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The potential of enzyme entrapment in konjac cold-melting gel beads

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The encapsulation of proteolytic enzymes in cold-melting hydrogel has been investigated. The objective was to enhance proteolysis during cheese production by maintaining enzymes in the beads during the clotting step and by releasing them during ripening through bead liquefaction (as temperature decreases). Konjac flour, a glucomannan, forms a gel after a mild alkali treatment and heating. The gel is liquefied easily at cold temperatures (≤10°C). A two-phase dispersion process has been adapted which allows the preparation of spherical konjac beads (50–500 μm diameter). The encapsulation yield of Protease B500 is about 50% based on residual proteolytic activity. A low leakage of enzyme at 30°C was found. This makes the system suitable for use during gel formation in cheese manufacture; however, at 4°C, the liberation of enzyme is 7% within 24 h, which is too low to ensure fast cheese ripening. Syneresis of the konjac gel beads under shear stress explains this low enzyme release. Evaluation of other gels is undertaken. © 1997 by Elsevier Science Inc.

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Introduction

The present paper deals with a new concept of enzyme-controlled release for cheese ripening. Enzymes are entrapped in cold-melting hydrogel beads. Beads are introduced within the milk during the clotting step which allows a homogeneous repartition in the curd. During the ripening step, beads are liquefied by the low temperatures and release the enzymes into the cheese.

Cheese production may be characterized as a three-step process. The milk is first transformed into curd by clotting enzymes. In a second step, the curd is drained to remove whey and molded into cheese forms. Finally, during the ripening phase, the components responsible for flavor and texture development evolve through complex microbial enzyme reactions. In order to reduce the cost during curing and the subsequent cheese storage (up to 10% of the production cost of Cheddar[1]), the use of exogenous enzymes has been investigated. The success of cheese treatment by enzyme has been greatly impaired by loss of enzymes in the whey (up to 90%), lost value of the contaminated whey (usually recycled in other food products), and unwanted yield-reducing proteolysis and premature flavor changes.[2]

Several authors have considered encapsulation of enzymes to achieve controlled release during the ripening period.[3] Most studies have been restricted to lipid vesicles (liposomes).[4] Lipid vesicles are not easily produced on a large scale; moreover, release from lipid vesicles is not easily controlled compared to other types of microcapsules.

The cold-melting hydrogel tested in the present study was konjac flour gel. Konjac is the generic name for the powder formed from grinding the tubers of Elephant yam plant *Amorphophallus konjac*.[5] Konjac forms a gel which is reversible at cold temperature. Konjac is recognized as safe (GRAS) in accordance with FDA regulations.

Konjac is mainly composed of a high molecular weight glucomannan in which mannose and glucose units in a ratio of 1.6:1 are connected by β–(1,4) linkages. The konjac has short side branches, and acetyl groups exist randomly at the C–6 position of the sugar units. Konjac solutions are deacetylated by cooking in a mild alkali to produce gels. Gelation occurs through a three-dimensional hydrogen-bounded network.[6]

Materials and methods

Materials

The proteolytic enzyme, Protease B500, was provided by Gist Brocades (Seclin, France). It is mainly constituted by neutral pro-

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tease synthesized by *Bacillus subtilis* (EC 3.4.24.4). Konjac powder was provided by FMC Corporation (Rockland ME). Potassium carbonate, bovine serum albumin, and D-glucose pentaacetate were purchased from Fluka (Saint Quentin Fallavier, France) and casein from Merck (Nogent-sur-Marne, France). All other reagents were from Prolabo (Paris, France).

Gel preparation

Konjac (1.5 g) is suspended in cold water (100 g) and heated at 85°C for 60 min. After replacing the water lost due to evaporation, potassium carbonate (K_2CO_3) is added in a ratio of 10% (w/w) of konjac powder (0.15 g). The konjac is then deacetylated by heating to 85°C. This temperature is held for 20 min which allows it to form a gel. The gel is placed in an ice bath for at least 4 h to cold-melt.

Enzyme entrapment in cold-melting hydrogel beads

The bead preparation procedure is adapted from κ–carrageenan bead production by dispersion in a two-phase system.[7] Konjac cold-melt sol (33.3 ml previously adjusted to pH 7.0 with 0.5 M HCl) is mixed with 6.7 ml of enzyme solution (50 g l^{-1}). The 40 ml of enzyme/konjac solution are rapidly poured into a 600-ml beaker containing 160 ml of canola oil. The water-in-oil dispersion is formed by stirring with a marine impeller (60 mm diameter, three blades) at 700 rpm for 15 min. Gelation of droplets is obtained by adding another 160 ml of hot canola oil (90°C). The resulting temperature in the reactor (50°C) is held for 20 min. The reactor is then cooled at 20°C by help of a cold waterbath. The bead-in-oil suspension is transferred in 100 ml of water. After complete partitioning of the beads to the aqueous phase, the oil is discarded and the beads are washed with water.

Acetyl group measurements

The acetylation degree of konjac is measured in accordance with the method described by Hestrin.[8] A 1-ml sample is added to 2 ml of alkaline hydroxylamine reagent (2 M hydroxylamine hydrochloride mixed with equal volume of 3.5 M sodium hydroxide). After 5 min, 0.1 ml of 4 M HCl and 1 ml of an iron solution (0.37 M iron chloride in 0.1 M HCl) are added. Samples are read at 540 nm against a blank. The calibration curve is made using D-glucose pentaacetate (0–3 mM). The standard deviation for acetyl group measurement was found to be ±2μM.

Protein measurement

Proteins are quantified by a method adapted from Lowry.[9] An alkaline solution is prepared by mixing 1 ml of a cupric solution (10 g l^{-1} Na/K tartrate and 5 g l^{-1} $CuSO_4 \cdot 5H_2O$ in 0.1 M NaOH) with 50 ml of carbonate buffer (20 g l^{-1} Na_2CO_3 in 0.1 M NaOH). Samples of 1 ml containing up to 1 mg of proteins are added with 5 ml of the alkaline solution. After 10 min, 0.5 ml of Folin phenol reagent is added. Absorbance measurement is made at 750 nm after 30 min. The calibration curve is established with a bovine serum albumin (BSA) solution (0–1 g l^{-1}).

Assay of proteolytic activity

Proteolytic activity is determined by measuring the rate of casein hydrolysis.[10] Casein solution (1%) is prepared by suspending 1 g of casein (Hammarsten quality) in 100 ml of 50 mM Tris–HCl buffer pH 7.2 with heating for 15 min in boiling water for complete dissolution.

For the calibration curve, aliquots (0.1–1 ml) of enzyme solution (0.5 g l^{-1} in Tris buffer) are pipetted into tubes, and the volumes are adjusted to 1 ml with the Tris buffer. After adding 1 ml of casein solution (at 37°C), each tube is then incubated at 37°C for 10 min. The reaction is stopped by adding 3 ml of 5% trichloroacetic acid solution. The solutions are filtered after settling for 1 h at 25°C. The hydrolyzed product concentration in the supernatant is determined by absorbance at 280 nm using buffer as the blank control. Proteolytic activities of unknown samples are determined using 1 ml aliquots.

Enzyme-leakage study

Fifty grams of beads are placed in 50 ml of water in a controlled temperature bath at 4°C (ripening temperature) or 30°C (clotting temperature). Protein concentration and proteolytic activities are determined on supernatants after 1, 4, and 24 h.

Rheological measurements

Flow tests on konjac were conducted in a coaxial rheometer Rheomat 120 (Contraves). The cold-melt konjac was heated up to 50°C during 20 min in the rheometer. The gel was allowed to cold-melt again at 20°C during 2 h before the measurement. The strain rate was increased from 0 to 200 s^{-1} in 60 s and dropped to 0 s^{-1} in 60 s. The apparatus measures the shear stress (Pa) vs strain rate (s^{-1}).

Results and discussion

Konjac gel preparation

The method described above was first used to prepare konjac gel. Some particles were not soluble, remained in suspension, and were easily visible. This phenomenon was attributable to incomplete dissolution of the polymer. Prolonged heating (2 h at 85°C instead of 1 h) with vigorous mixing (up to 950 rpm) did not increase solubility of aggregates.

Some authors[6,11] have reported that konjac flour contains fine oval whitish sacs (100–500 μm) with polymer inside. By heating the flour in water, the vesicles swell and rupture, thus releasing the glucomannan polymer. The presence of residual envelopes is not a nuisance in food applications; however, it is undesirable for the production of microspheres with diameters ranging from 200–500 μm. Since high viscosity fluids are difficult to filter, undesirable particles are separated on centrifugation (1.4×10^4 g for 20 min at 5°C). The residue represents approximately 5% of the dry konjac powder and is mainly composed of proteins.

As described in MATERIALS AND METHODS, the konjac solution easily forms a gel after alkaline and heat treatment. The konjac gel is able to liquefy at 4°C (within 4 h); however, it was observed that after 3 days at 4°C, the cold-melt sol splits into two phases. For this reason, the gel is always prepared before use.

Gelation of cold-melt konjac

The cold-melt konjac solution is able to regelify after heat treatment. A minimum temperature of 50°C for 20 min is required for gelation of cold-melt konjac. The gelified material remains solid at room temperature (20°C).

Acetyl group measurement

Maekaji in Nishinari et al.[12] suggests that deacetylation of konjac occurs during alkaline treatment and deacetylation is necessary for the gelation. Although the chemical change is small, the physical change in gelation is quite remarkable. In order to quantify konjac deacetylation, acetyl group measurements have been made on several samples of konjac solutions. Both effects of alkali concentration (K_2CO_3) and heating time were studied. The results are reported in *Table 1*. The initial konjac solution which has not been treated with alkali does not form a gel. For complete gelation of the konjac solution, an alkali treatment with 10% potassium carbonate and 10 min heating time is required.

Konjac gel bead production

The two-phase dispersion process allows the preparation of whitish hydrogel beads which are spherical in shape. The mean particle diameter is about 200 μm with a size ranging between 50–500 μm. Broad size distribution may be attributed to a non-homogeneity in the liquid–liquid dispersion and a slow gelation process. It has also been observed with other types of gel beads that fast gelation produces narrower particle size distributions.[13] The protease encapsulation yield in konjac gel beads is between 40–60% based on activity of the surrounding medium.

Leakage studies

Ideally, to succeed in controlling cheese ripening, the encapsulation method must insure no enzyme leakage at 30°C (or at least a very slow one). This is to avoid a premature proteolysis; however, enzymes must be released during the ripening stage at 4°C.

At 30°C, protein release from konjac beads remains lower than 3% of the total immobilized within 4 h (*Table 2*). The low enzyme release in whey (2.2% instead of 90% with free enzymes) leads to operating cost reduction, production yield improvement, premature proteolysis decrease, and prevents the development of a bitter taste in the curd.

Seven percent of the total immobilized proteins were released in the bulk solution at 4°C after 24 h. Liquefaction of the beads in a 50 mM Tris–HCl buffer pH 8.0 at 10°C was not observed even after 20 days. Konjac beads did not follow the expected behavior of thermal reversible gels. This phenomenon may be the consequence of the contact with the oil phase and/or the shear applied during emulsification.

Table 1 Acetyl group measurements by the Hestrin method on 1.5% konjac sols

Sample	K_2CO_3 (%)	Heating 85°C (min)	Acetyl groups (mM)	Acetylation (%)	Gelation quality
1	0	20	0.110	100	No gelation
2	5	20	0.012	11	Bad
3	10	10	0.002	2	Acceptable
4	10	20	0.000	0	Very good

Table 2 Leakage study results (% total proteins entrapped leaked in the surrounding medium)

Time (h)	30°C	4°C
1	2	5.2
4	2.2	5.3
24	–	6.1

Effect of the shear on konjac

Rheological behavior of konjac was investigated using coaxial cylinder rheometry. When shear stress was applied, the konjac solution separated into a two-phase system with white solid blocks and a transparent liquid phase. This phenomenon prohibits any analysis of the obtained curves (shear stress vs strain rate). Maekaji[14] suggests that the stress of konjac gels is relaxed by a contraction in volume of the gel with a resultant release of squeezed water (syneresis). High mixing (700 rpm) is required to produce the beads and may be responsible for gel shear damage. The contraction of the konjac gel solidifies the droplets, thus prohibiting liquefaction of the beads and enzyme release.

Conclusions and the Future

Use of cold-leaking entrapment systems is a promising and innovative method to accelerate cheese ripening. The konjac flour polymer shows interesting cold-melting properties; however, its sensitivity toward shear stress prohibits its use as a cold-melting gel matrix for enzyme entrapment.

Konjac flour develops a strong synergism with κ–carrageenan. Konjac/carrageenan mixture (40/60 ratio) gives a stronger gel than a pure κ–carrageenan gel[5] (*Figure 1*). An addition of κ–carrageenan in konjac solution may avoid contraction of the gel network during bead production while not affecting the cold-melting properties of the konjac. This assumption is currently being investigated.

Poly–N–vinyl caprolactam also exhibits cold-melting properties. This polymer is water soluble, nontoxic, and

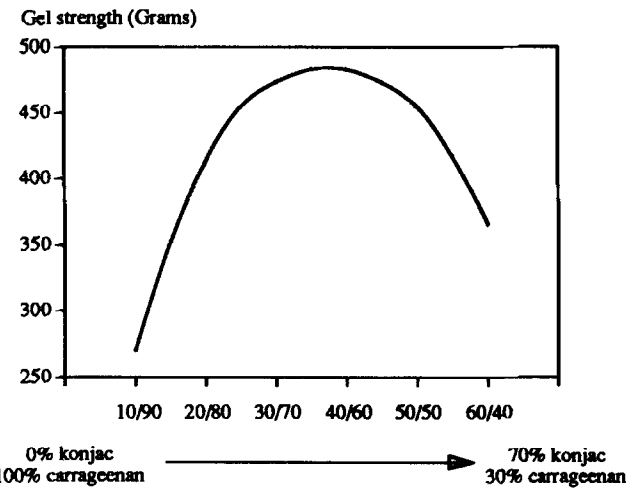

Figure 1 Ratio of konjac to carrageenan

cheap. The solution is thermally thixotropic (i.e., it gelifies at high temperature and has a low viscosity when the temperature decreases) and it is suitable to produce stable beads. Collaborative work is being carried out with Professor Zubov's group (Moscow, Russia) for enzyme encapsulation within poly–N–vinyl caprolactam beads.[15–17]

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