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Immobilization of α-chymotrypsin in κ-carrageenan beads prepared with the static mixer

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Abstract

The α-chymotrypsin-loaded κ-carrageenan beads preparation and properties are described. The beads were obtained by emulsification/thermal gelation with a sunflower oil at ambient temperature as the continuous phase using a Sulzer SMX static mixer. The mean Sauter bead diameter was about 300 μm. The α-chymotrypsin encapsulation efficiency may be increased in two times by preliminary enzyme cross-linking by glutaraldehyde. The stability upon storage is higher for beads-containing cross-linked α-chymotrypsin (no activity loss during at least 2 weeks). The α-chymotrypsin-loaded κ-carrageenan beads may be used in repeated batch runs and the operational biocatalyst stability may be enhanced by κ-carrageenan beads the coating by chitosan (no significant activity decrease in seven runs).

Keywords: α-Chymotrypsin; Encapsulation efficiency; Emulsification/thermal gelation; κ-Carrageenan; Static mixer

1. Introduction

The enzymes' properties like specificity, high activity, make them of great interest as industrial catalysts. The use of enzymes in this field requires their easy recovery from the reaction mixture at the end of the procedure, the catalytic activity remaining stable over long time period and catalyst applicability to continuous processes. All these problems may be solved by enzyme entrapment within a gel matrix.

For this application, polysaccharides (carrageenans, agarose or alginate) forming hydrogels under mild conditions may be employed. Such preparation may be even applied to the multi-enzyme systems [1,2], cofactors-dependent enzymes acting in water-organic media [3,4] and enzyme immobilization for food product treatment [5].

One of the most widely used immobilization materials is κ-carrageenan, linear, sulfated polysaccharide extracted from marine red algae. The primary structure is made up of alternating α(1,3)-D-galactose-4-sulphate and β(1,4)-3,6-anhydro-D-galactose residues. κ-Carrageenan forms gels, undergoing a coil (disordered sol state) to helix (ordered state) transition, triggered by a reduction in temperature and/or through ionic interactions [6].

Several encapsulation protocols for immobilization in κ-carrageenan hydrogel are based on the emulsification of sol phase into a hydrophobic phase. The emulsified sol droplets are then caused to gel in a separate step by dropping the temperature. Emulsification is generally carried out on a batch basis in a standard baffled turbine reactor [7], but at large scale the static mixer technology may be employed [8]. A static mixer consists of a series of specially designed stationary elements placed transversely in a tube (Fig. 1). These elements form crossed channels that promote division and longitudinal recombination of the liquid flowing through the static mixer and, consequently, the emulsification. One of the benefits of this technology is the potential for the continuous processing. The standard method for κ-carrageenan beads production with static mixer involves heating both the dispersed κ-carrageenan sol and continuous oil phases before mixing and cold oil injection in the outlet of the static mixer to initiate gelation [9]. In our previous study an alternative approach was proposed: the continuous oil phase at ambient temperature was used and the choice of best operational parameters for this procedure was determined [10].

The aim of the present work was to apply this last procedure of κ-carrageenan beads production by static mixer for the enzyme encapsulation and to obtain the active and stable enzyme preparation. Proteolytic enzyme α-chymotrypsin (EC 3.4.21.1) (Cht) was chosen as model enzyme.

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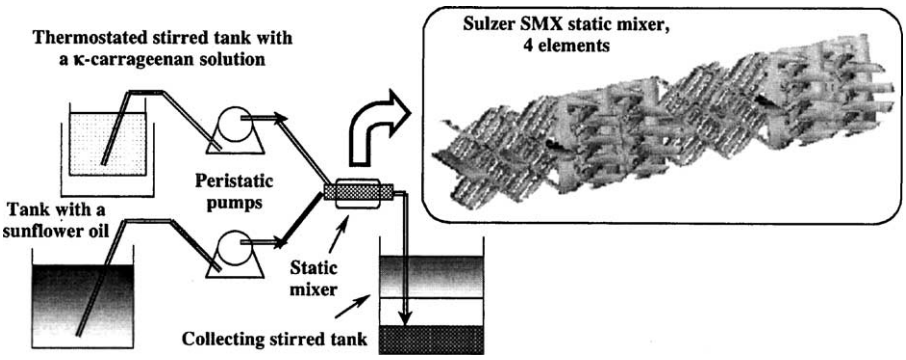

Fig. 1. Experimental installation for κ-carrageenan beads production using the static mixer.

2. Materials and methods

2.1. Reagents

α-Chymotrypsin (M = 25,000 Da, 51 U/mg), *N*-benzoyl-L-tyrosine *p*-nitroanilide (BTNA) and calcium chloride were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). κ-Carrageenan in the form of potassium salt (kindly provided by Degussa, Baupte, France) and chitosan (type 222) (kindly provided by France Chitin, Marseille, France) were used without additional purification. Sunflower oil (Alba, Lesieur, France) was a commercial grade product. Glutaraldehyde (Glut) (25% water solution), L-lysine hydrochloride, sodium acetate, potassium chloride, tris(hydroxymethyl)aminomethane (Tris), acetic acid, urea and dimethylsulfoxide (DMSO) were from Fluka Chemie GmbH (Buchs, Germany).

2.2. Preparation of κ-carrageenan/α-chymotrypsin solutions

2.2.1. Native α-chymotrypsin

The native α-chymotrypsin solutions were prepared directly before the bead preparation procedure. The known weight of α-chymotrypsin (25–75 mg) was dissolved in 10 ml of 0.01 M $CaCl_2$.

2.2.2. Cross-linked α-chymotrypsin

The cross-linking of α-chymotrypsin by glutaraldehyde was carried out according to the method, described in [11]. To α-chymotrypsin (100–200 mg) solution in 8.6–8.2 ml of 0.02 M acetate buffer (pH 5.0) an aqueous 2.5% glutaraldehyde (0.4–0.8 ml) was added dropwise under gently stirring. The ratio of glutaraldehyde to protein concentration was kept equal to 10% (w/w) for all preparations. The reaction mixture was left at room temperature without stirring for 3 h. The cross-linking was stopped by addition of 1 ml of 1 M L-lysine hydrochloride solution (to the final concentration 0.1 M) and the solution obtained (10 ml) was used for the bead preparation.

2.2.3. κ-Carrageenan/α-chymotrvpsin solutions

In all studies the 1.5% (w/w) κ-carrageenan solution was used. κ-Carrageenan powder (1.5 g) was suspended in 90 ml 0.01 M $CaCl_2$ at room temperature, heated to 70 °C, stirred and held at 70 °C for 20 min to completely dissolve the polysaccharide. Then the solution was cooled to 40 °C and mixed with the 10 ml of native or cross-linked α-chymotrypsin solution. The mixing was carried out directly before start the procedure of bead production.

2.3. Viscosity measurements

Viscosity measurements were performed with the rheometer AR 1000-N (TA Instruments, UK). Hot (55 °C) α-chymotrypsin-containing κ-carrageenan solution was poured directly on the rheometer plate, which was pre-equilibrated at the same temperature. The sample was immediately covered by a thin paraffin oil layer to avoid water evaporation during the measurements. The samples were then cooled (from 55 to 30 °C, 5 °C/min). The viscosity at shear rate 100 s^{-1} was determined.

2.4. Differential scanning microcalorimetry (DSC) measurements

DSC measurements were performed with a Micro-DSCIII calorimeter (SETARAM, France) equipped with 1 ml sample and reference cells. A known weight of α-chymotrypsin-containing κ-carrageenan solution was transferred to the DSC sample cell and an equivalent weight of pure water added to the reference cell. The cells were cooled to 10 °C, equilibrated for 10 min, then heated to 50 °C. After heating and equilibrating at 50 °C for 10 min, the samples were cooled to 10 °C. The rate of heating/cooling was varied from 0.5 to 3 °C/min.

2.5. Production of α-chymotrypsin-containing κ-carrageenan beads with a static mixer

The experimental setup used is shown schematically in Fig. 1. κ-Carrageenan solution was held at controlled tem-

perature (40 °C) with continuous agitation (magnetic stirrer with temperature control AM 3003 Control, Bioblock Scientific, France) and the oil was held at room temperature (22 °C). The static mixer was fed by two peristaltic pumps (Masterfiex, Cole-Parmer Instrument Company, USA). The total volume flow rate Q_t was 300 ml/min the volume fraction of κ-carrageenan in oil was 0.05.

Mixing elements of type SMX DN6 with the hydraulic diameter 1.59 mm manufactured by Sulzer Chemtech AG (Switzerland) were used (Fig. 1). A series of eight mixing elements (diameter 6 mm, length of each element 6 mm) were inserted into a plexiglass turbular housing with diameter and length of 6 and 50 mm, respectively.

At the outlet of the static mixer, the liquid–liquid dispersion was collected in a stirred tank, containing cold (10 °C) 0.05 M Tris buffer containing 0.2 M KCl (pH 8.0). During phase separation, the resulting gel beads were stored in 0.05 M Tris buffer (0.2 M KCl, pH 8.0) at 4 °C for 14–16 h in order to complete transfer of beads to the aqueous phase. The microbeads were then collected by decantation (residual traces of oil removed with absorbant paper), recovered on a nylon sieve (10 μm), washed by 0.05 M Tris buffer (0.2 M KCl, pH 8.0), drying on air at room temperature (during 24 h), weighed and used for further experiments. The weight yield of beads production was calculated basing on the total initial weight of enzyme-containing κ-carrageenan solution. The supernatant was collected to examine the encapsulation efficiency.

2.6. Microbead size analysis

Particle size analysis was performed using a laser diffraction particle sizer (Malvem MasterSizer, Malvem Instruments S.A., France). The Sauter mean diameter (d_{32}) was determined. Reported values represent the average of at least three replicates for each sample.

2.7. Coating of α-chymotrypsin-containing κ-carrageenan beads by chitosan

Chitosan (0.2% w/v) solution was prepared in 1% (v/v) acetic acid. The pH of the solution was adjusted to 6.0 using concentrate sodium hydroxide. Beads (25 g) were incubated in 250 ml of chitosan solution at 20 °C and pH 6.0 for 30 min under gentle agitation. After incubation, the beads were collected on the nylon sieve (10 μm) and washed by 0.05 M Tris buffer (0.2 M KCl, pH 8.0).

2.8. α-Chymotrypsin activity assays

The α-chymotrypsin activity was determined as initial rate of BTNA hydrolysis (at 390 nm, spectrophotometer UV1 UNICAM, UK) at substrate concentration 0.04 mM in 0.05 M Tris buffer, containing 0.2 M KCl, pH 8.0 (20 °C).

2.8.1. In bead collecting solution

Three milliliters of supernatant recovered after the bead collection were placed in the spectrophotometric cuve and mixed with 0.03 ml of 4 mM BTNA solution in DMSO. The activity was measured and using the calibration graph the α-chymotrypsin concentration was calculated.

2.8.2. In beads

A known weight of beads (2–3 g) was suspended in 30 ml of 0.05 M Tris buffer (0.2 M KCl, pH 8.0) and mixed with the BTNA concentrated solution (in DMSO). Each 15–60 s the aliquote (2 ml) was taken and immediately mixed in spectrophotometric cuve with 1 ml of 8 M urea solution (to stop the hydrolysis). After beads sedimentation (within 3 min), the absorption at 390 nm was measured, plotted as time function and the α-chymotrypsin activity were calculated.

2.8.3. Operational stability

A known weight of beads (20–25 g) was suspended in 250 ml of 0.05 M Tris buffer (0.2 M KCl, pH 8.0), a concentrate BTNA solution in DMSO was added (to obtain the final BTNA concentration 0.04 mM) and the activity of preparation was measured as described in Section 2.8.2. After the reaction being completely finished (near 15 min), the beads were separated from the reaction media (on the 10 μm nylon sieve), washed several times by 0.05 M Tris buffer (0.2 M KCl, pH 8.0), weighed, resuspended and the following batch was carried out.

3. Results and discussion

3.1. Beads preparation

For emulsification/thermal gelation with a continuous phase at ambient temperature, the choice of κ-carrageenan injection temperature had to be selected to get the discrete and spherical gel beads [10]. This choice was defined primarily by κ-carrageenan solution viscosity and gelation temperature. As can be seen from Figs. 2 and 3, physical properties of solution containing both native and cross-linked enzyme did not significantly distinguish from the α-chymotrypsin free κ-carrageenan solution. So, the κ-carrageenan injection temperature (40 °C) and other operational conditions (total volume flow rate and κ-carrageenan volume fraction) were selected according criteria discussed in [10]. In a preliminary experiment, it was shown that for both native and cross-linked α-chymotrypsin at 40 °C in 0.01 M $CaCl_2$ the activity remained stable during at least 1 h.

The α-chymotrypsin-containing beads properties are summarized in Table 1. The weight yield of beads production varied from 58 to 66%. There was not a correlation between immobilized α-chymotrypsin concentration and beads size, but the beads with cross-linked α-chymotrypsin were slightly larger than the ones with the native enzyme.

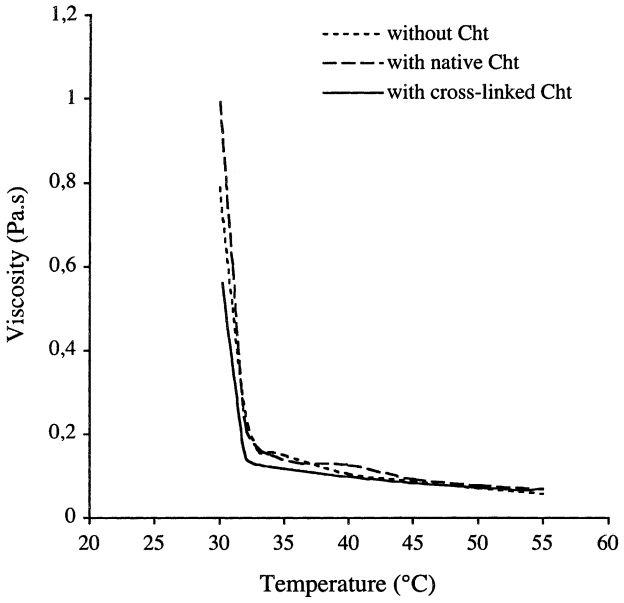

Fig. 2. Viscosity dependence from temperature (cooling rate 5 °C/min, shear rate 100 s^{-1}) for 1.5% (w/v) κ-carrageenan (0.01 M $CaCl_2$) without α-chymotrypsin, with native α-chymotrypsin (0.75 mg/ml) and with cross-linked α-chymotrypsin (1.0 mg/ml).

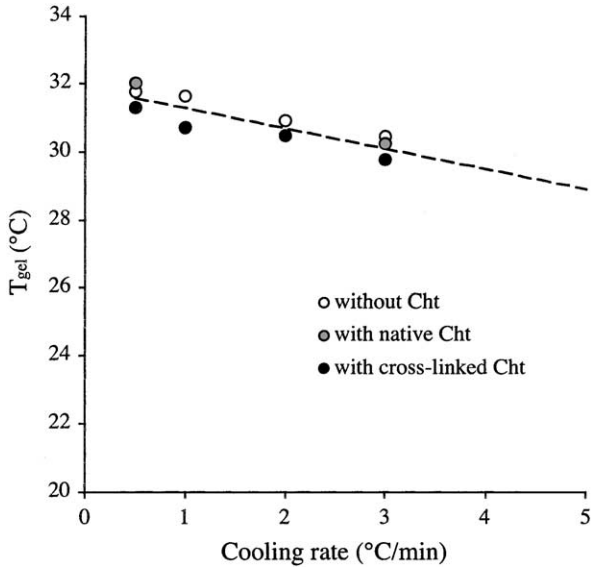

Fig. 3. Gelation temperature (T_{gel}) dependence from cooling rate for of 1.5% (w/v) κ-carrageenan (0.01 M $CaCl_2$) without α-chymotrypsin, with native α-chymotrypsin (0.75 mg/ml) and cross-linked α-chymotrypsin (1.0 mg/ml).

3.2. Encapsulation efficiency

Due to the increased size of the chemically cross-linked enzyme aggregates the enzyme retention in the beads must be higher. The combination of glutaraldehyde and chitosan (spacer) might be also used [12] but the presence of chitosan admixture in κ-carrageenan might make more difficult the choice of operational parameters. As can be seen from Fig. 4, the preliminary α-chymotrypsin treatment by glutaraldehyde gave rise the encapsulation efficiency enhancing from 12 to 13% for native to 20–25% for cross-linked enzyme-containing beads (calculated values are based on the α-chymotrypsin activity before immobilization taken as 100%).

However, there was some part of enzyme activity (20–50%) that was not detected in beads or in beads collecting solution. Three possible explanations may be given.

Firstly, it was shown that the immobilized enzyme loss (leakage) during hardening the κ-carrageenan beads in

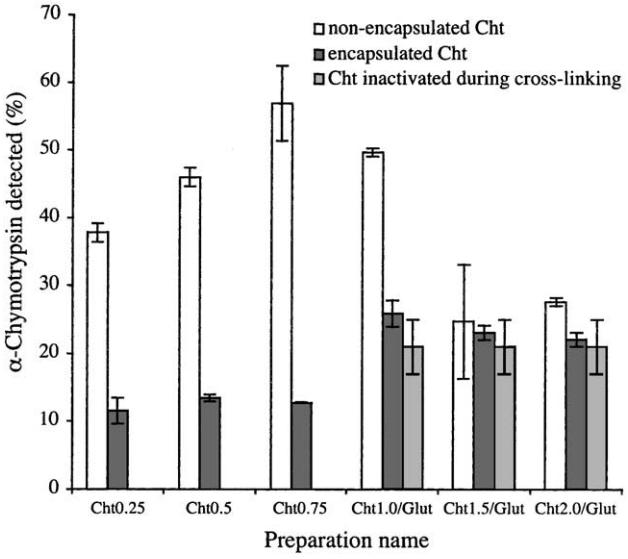

Fig. 4. Analysis of encapsulation efficiency for different α-chymotrypsin-loaded beads preparations.

Table 1
Properties of α-chymotrypsin-loaded κ-carrageenan beads

Preparation name	Cht_0 (mg/ml)[a]	Cross-linking	Weight yield (%)	Cht_{imm}, (mg/g_{beads})[b]	d_{32} (μm)
Cht0.25	0.25	−	63	0.045	305 ± 3
Cht0.5	0.5	−	62	0.107	277 ± 13
Cht0.75	0.75	−	59	0.159	288 ± 47
Cht1.0/Glut	1	+	66	0.384	343 ± 48
Cht1.5/Glut	1.5	+	58	0.591	346 ± 17
Cht2.0/Glut	2	+	61	0.717	319 ± 20

[a] α-Chymotrypsin concentration used for beads preparation.
[b] α-Chymotrypsin concentration in beads.

aqueous solution may reach out 50% [4]. The α-chymotrypsin leakage during beads collection could be followed by autholysis (since the beads are small, the completely beads transition in the aqueous phase occurred in 14–16 h) that decreased the active enzyme concentration in beads collecting solution.

Secondly, it is known that immobilization procedure may lead to Michaelis constant increase, for example two times increase of Michaelis constant for α-chymotrypsin after immobilization was determined [13]. Since the amount of encapsulated α-chymotrypsin was calculated basing on the beads activity data (determined as initial hydrolysis rate), the rise in Michaelis constant after immobilization might affect on resulting value.

Thirdly, the encapsulated enzyme activity determination might be influenced by internal diffusion limitations like it was shown for example for trypsin-containing microcapsules [14].

3.3. Activity change upon storage

The change of α-chymotrypsin-loaded beads activity upon storage is illustrated in Fig. 5 (the initial beads activity was taken as 100%). While for the cross-linked α-chymotrypsin-containing beads, no activity loss during at least 2 weeks was found, the preparations with the native enzyme shown an activity decrease down to 50% of initial value. Because the κ-carrageenan matrix had a relatively low concentration (1.5% w/w), single α-chymotrypsin molecules might leak out from beads and/or diffuse inside beads, so the autholysis process may occur. As can be seen form Fig. 5, the activity loss for native enzyme-containing beads slightly increased with the α-chymotrypsin concentration that confirms the possibility of autholysis inside of beads. Since the increased size for cross-linked α-chymotrypsin aggregates made the possibilities of leakage and diffusion inside of beads more difficult, cross-linked preparations activity was stable.

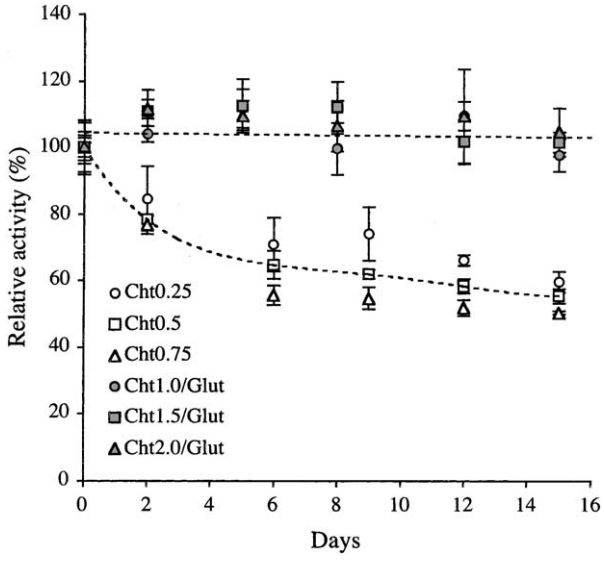

Fig. 5. Enzymatic activity change upon storage.

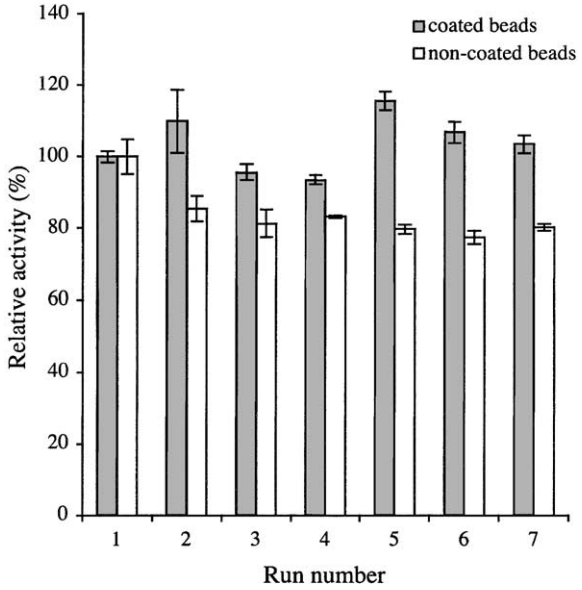

Fig. 6. Variation of α-chymotrypsin-loaded κ-carrageenan beads activity in repeated batch runs (Cht2.0/Glut with or without chitosan coating).

3.4. Operational biocatalyst stability

The beads with cross-linked α-chymotrypsin were used in a repeated batch runs to establish the operational biocatalyst stability (Fig. 6). Coating by chitosan was used to avoid the enzyme leakage during reaction. As can be seen from Fig. 6, even with a low concentrate κ-carrageenan matrix (1.5% w/v), the cross-linked α-chymotrypsin preparations demonstrated a high operational stability. The non-coated beads activity decreased in 20% after first batch (the leakage of enzyme during reaction and beads wash took place) remaining stable for the following. With coated beads at least seven runs might be carried out without any activity decrease. This result was of the same level that the operational stability of α-chymotrypsin covalently attached to the support [13].

4. Conclusions

The α-chymotrypsin-containing κ-carrageenan beads were prepared by emulsification/thermal gelation with a continuous phase at ambient temperature using static mixer technology. The encapsulation efficiency may be increased in two times by preliminary enzyme cross-linking by glutaraldehyde. For beads-containing cross-linked α-chymotrypsin no activity loss upon storage was detected during at least 2 weeks. The beads were used in repeated

batch and the operational biocatalyst stability was perfected by beads coating with chitosan.

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