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In Vitro Analysis of Protection of the Enzyme Bile Salt Hydrolase against Enteric Conditions by Whey Protein−Gum Arabic Microencapsulation

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The interest in efficient intestinal delivery of health-promoting substances is increasing. However, the delivery of vulnerable substances such as enzymes requires specific attention. The transit through the stomach, where the pH is very low, can be detrimental to the enzymatic activity of the protein to be delivered. Here, we describe the microencapsulation of the model enzyme bile salt hydrolase (Bsh) using whey protein−gum arabic microencapsulates for food-grade and targeted enzyme delivery in the proximal region of the small intestine. Furthermore, the efficacy of enteric coating microencapsulates for site-specific enzyme delivery was compared in vitro with living *Lactobacillus plantarum* WCFS1 bacteria that endogenously produce the Bsh enzyme. Microencapsulates allowed highly effective protection of the enzyme under gastric conditions. Moreover, Bsh release under intestinal conditions appeared to be very efficient, although in the presence of pancreatin, the Bsh activity decreased in time due to proteolytic degradation. In comparison, *L. plantarum* appeared to be capable to withstand gastric conditions as well as pancreatin challenge. Delivery using encapsulates and live bacteria each have different (dis)advantages that are discussed. In conclusion, live bacteria and food-grade microencapsulates provide alternatives for dedicated enteric delivery of specific enzymes, and the choice of enzyme to be delivered may determine which mode of delivery is most suitable.

KEYWORDS: Bile salt hydrolase; encapsulation; microencapsulates; intestinal delivery; whey protein; gum arabic

INTRODUCTION

Microencapsulation technology can be used to protect substances from the environment by entrapment into another material. This technology has been used in the food and pharmaceutical industry for controlled release, enhancement of stability, and taste masking in applications such as drugs, vitamins, and minerals (for reviews, see refs *1* and *2*). Nowadays, the food industry is increasingly focusing on the incorporation of components that provide a health benefit to the customer in food products. The most common and noninvasive mode of administration of health-promoting substances is via the oral route. Therefore, the availability of systems for delivery of specific components into the intestinal tract is of importance. The intestinal delivery of enzymes deserves special attention, since only the slightest change in conformation of a protein can be detrimental to its enzymatic properties. However, the intestinal delivery of proteins or enzymes is severely hampered by denaturation and degradation of the protein in the highly acidic environment in the stomach.

Several methods have been proposed to protect therapeutic proteins during passage of the stomach, such as delivery using viable bacteria (*3*) and alginate−chitosan encapsulation (*4, 5*). However, not all encapsulation techniques can be used, as some require the use of high temperatures or organic solvents during the manufacturing process that in many cases prove to be detrimental to the enzyme. A promising system for enzyme encapsulation for intestinal delivery is the use of whey protein−gum arabic encapsulates using activated whey proteins (*6*) (*7*). Prior to the encapsulation procedure, activation of the whey proteins with a heat treatment leads to natural cross-linking via disulfide bridges. At the low pH conditions encountered during gastric transit, the activated whey proteins layer remains insoluble. Furthermore, the whey protein and gum arabic layers form an electrostatic complex below the isoelectric point of the protein (i.e., 5.2 for native whey proteins), leading to a tight network protecting the encapsulated enzyme at low pH. Subsequently, at the neutral pH of the duodenum, the coacervate network dissolves and the water-insoluble network of activated whey proteins slowly releases the encapsulated enzyme, with the release profile depending on the extent of cross-linking of

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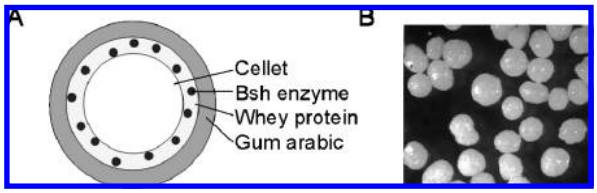

Figure 1. (**A**) Schematical representation of the Bsh−whey protein−gum arabic encapsulate. (**B**) Stereoscopic image of the Bsh−whey protein−gum arabic encapsulate (40× magnification).

the whey protein layer and on the presence of proteases in the environment. Furthermore, activated whey protein−gum arabic encapsulates are label-friendly and food-grade and are not associated with health and religion-derived dietary issues, which can not be claimed for the more commonly used gelatin−gum arabic coacervates (for examples, see refs *8*−*11*).

Using whey protein−gum arabic microencapsulates, a wide range of enzymes could be delivered into the small intestine. In this work, the delivery of enzymes protected by whey protein−gum arabic microencapsulates in the proximal small intestine was studied in vitro, using the model enzyme bile salt hydrolase (Bsh). Bsh is produced by the commensal bacterial inhabitants of the intestinal tract (*12*) (Lambert, unpublished results) and deconjugates the amino acid moiety of the bile salts that are produced in the liver and secreted into the proximal small intestine in vertebrates. Thereby, Bsh is an attractive model enzyme to study delivery in the proximal small intestinal tract, since its effective and active delivery would immediately affect the conjugation state of bile acids in this region of the small intestine. Thus, the Bsh enzyme was chosen to study the enteric delivery properties of whey protein−gum arabic microencapsulates, using in vitro conditions mimicking physicochemical conditions encountered during gastric transit and upon proximal small intestinal entry. This food-grade delivery concept aims to protect the enzyme during gastric conditions, while allowing dedicated release into the lumen of the proximal small intestine. Moreover, the delivery efficacy of Bsh by these microencapsulates was compared with delivery by the living model bacterium *L. plantarum* WCFS1 (*13*), which is a natural Bsh-producing strain (*14*). These experiments allow for the comparison and evaluation of enzyme delivery using whey protein−gum arabic microencapsulates and viable bacteria.

MATERIALS AND METHODS

Chemicals and Enzymes. Bsh enzyme of *Clostridium perfringens* (see refs *15* and *16* for enzymatic properties), pepsin (43 U/mg solid), and pancreatin (8× USP) were purchased from Sigma (St. Louis, MO). Whey protein isolate (Bipro) was purchased from Davisco (Eden Prairie, United States), and gum arabic (IRX 40693) was purchased from Colloïdes Naturels International (Rouen, France).

Preparation of Bsh−Whey Protein−Gum Arabic Encapsulates. Stomach-resistant Bsh encapsulates were prepared using a fluidized bed coater (FB-1, Glatt, Binzen, Germany) consisting of a core of inert 350−500 μm microcrystalline cellulose spheres (Cellets 350, Syntapharm, Mülheim an der Ruhr, Germany) on which Bsh, whey protein, and gum arabic were sprayed (**Figure 1A**). First, 60 g of a mixture of 7500 U (56 mg) powdered Bsh and 8% (w/v) heat-denatured whey protein (preheated at 90 °C at pH 7 for 30 min) was coated onto 300 g of Cellets at a product temperature of maximally 42 °C, 70−80 m^3/h of air flow, and 150 kPa of spraying pressure. Subsequently, a coating of 30 g of 20% (w/v) gum arabic (pH 7) (Colloïdes Naturels International, Rouen, France) was added at a product temperature of maximally 42 °C, resulting in the end product (**Figure 1B**).

Simulated Gastrointestinal Conditions. Gastric conditions were simulated by the incubation of 0.5 g of microencapsulate, 1 U of free Bsh enzyme, or the Bsh-producing strain *L. plantarum* WCFS1 (*13*) (for preparation, see below) in 1 mL of simulated gastric fluid (SGF), pH 4.5 (5 mM acetate buffer, pH 4.5; ionic strength, 70 mM), or SGF, pH 2 (5 mM phosphate buffer, pH 2; ionic strength, 70 mM), with or without 0.07 g/L pepsin (adapted from ref *17* and K. Venema, personal communication) for 15 min at 37 °C. Subsequently, the samples were transferred to intestinal conditions by the addition of 1 mL of prewarmed simulated intestinal fluid (SIF; 50 mM phosphate buffer, pH 7; ionic strength, 111 mM; *18*) with or without 1.25 g/L pancreatin at 37 °C. When no gastric incubation was performed, intestinal conditions were simulated by incubation in a mixture of 1 mL of SGF, pH 4.5, and 1 mL of SIF (which is a stronger buffer than SGF) with or without 1.25 g/L pancreatin at 37 °C.

***L. plantarum* Preparations.** For *L. plantarum* WCFS1 Bsh activity assays, an overnight culture grown in MRS broth (Difco, West Molesey, United Kingdom) at 37 °C without aeration was inoculated 1:20 in fresh MRS broth and grown to an OD_{600} of 3. Cells were harvested by centrifugation for 10 min at 4500 rpm at room temperature (Megafuge 1.0R, Heraeus, Hanau, Germany) and resuspended in the appropriate SGF buffer at a final OD_{600} of 50. Of this preparation, 1 mL was used for further assays. When no gastric incubation was performed, a mixture of equal amounts of SGF, pH 4.5, and SIF was used for resuspension of *L. plantarum* cells, and 2 mL of this preparation was used for further assays.

Bsh Activity Assays. To determine the Bsh activity level of Bsh microencapsulates, 20 μL of 0.1 M bile salt glycocholic acid (GC) was added immediately after transfer to intestinal conditions. At regular time intervals, 40 μL of sample was transferred to 120 μL of 70% (v/v) acetonitrile to stop the Bsh reaction and to precipitate proteins. High-performance liquid chromatography (HPLC) analysis of GC deconjugation was carried out as described below.

Influence of Pancreatin on Bsh Activity. The influence of pancreatin on the Bsh activity of microencapsulates, free enzyme, and *L. plantarum* WCFS1 was started either immediately or at regular time points after transfer to simulated intestinal conditions by addition of 20 μL of 0.1 M GC. After 30 min of incubation with GC at 37 °C, 40 μL of sample was transferred to 120 μL of 70% (v/v) acetonitrile to stop the Bsh reaction and to precipitate proteins. HPLC analysis of GC deconjugation was carried out as described below.

Release Properties of Bsh Encapsulate. The release of Bsh from the Bsh microencapsulates was studied by comparison of the input of enzymatic units of Bsh activity at encapsulate assay conditions and the output of activity during simulated intestinal conditions. For this, 0.5 g of Bsh encapsulate and 1 U of free Bsh enzyme (which was used as a reference), respectively, were incubated in 2 mL of prewarmed SIF for 90 min at 37 °C. Subsequently, 20 μL of 0.1 M GC was added. At several time points during incubation, 40 μL of sample was transferred to 120 μL of 70% (v/v) acetonitrile to stop the Bsh activity and to precipitate proteins. HPLC analysis of GC deconjugation was carried out as described below.

HPLC Analysis of GC Deconjugation. Prior to HPLC analysis, samples obtained during GC deconjugation assays were cleared of proteins and encapsulates by repeated centrifugation at 13200 rpm for 5 min at room temperature (Eppendorf AG, Hamburg, Germany). Subsequently, the supernatant was diluted 3.5-fold in double distilled water. GC deconjugation was determined by HPLC as described previously (*19*). Separations were carried out with a reversed-phase resin-based column (PLRP-S, 5 μm, 300 Å, 250 × 4.6 mm i.d., Polymer Laboratories, Shropshire, United Kingdom) and matching precolumn, using an acetonitrile gradient. Bile salts were detected using a pulsed amperometric detector (EG&G Princeton applied research, Princeton, NJ), equipped with a gold working electrode and a reference electrode (Ag/AgCl). Chromatograms were analyzed and integrated using the Chromeleon program (Dionex, Sunnyvale, United States), and the Bsh activity level was calculated on the basis of the disappearance of GC.

Microencapsulate Imaging Techniques. For stereoscopic imaging, Bsh microencapsulates were magnified 40 times using a Leica MZ16 stereoscope; images were captured using Leica IM imaging software (Leica, Rijswijk, The Netherlands). For microscopic imaging, microencapsulates were treated with SGF with or without pepsin or left untreated. Subsequently, at regular time intervals after transfer to

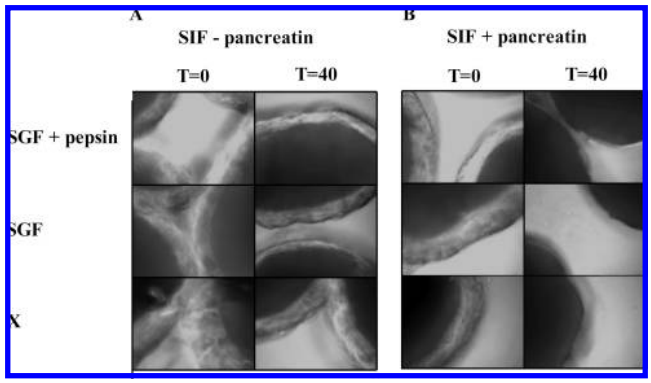

Figure 2. Microscopic images (312.5× magnification) of the Bsh−whey protein−gum arabic coat of the Bsh encapsulates. The Bsh−whey protein−gum arabic coating was visible as a transparent layer around the nontransparent cellets. When appropriate, Bsh encapsulates were pretreated in SGF with or without pepsin, and images were made at the start of the SIF incubation ($T = 0$) and after 40 min of SIF incubation ($T = 40$) in the absence (**A**) or presence (**B**) of pancreatin. Independent of the gastric pretreatment condition, the encapsulate coat was dissolved only in the presence of pancreatin during the SIF treatment. X, no pretreatment.

intestinal conditions, some microencapsulates were placed onto a glass slide, immersed in a small amount of SIF, and magnified 312.5 times using a Leica Dialux 20 microscope; images were captured using a COHU High Performance CCD camera and Leica QFluoro imaging software (Leica, Rijswijk, The Netherlands).

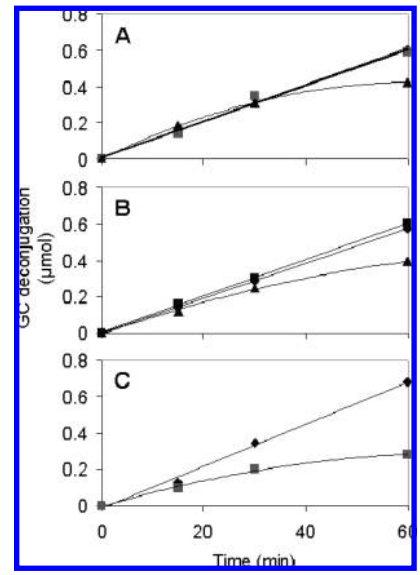

Figure 3. (**A**) GC deconjugation by 0.5 g of Bsh encapsulate in time following treatment in SGF with or without pepsin at pH 2. (**B**) GC deconjugation by 0.5 g of Bsh encapsulate in time following treatment in SGF with or without pepsin at pH 4.5. (**C**) GC deconjugation by 0.5 g of Bsh encapsulate in time without prior treatment in SGF. (**A** and **B**) Treatment with SGF, activity in SIF (◆); treatment with SGF with pepsin, activity in SIF (■); and treatment with SGF, activity in SIF with pancreatin (▲). (**C**) Activity in SIF (◆); and activity in SIF with pancreatin (■).

RESULTS AND DISCUSSION

Bsh Microencapsulation. Bsh microencapsulates were manufactured using the fluidized bed coating technique, applying a coat of a mixture of Bsh enzyme and heat-denatured whey protein onto microcrystalline cellulose cellets. The heat treatment caused the whey proteins to form a coating linked by disulfide bonds, lowering the total porosity of the whey protein layer and providing lowered solubility of the coating in water (*7, 20*). Subsequently, a coat of gum arabic was added (**Figure 1**), enabling the reversible formation of a protein−gum arabic coacervate (*6*) at low pH.

Microencapsulate Protection against Gastric Conditions. The influence of various gastrointestinal treatments on the physical appearance of the Bsh microencapsulates was monitored by microscopical analysis (**Figure 2A**). The physical appearance of the microencapsulates did not appear to be altered under gastric conditions (SGF) in either the presence or the absence of gastric pepsin (**Figure 2A**). Gastric conditions were applied by incubation of the Bsh encapsulates in SGF at pH 2 or pH 4.5, respectively, representing either the fasting or the fed state in humans (*17, 21*). In agreement with the physical appearance of the microencapsulates, the Bsh activity of the microencapsulates under simulated intestinal conditions in absence of pancreatin appeared to be independent of the type of gastric incubation condition used (i.e., incubation at pH 2 or pH 4.5 and in presence or absence of pepsin, or no gastric incubation), with Bsh activity ranging from 10 to 12 nmol/min (**Figure 3**). These findings indicate a strong protective effect of the whey protein−gum arabic coacervate against stomach conditions.

Intestinal Bsh Activity from Microencapsulates. In the absence of pancreatin during intestinal conditions, the amount of GC deconjugated increased linearly in time (**Figure 3**), indicating a constant rate of GC deconjugation. No lag phase in Bsh activity was observed; thus, it can be concluded that Bsh activity from the microencapsulates was available immediately following transfer to intestinal conditions, as desired. Notably, these findings were not reflected macroscopically (**Figure 2A**). In the absence of pancreatin, the encapsulate coating was not dissolved during intestinal conditions. Upon entrance of the encapsulates into the intestine, the increase in pH led to the decomplexation of the gum arabic and whey protein layer complex. In addition, at the pH of the intestine, the activated whey protein provided a less tight network than at gastric pH, enabling the bile salt substrate to permeate into the microencapsulate layer, without total release of the relatively large Bsh enzyme from the microencapsulate being necessary. Apparently, intestinal delivery of Bsh activity from microencapsulates is highly efficient, regardless of physical release from the microencapsulate.

Furthermore, Bsh activity from the encapsulates under intestinal conditions in absence of pancreatin was approximately 10% of the theoretical input. Because intestinal delivery of Bsh from the microencapsulates was highly efficient, the loss of activity was very likely due to processing conditions during the fluid bed coating procedure. The relative instability of the Bsh enzyme in solution (data not shown) may be of importance here, and activity retainment could probably be improved by reduction of processing time. In addition, the process temperature could be decreased to limit activity loss by heat damage, but as a consequence, preparation time would be increased. Alternatively, instead of the spraying of the Bsh enzyme onto cellets, where the Bsh enzyme remains in solution for a relatively long time, the Bsh enzyme could be incorparated into core particles prepared by an encapsulation method that involves reduction of the amount of water in the particles (such as extrusion). Subsequently, the encapsulate core containing the stabilized Bsh enzyme would be coated with a protective layer of activated whey protein and gum arabic. Furthermore, after 1 month of

dry storage at 4 °C, Bsh activity in the microencapsulates was fully retained (data not shown), illustrating the excellent shelf life properties of these encapsulates.

Influence of Pancreatin on Intestinal Bsh Activity. The presence of pancreatin during intestinal incubation had a major effect on the physical appearance of the Bsh encapsulates, as determined by microscopical analysis (**Figure 2A**,**B**). The digestive action of pancreatin totally dissolved the Bsh−whey protein−gum arabic layer of the microencapsulates, whereas in the absence of pancreatin, this layer remained intact. Thus, pancreatin may enhance release of enzymes from the microencapsulate layer.

In contrast, in the presence of pancreatin during intestinal conditions, the Bsh activity from microencapsulates was found to decrease parabolically (**Figure 3**). The pancreatin-dependent decrease of Bsh activity in time of the microencapsulates probably indicated that the Bsh enzyme was degraded by proteolytic enzymes present in pancreatin. Thus, pancreatin may have a dual action in delivery of enzymes in the small intestine. Pancreatin may enhance the release of enzymes from the microencapsulate layer, but it will also degrade the released enzyme. This is in agreement with the U.S. Pharmacopeia (*22*), where the use of pancreatin was found to be of importance for in vitro oral drug release assays.

Notably, the Bsh activity from the microencapsulates in presence of pancreatin did not exceed the activity in absence of pancreatin (**Figure 3**). Thus, in our experiments, total release of the Bsh−whey protein−gum arabic layer as observed during microscopical analysis (**Figure 2B**) appeared nonbeneficial for obtaining higher Bsh activity during intestinal conditions in the presence of pancreatin. This is in agreement with the finding that Bsh activity is available immediately after transfer to intestinal conditions in the absence of pancreatin and thus excluding physical release of the microencapsulate layer, as described above.

Furthermore, the formation of a coacervate layer during gastric incubation may provide protection against the proteolytic activity of pancreatin while still allowing for Bsh activity upon entrance into intestinal conditions. This suggestion is supported by the fact that a slightly larger decrease in bile salt deconjugation was found in response to the presence of pancreatin during intestinal conditions with no gastric preincubation as compared to gastric preincubation at pH 2 or pH 4.5 (**Figure 3**) (0.28, 0.42, and 0.39 μmol of GC hydrolyzed in 60 min, respectively). The protective effect of the formation of a whey protein−gum arabic coacervate layer on Bsh activity against pancreatin pressure was also found in a pancreatin resistance assay, where Bsh activity was monitored after various pancreatin incubation times with gastric preincubation at pH 2 or pH 4.5 in the presence of pepsin (**Figure 4A**). Here, gastric preincubation at pH 2 appeared to confer a slight advantage over preincubation at pH 4.5. However, more investigation is needed to establish this effect further and eventually unravel the underlying mechanism.

Gastrointestinal incubation of free Bsh (**Figure 4B**) clearly shows that only a small amount of Bsh activity was left after gastric incubation in the presence of pepsin (at assay starting time 0), with a more severe reduction of activity at gastric incubation at pH 2 as compared to pH 4.5. Clearly, the enzyme is broken down further by pancreatin activity. As expected, the use of free enzyme is not recommended for delivery of Bsh activity in the proximal small intestine.

However, an alternative to encapsulation of enzyme is the use of viable bacteria for delivery of enzymes in the small intestine (*3*). To evaluate this alternative, the bacterial strain *L. plantarum* WCFS1 that natively produces the Bsh enzyme intracellularly (*14*) and that is capable of surviving the gastrointestinal tract (*23*) was used as a model. Notably, the native amount of Bsh activity produced per ml of *L. plantarum* WCFS1 culture at an OD_{600} of 3 was 20 times lower as compared to activity per gram of encapsulate. Importantly, *L. plantarum* WCFS1 appeared to be highly pancreatin-resistant in time (**Figure 4C**). This characteristic may provide a particular advantage over encapsulate delivery. For *L. plantarum*, gastric incubation at pH 2 initially appeared to provide a minor negative effect on Bsh activity as compared to pH 4.5, with gastric incubation decreasing Bsh activity by approximately 50%. However, after 60 min of incubation, *L. plantarum* appeared to have recuperated from gastric conditions, showing no difference in Bsh activity for both gastric incubation treatments (**Figure 4C**). Thus, although bacterial delivery may be limited quantitatively, it appears to be a more stable means of delivery under the conditions of the small intestine as compared to delivery using encapsulates.

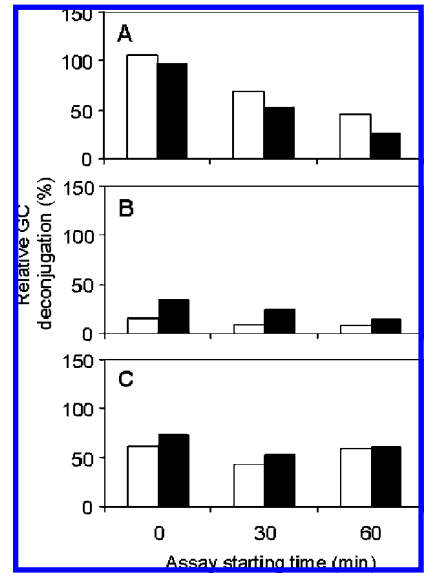

Figure 4. GC deconjugation in response to pancreatin pressure during simulated intestinal conditions. Following treatment with SGF containing pepsin for 15 min, the GC deconjugation levels of (**A**) Bsh encapsulate, (**B**) free Bsh enzyme, and (**C**) *L. plantarum* WCFS1, respectively, were determined in SIF containing pancreatin. To determine the effect of various pancreatin treatment times, the GC deconjugation assay was started at various time points after transfer to simulated SIF containing pancreatin. GC deconjugation levels are shown relative to the GC deconjugation in SIF without prior SGF treatment, which was taken as a reference condition (that is, 20 nmol/g/min for encapsulate, 1 nmol/ml/min for *L. plantarum* culture at an OD_{600} of 3, and 40 nmol/g/min for free Bsh, respectively). White bars represent pretreatment with SGF, pH 2; black bars represent pretreatment with SGF, pH 4.5.

Conclusion. Whey protein−gum arabic encapsulation is an excellent tool to protect enzymes during transit through gastric conditions and release enzyme activity in the proximal small intestine. However, following transfer to intestinal conditions, the enzyme is released by and exposed to the activity of pancreatin and other proteolytic activities in vivo. Because *L. plantarum* WCFS1 is capable of surviving gastric conditions and is able to withstand pancreatin pressure, it may be a good alternative for intestinal delivery of enzymes. However, several issues need to be considered. For example, the ease of handling of encapsulates in terms of storage and shelf life may be

preferred as compared to viable bacteria. Furthermore, the exact site of delivery using live bacteria is less well predictable as compared to microencapsulates. In addition, the expression level of enzymes is limited by the constraints on protein production in live bacteria. In microencapsulates, the amount of enzyme that can be incorporated is virtually unlimited due to the usually high activity-to-weight ratio of enzymes. However, especially in industrial systems, the cost of purified enzymes to be used in microencapsulates may be an important issue; use of crude enzyme extracts could in many cases provide cost reduction. Furthermore, the use of genetically modified organisms (GMOs) for both production and delivery of enzymes in the intestinal tract could offer several advantages. The expression level of enzymes could be improved using overexpression systems such as the food-grade NICE system (*24*), in combination with an appropriate food-grade selection marker (*25*), when desired. In addition, enzyme sequences could be optimized by strategies such as site-directed mutagenesis to improve stability during microencapsulation procedures, protease resistance, or activity level under intestinal conditions. GMO strategies may also be preferred for the delivery of human enzymes. Furthermore, the perception of oral intake of viable bacteria may be perceived differently as compared to encapsulates for use in the food industry. However, application of GMO-derived products in food, even when genetic modifications are performed using food-grade cloning strategies, are still subject to debate. Moreover, expression of some enzymes may only be achievable using genes obtained from nonfood-grade hosts. Thus, the system to be used for intestinal delivery of enzymes largely depends on the enzyme to be delivered.

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