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Protein loss by the microencapsulation of an enzyme (lactase) in alginate beads

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Abstract

Alginate gel beads are used in many applications as matrices for release or immobilisation. The problems of enzyme stability and low entrapment efficiency by this microencapsulation procedure are well known, especially with water-soluble substances like peptides. Microencapsulation of the enzyme lactase into alginate beads resulted in a protein loss of about 36%. During the crosslinking step, the bead weight decreased due to the 'syneresis' phenomenon: the carboxylate groups of the guluronate monomers complex the calcium cations. The water leakage was 44% when 1% sodium alginate was used. The contraction phenomenon by the alginate crosslinking and subsequent water leakage is the reason for the loss of water soluble peptides. A reduction of water leakage was achieved decreasing the pH of the alginate solution from 6.5 to 4.3. To hold back the water in the beads, 1% of bentonite was added and the water leakage was reduced to 28%. The protein adsorption onto alginate beads was shown to be affected by pH. Namely, the maximum of protein adsorption was found at a pH slightly below the isoelectric point of lactase (4.61). On charging the protein positively, a binding to anionic alginate occurred. On decreasing the pH of the alginate solution and adding bentonite, the protein loss was reduced from 36 to 3% without lowering of the enzyme activity.

Keywords: Enzyme; Microencapsulation; Alginate beads; Entrapment efficiency

1. Introduction

The immobilisation of enzymes within microcapsules has several advantages. The microcapsules can easily be separated from the reaction products by filtration, microencapsulated enzymes can be used for selective cleavage of low molecular weight substrates in the presence of high molecular weight substrates and immobilized enzymes can be used many times. One of the most widespread methods to immobilize enzymes or microorganisms is their inclusion into semiperme-

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able microcapsules (Lim and Sun, 1980; Poncelet et al., 1992; Monshipouri and Price, 1995). When an aqueous solution of sodium alginate is added dropwise to a solution containing divalent metal ions, spherical alginate beads are produced. An insoluble alginate matrix is formed by cation exchange. The L-guluronan blocks of alginate react with Ca^{2+} ions according to a cooperative mechanism, giving rise to the formation of junction zones referred to as the eggbox model. Alginate beads have the advantage of being nontoxic as biological entities.

The problems of enzyme stability and low entrapment efficiency by this microencapsulation procedure is well known, especially with water-soluble substances like peptides. Arginase microencapsulation was reported by Kondo (1976) and he concluded that enzyme inactivation during microencapsulation was caused by the contact of arginase with an organic solvent and incorporation of the enzyme into the membrane. Wood and Whateley (1982) investigated the loss of enzyme activity during an interfacial polymerization microencapsulation process. The low yields of activity (about 40%) found in polyamide microcapsules for chymotrypsin are typical (Aisina, 1992).

Significant protein loss was observed by the microencapsulation in alginate beads. Pommersheim et al. (1994) reported that during the formation of initial gel beads, 34.4% of the enzyme was lost by diffusion into solution without denaturation. The main loss (30.2%) happened in the first bath when the droplets are hardened to gel beads. Thus, the enzyme is well protected against diffusion by the gel beads even without an additional membrane (Pommersheim et al., 1994).

Protein adsorption onto alginate beads was shown to be affected by pH. The maximum of adsorption was found at a pH slightly below the isoelectric point of the protein (Velings and Mestdagh, 1994).

The enzyme lactase (β-galactosidase) belongs to the group of sugar converting enzymes in the family of hydrolases as well as other hydrolytic enzymes, for instance, lipases, esterases, peptidases etc.

Lactases have been isolated from different organisms, eukaryotes as well as procaryotes. Thermostable lactases were found in bacterial strains of the genus *Thermus* (Berger et al., 1995) and *Bacillus* (Choi et al., 1995). Lactases from *Kluyveromyces fragilis* (Carrara and Rubiolo, 1996), *K. lactis* (Cavaille and Combes, 1995), *Bifidobacterium bifidum* (Passerat and Desmaison, 1995) and *E. coli* are examples for other important bacterial enzymes.

The β-galactosidase from *Xanthomonas manihotis* exhibits strong homology to several eucaryotic β-galactosidases from plants, animals and fungi. For this enzyme the strongest similarity was found with lactases of human and mouse lysosomes with 42 and 41% identity respectively (Taron et al., 1995). Fungal β-galactosidase from *Aspergillus* species is prepared for technical applications (Iwasaki et al., 1996).

Lactases are important tools for the treatment of milk intolerances in adult humans, which are caused by the disaccharide, lactose. The enzymes are used to pre-hydrolyze milk. Lactase preparations are commercially available to be added to milk (Passerat and Desmaison, 1995; Suarez et al., 1995).

The objective of this study was to investigate the reasons which lead to protein loss during enzyme microencapsulation into alginate beads and to find measures to overcome this loss.

2. Materials and methods

2.1. Materials

Sodium alginate (Kelco International Ltd. Tadworth, UK), lactase (Amano Pharmaceutical Co. Ltd., Japan), isolated from *Aspergillus oryzae*, bentonite (Sigma, St. Louis, MO, USA) were used.

All other chemicals were of analytical grade and used without any further purification.

2.2. Methods

2.2.1. Formation of microcapsules

For the alginate bead preparation the method of Lim and Sun (1980) was applied. For drop formation the DropJet System MJ-K-120 (Microdrop GmbH, Norderstedt, Germany) was used.

2.2.2. Water leakage

Water leakage was determined as follows: 300 ml of Na alginate solution (0.25, 0.5, 0.75, 1% w/v) was extruded into 500 ml of 1.5% (w/v) calcium chloride to form the beads. After separation of the beads, the volume of the liquid was determined. An increase in volume as a percentage of the initial volume (300 ml) of alginate solution was calculated.

2.2.3. Protein determination

Protein determination was performed by the Bradford test (Bradford, 1976) using the protein dye reagent (Sigma, St. Louis, MO, USA).

2.2.4. Protein adsorption

Protein adsorption was calculated as the percentage of protein concentration decrease after agitation with empty alginate microbeads for 10 min (initial concentration was kept at 0.8 mg/ml).

2.2.5. Kinematic viscosity

Capillary viscometers (Schott Geräte, Hofheim, Germany) with different capillary diameters, according to the viscosity of experimental liquids, were used. The efflux time of the liquids was determined and the kinematic viscosity (mm^2/s) was calculated.

3. Results and discussion

When drops of alginate solution fall into a $CaCl_2$ solution, beads are immediately formed. For a short time, the beads stay on the top of the solution. After a while they sink due to an increase in the density. This period is known as the maturation step (Velings and Mestdagh, 1995). The bead's weight decreases due to the 'syneresis' phenomenon: the carboxylate groups of the guluronate monomers complex with the calcium cations. This network formation reduces the space occupied by the alginate and, therefore, decreases the volume of the beads.

Comparing weight and volume losses during alginate bead formation, Velings and Mestdagh (1995) have found a linear correlation with a slope of 1.08 mg/ml being close to the value of the water density at room temperature. Therefore, they supposed that water release is essentially a 'syneresis' phenomenon, which takes place during the maturation of the beads. The water leakage was 44%, when 1% (w/v) sodium alginate was used (Fig. 1). Water soluble compounds, in our case enzymes, can be lost along with the liquid if the interchain space is large enough and allows the peptide to diffuse out. Therefore, either reduction of water to be lost by the crosslinking or binding of peptide into the interchain structure can be useful to reduce the protein loss. To check this assumption, the following experiments were carried out.

3.1. Binding of protein to alginate

The alginate is polyanionic: a protein can be charged either positively or negatively, depending on the pH of the environment and the isoelectric point of the peptide. At pH values below the isoelectric point, proteins are positively charged. Under this condition, an effective binding of encapsulated enzyme with alginate can be achieved. To clarify this fact, the influence of the pH value and the bead's surface area (by varying the particle size) on the adsorption of lactase, having an isoelectric point of 4.61, (Ullmann's Encyclopedia of Industrial Chemistry, 1995) was investigated using placebo alginate beads. The adsorption was more effective at the pH 4.3 (Fig. 2). The adsorption data for small (500 μm) and large (5000 μm)

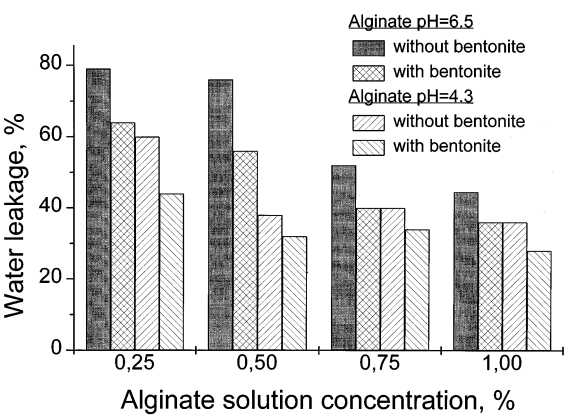

Fig. 1. Water leakage during alginate beads formation.

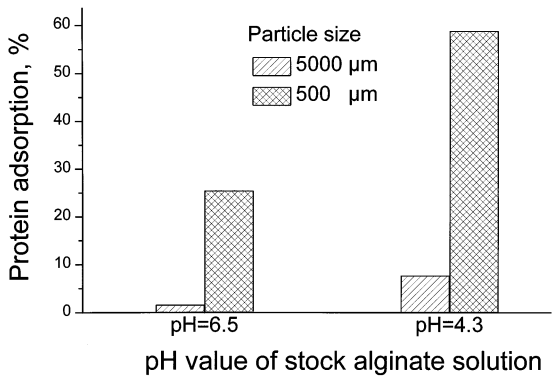

Fig. 2. Effect of pH of alginate solution on protein adsorption onto alginate beads.

beads (difference in outer surface area is 100 times) show that peptides are adsorbed only on the surface of the beads without penetration into the gel structure.

3.2. Water leakage

The results of trials to hold back the water in the gel structure by the alginate crosslinking are shown in Fig. 1. The concentration in the range 0.25–1% w/v and pH values from 6.5 to 4.3 of stock alginate solution were used. A fine powder of bentonite (1% to the volume of the alginate solution) was added to bind the water into a gel structure. Bentonite is known to be insoluble in water, but swells into a homogeneous mass occupying about 12 times the volume of the dry powder (Martindale, 1982). Lowering the alginate concentration from 0.25 to 1% w/v leads to a decrease in measurable water loss. Investigating the influence of pH value, the water loss at pH 4.3 was found to be significantly smaller. This condition corresponds to pre-coagulation, where the polymer chains are rolled up, still being in solution. The addition of bentonite to the alginate solution (pH 6.5) causes almost the same effect as the lowering of the pH. With the use of bentonite, along with alginate solution (pH 4.3), the water loss can be reduced to 28%.

3.3. Viscosity of alginate solutions

The viscosity of the solutions to be crosslinked is important. It limits the ability of such solutions to be extruded through the nozzle in order to form small droplets and the viscosity of the droplet content can determine the rate of water leakage during crosslinking. The results of the measurement of kinematic viscosity of the above-mentioned liquids are shown in Fig. 3. All samples are non-Newtonian liquids with a non-linear dependence of kinematic viscosity on concentration. The sharper increase in the curve for the alginate solution (pH 4.3) in the presence of bentonite additionally shows the better properties for regular bead formation by extrusion because of the greater resistance of the droplets to mechanical deformation.

3.4. Protein loss

The results of the protein determination in the precipitation bath are presented in Fig. 4. In correlation with the above-mentioned results, the change in the pH of the alginate solution from 6.5 to 4.3 lowers the protein loss from 35 to 18%. The addition of bentonite to the alginate solution (pH 6.5) gave no significant improvement. But the protein loss was only about 3% when a combination of alginate solution (pH 4.3) with bentonite was used. As reported presently, in the case of lactase microencapsulation, the reduction of pH value of alginate solution from 6.5 to 4.3 led to an

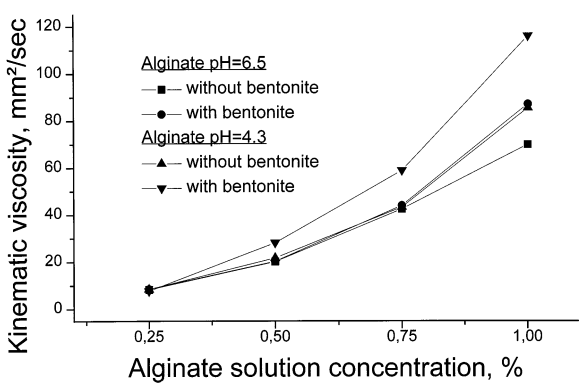

Fig. 3. Kinematic viscosity of algiante solutions.

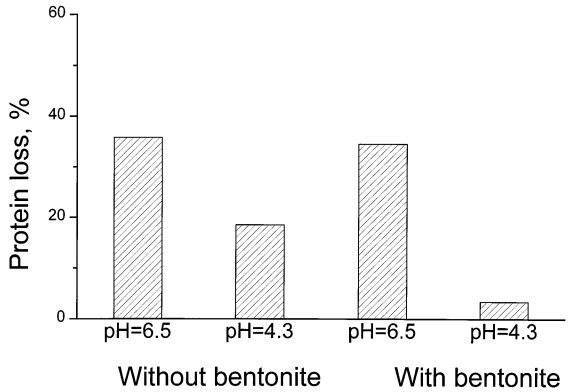

Fig. 4. Protein loss during alginate bead formation and the dependence on the pH of the alginate solution and bentonite addition.

improvement in enzyme activity (Dashevsky et al., 1996).

In conclusion, a considerable water leakage during the formation of alginate beads was shown. It can be explained by the contraction phenomenon during the crosslinking. This can be a reason for the drastic protein loss by this microencapsulation method. To prevent protein loss, a binding of positively charged protein with anionic alginate was shown to be effective. The water leakage during the bead formation was reduced from 44 to about 36%, using either a lower pH of the alginate solution or bentonite addition, and up to 28% when both measures were used. The protein loss was reduced from 36 to 3%.

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