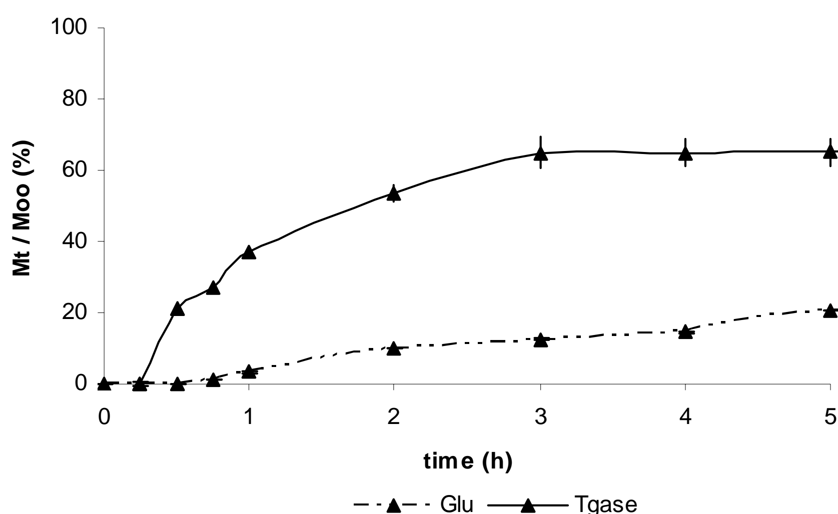

Figure 3. Release curves of KD from microparticles reticulated with glutaraldehyde or transglutaminase using SDS (5%wt./v) as the release medium.

Conclusions: The enzyme transglutaminase or glutaraldehyde were able to reticulate gelatin used to produce microparticles by complex coacervation. Glutaraldehyde was more efficient to control the release of KD encapsulated compared to the action of the enzyme. The different effects could be produced by the size of agents used to produce the reticulation. Transglutaminase presents constant size and glutaraldehyde suffer polymerization and can be a mixture of different molecular sizes improving the capacity of crossking on the protein.

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