Preparation and Characteristics of Rumen-Bypass Microcapsules for Improvement of Productivity in Ruminants

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Rumen-bypass microcapsules were prepared by a spray-dry method for protection against microbial hydrogenation in the rumen (neutral pH). Porous starch was used as the core material, and the microcapsules were prepared by a triple coating of Eudragit E100, AS-HF, and shellac. Capsules were generated with yield of about 48% and a mean particle diameter of 20−30 μm. The microcapsules had high stability in a neutral solution that mimicked a ruminal pH (pH 6.5). Moreover, when microcapsules were incubated in the presence of ruminal microorganisms, about 65% of the microcapsules were resistant to digestion in ruminal fluids, and protection of the inclusion substance was observed. In addition, the efficiency of release of these microcapsules was about 85% within only 30 min in the abomasal environment (pH 3.0).

Keywords: *Microcapsule; spray-dry method; rumen-bypass; shellac; resistance to ruminal microorganisms*

INTRODUCTION

Postruminal supplements of proteins and amino acids may increase growth and productivity in ruminants. McDonald (1948) showed that rumen microorganisms readily degrade some proteins to ammonia, a large part of which may then be absorbed and excreted as urea. Ammonia, which is produced by degradation of some proteins in the diet and of urea going into the rumen from the saliva and the vascularization of blood supply to the ruminal wall, is utilized as a potential nitrogen source for protein synthesis by most rumen microorganisms. Therefore, in ruminants, microbial protein synthesized in the rumen is a major protein source instead of the protein in the diet. That is, utilization of nutrients by the body in ruminants is very different from that in other mammals, and nonprotein nitrogen (NPN) in the diet can be used for all or part of the protein requirement of ruminants. A great number of feeding experiments have been made in different countries to find out how much protein can be successfully replaced by urea, which is readily decomposed to ammonia in the rumen. However, in the case of normal feeding with plenty of protein, the microorganisms in the rumen decompose the different proteins of the feed to ammonia, and microbial protein is again partly synthesized from the ammonia to maintain constant quantities (a leveling effect). Accordingly, since substantial amounts of dietary protein are lost to the animal, high-producing or rapidly growing animals may require more high-quality protein than that provided by rumen microorganisms. A means of increasing the amount of protein reaching the intestines, without reduction in its subsequent utilization by the host, would be of great potential value in ruminant nutrition. A possible way to achieve this would be protection of dietary protein from microbial attack in the rumen. That is, any protection method would need to leave the protein in a form capable of being digested and absorbed further along the alimentary tract so that the protein could be utilized. Partial protection of proteins has been achieved by heating (Chalmers et al., 1964), and it has been claimed that treatment with formaldehyde is effective (Minson, 1981). However, the nutritional value of both heated proteins (Danke et al., 1966) and formaldehyde-treated proteins (Schmidt et al., 1973) may be impaired.

On the other hand, coating individual particles with a protective envelope is a method that enables the intact proteins to reach the small intestine. Scott et al. (1969) have shown that it is possible to increase the proportions of polyunsaturated fatty acids in ruminant milk fat by feeding encapsulated fats and oils. Mowat and Deelstra (1972) have shown that encapsulation of methionine with kaolin is successful. In a previous study (Yoshimaru et al., 1997), we revealed that the microcapsulation method (spray-dry) using porous starch allows us to prepare target-specific microcapsules by selection of an appropriate coating agent. Therefore, we tried to prepare rumen-bypass microcapsules that can protect against microbial hydrogenation in the rumen (neutral pH), followed by the release of their contents in the abomasum (acidic pH).

EXPERIMENTAL PROCEDURES

Materials. Porous starch was supplied by San-ei Sucrochemical Co., Ltd. (Aichi, Japan). Eudragit E100, a synthetic acrylic copolymer, was supplied by Röhm Pharma GmbH (Darmstadt, Germany) and AS-HF was from Shin-etsu Chemi-

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cal Co., Ltd. (Tokyo, Japan). These are coating agents that are soluble under acidic and alkaline conditions, respectively. Shellac was obtained from Gifu-Shellac Co., Ltd. (Gifu, Japan). Protease YP-SS from *Aspergillus niger*, obtained from Yakult Medical Co. (Tokyo, Japan), was selected to evaluate resistance and release efficiency of the rumen-bypass microcapsule in a particular environment. All other chemicals were obtained from Nacalai Tesque (Kyoto, Japan) and were of analytical reagent grade.

Preparation of Rumen-Bypass Microcapsules. To protect the contents of rumen-bypass microcapsules from degradation in the rumen, we attempted to microencapsulate protease YP-SS from *Aspergillus niger* by the spray-dry method because of its convenience and high degree of reproducibility. To prepare the microcapsules, 300 g of porous starch with numerous holes of a few micrometers in diameter in each particle was added to 400 mL of a 50% solution (w/v) of protease YP-SS, and the mixture was stirred for 2 h. The solution was then sonicated for 5 min to integrate the protease YP-SS into the porous starch. After freeze-drying, the freeze-dried materials were coated with Eudragit E100 by combining with 600 mL of a 5% solution (w/v) of Eudragit E100 in ethanol. Further, the spray-dried microcapsules were coated with AS-HF to enhance stability in neutral conditions (AS-HF/E100 microcapsule). Finally, AS-HF/E100 microcapsules were coated with shellac, which has an excellent reputation as a coating material (Labhasetwar et al., 1989), to protect protease YP-SS against microbial proteolysis in the rumen (shellac/AS-HF/E100 microcapsule). The microencapsulated protease YP-SS was prepared using a CL-8 spray dryer (Ohgawara-Kakouki Co., Tokyo, Japan) equipped with a rotary atomizer nozzle, a nozzle speed of 10 000 rpm, and inlet and outlet air temperatures of 105 and 62−79 °C, respectively.

The external appearance of spray-dried microencapsulated protease YP-SS was examined in a scanning electron microscope (SEM; Hitachi, S-400). To mount microencapsulated particles for SEM, double-sided sticky tape was first attached to the specimen holder and then the tape was lightly sprinkled with the microcapsules. Specimens were coated with a gold−palladium layer using a SEM sputter coater (Hitachi, E102 Ion Sputter). Images of the specimen were obtained at an accelerating voltage of 3.0 kV.

Assay of Enzymatic Activity. Enzymatic activities were determined by a spectrophotometric method (Anson, 1938) by reference to a calibration curve for standard tyrosine solutions (20−80 μg/2 mL of 0.2 M HCl). For each assay, 1 mL of enzyme solution (suitably diluted so that the absorbance was in the range of the calibration curve) was incubated with 5 mL of a 0.6% (w/v) solution of Hammersten-casein (Wako Pure Chemicals Ltd., Osaka, Japan), which had been dissolved in a 0.75% solution of lactic acid (pH 3.0) as substrate, at 37 °C for 10 min. The reaction was then stopped by addition of 5 mL of a 0.11 M solution of trichloroacetic acid. After incubation at 37 °C for 30 min, the solution was filtered through filter paper (No. 5B, Advantec Toyo Co., Tokyo, Japan). Then 5 mL of a 0.55 M solution of Na_2CO_3 and 1 mL of Folin-Ciocalteu's reagent (Nacalai Tesque Co., Kyoto, Japan) were added to 2 mL of the filtrate, and the absorbance was measured at 660 nm with a spectrophotometer (UV-1200, Shimadzu Co., Kyoto, Japan). Blank assay was performed as follows. One milliliter of 0.1 M citrate buffer, which is a solvent of enzyme solution, was mixed with 5 mL of a 0.11 M solution of trichloroacetic acid, and a 0.6% (w/v) solution of Hammersten-casein was added. After incubation at 37 °C for 30 min, this solution was filtered, and absorbance was measured as above in order to obtain a blank value. Finally, the absorbance in the enzyme solution was corrected by subtracting the value obtained in a blank assay. One unit (U) was defined as the enzymatic activity that, in 1 min under the present assay conditions, liberated Folin-positive amino acids and peptides equivalent to 1 μmol tyrosine.

Efficiency of Encapsulation of Protease YP-SS into Microcapsules. Before measurement, 50 mg of microencapsulated protease YP-SS was ground by a mortar and a pestle in 1 mL of 0.1 M citrate buffer (pH 3.0). A suitable dilution of the resultant solution was assayed for enzymatic activity as described above. The encapsulation efficiency was calculated by expressing the amount of protease YP-SS encapsulated as a percentage of the initial amount of protease YP-SS used to prepare the microencapsulated enzyme.

Stability and Release Efficiency of Rumen-Bypass Microcapsules. The stability of microencapsulated protease YP-SS under pH and temperature conditions that are similar to those of the rumen was investigated. A 100 mg aliquot of the microencapsulated protease YP-SS was incubated at 38 °C with reciprocal shaking (100 strokes/min) in 15 mL of 0.1 M phosphate buffer (pH 6.5). The microcapsules were then collected by filtration and washed with distilled water. The collected microcapsules were dissolved completely in citrate buffer (pH 3.0) and assayed for the protease YP-SS activity that was retained after treatment with the neutral solution. The resistance of protease YP-SS was expressed as the amount of protease YP-SS activity retained as a percentage of the initial protease YP-SS activity.

The release of protease YP-SS from the microcapsules was evaluated as follows. A 100 mg aliquot of the microencapsulated protease YP-SS was incubated at 38 °C with reciprocal shaking (100 strokes/min) in 15 mL of 0.1 M citrate buffer (pH 3.0). At appropriate times, the solution was filtered and protease YP-SS activity in the filtrate was assayed.

Stability of Microcapsules during Anaerobic Culture with Ruminal Microorganisms. The stability of microcapsules to ruminal microorganisms was determined by the method of Tilley and Terry (1963). First, rumen fluid was collected from a cow fed a diet consisting of 60% forage and 40% commercial diet before the morning feed (at 9:00) and filtered immediately through a double layer of gauze. The rumen fluid was diluted 5 times with McDougall's artificial saliva that had been preaerated with CO_2 gas and maintained at 38 °C in a CO_2 atm. Anaerobic culture of ruminal microorganisms was performed as follows. A 500 mg aliquot of the microcapsules was incubated with 50 mL of the rumen fluid (diluted 5 times with McDougall buffer) at 38 °C in an Erlenmeyer flask stoppered with a Bunsen valve to prevent aerobiosis of the rumen fluid. At appropriate times, the solution was treated with 10 mL of formalin overnight, and the microcapsules were collected by filtration and dried under reduced pressure for 4 h at 60 °C. The collected microcapsules were then weighed and assayed for protease YP-SS activity. The resistance of microcapsules to ruminal microorganisms was evaluated from the quantity of microcapsules and protease YP-SS activity that was retained.

RESULTS AND DISCUSSION

Efficiency of Encapsulation and Morphology of Microcapsules. To prepare microcapsules that can pass through the rumen (neutral pH) with little degradation, we used porous starch (Suzuki, 1995) as the core material for its convenience and simplicity of microencapsulation. We have already reported that the microencapsulation method using porous starch allows preparation of target-specific microcapsules. However, despite preparation of microcapsules coated with Eudragit E100 that were easily soluble in acidic fluid (Kawata et al., 1986), the stability of these microcapsules was drastically reduced due to swelling in neutral media. Thus, we prepared rumen-bypass microcapsules that were further coated with AS-HF and shellac to improve stability and to protect then from ruminal microorganisms.

First, we investigated the efficiency of encapsulation of protease YP-SS into the microcapsules. As shown in Table 1, we found that protease YP-SS was generated with a yield of 52% in the case of AS-HF/E100 microcapsules and 48% in shellac/AS-HF/E100 microcapsules. From the efficiency (about 70%) of encapsulation of

Table 1. Encapsulation Efficiency of Protease YP-SS into the Microcapsules

condition of microcapsule	protease YP-SS activity (U/g)	trap I[a] (%)	trap II[b] (%)
start	20175	100	–
E100 microcapsule	14123	70	100
AS-HE/E100 microcapsule	10463	52	74
shellac/AS-HE/E100 microcapsule	9640	48	68

[a] Efficiency of encapsulation on the basis of protease YP-SS administered. [b] Efficiency of encapsulation on the basis of protease YP-SS into microcapsules coated with Eudragit E100.

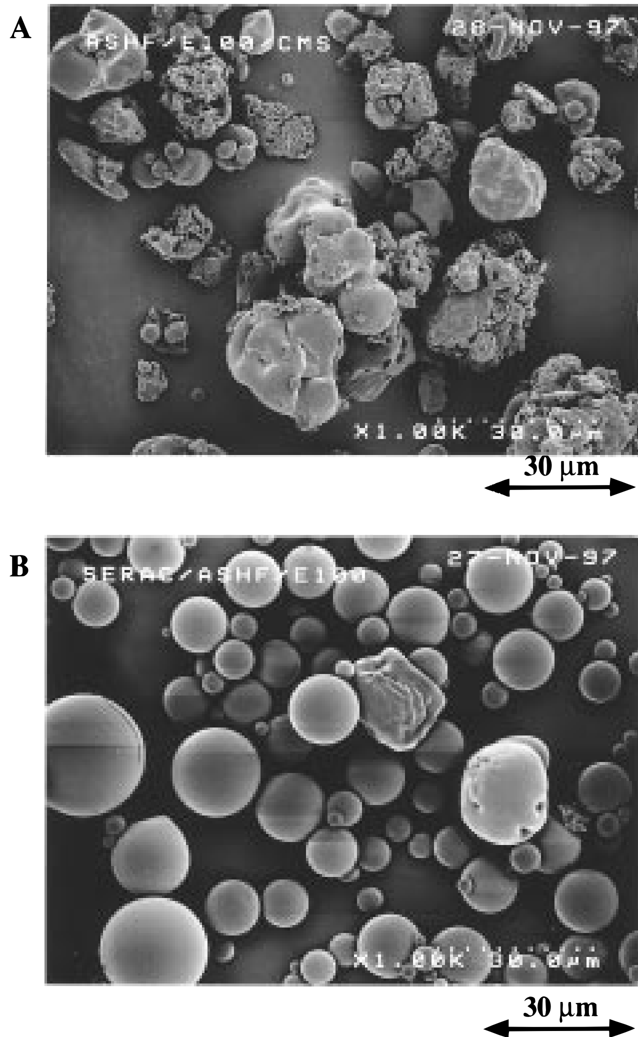

Figure 1. SEM of AS-HF/E100 microcapsules (A) and shellac/AS-HF/E100 microcapsules (B) prepared by the spray-dry method.

protease YP-SS into E100-microcapsules, we assumed that coating with AS-HF or shellac by the spray-dry method would efficiently preserve protease YP-SS and that protease YP-SS activity would hardly be affected by the ethanol used as the solvent for the shellac. Thus, the spray-dry method seems to be effective and applicable to the feed industry because of both its convenience and safety.

Typical scanning electron micrographs (SEM) of AS-HF/E100 microcapsules and shellac/AS-HF/E100 microcapsules are shown in Figure 1. Both prepared capsules had a mean particle diameter of 20–30 μm; shellac/AS-HF/E100 microcapsules were spherical and their surfaces almost smooth, while those of AS-HF/

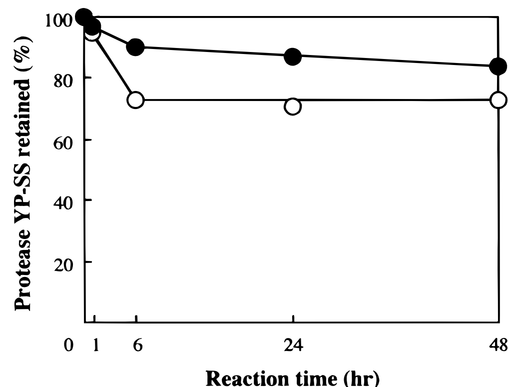

Figure 2. Stability of rumen-bypass microcapsules under conditions that mimic the rumen (38 °C and pH 6.5): AS-HF/E100 microcapsules (○); shellac/AS-HF/E100 microcapsules (●).

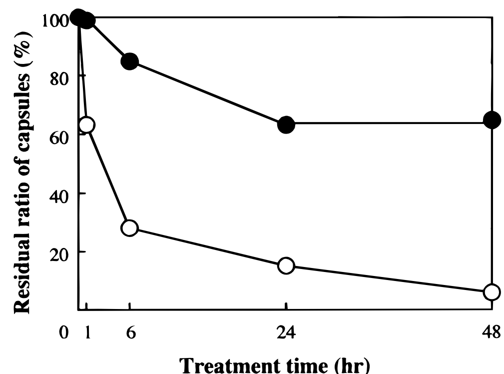

Figure 3. Time course of digestion of microcapsules by ruminal microorganisms: AS-HF/E100 microcapsules (○); shellac/AS-HF/E100 microcapsules (●).

E100 microcapsules were rough. In general, feeds must have a particle diameter of ≤1 mm in order to pass through the omasum. Therefore, since the microcapsules prepared by the spray-dry method were fine particles with a mean diameter of 20–30 μm, the particle size was not expected to hinder absorption by the omasum.

Evaluation of Rumen-Bypass Microcapsules for Resistance to Microbial Degradation in the Rumen. To investigate the resistance of rumen-bypass microcapsule to degradation in the ruminal environment, AS-HF/E100 microcapsules and shellac/AS-HF/E100 microcapsules were treated with a phosphate buffer (pH 6.5) that mimicked the environmental pH of the rumen. As shown in Figure 2, we observed high stability of each microcapsule in the neutral solution, even though the protease YP-SS activity retained in the microcapsules was slightly reduced during a 48 h incubation. Furthermore, protease YP-SS activity inside microcapsules was barely affected by various pH values around neutrality (pH 5.5–8.0) during the 48 h incubation. These results indicate that the inclusion substance should not be susceptible to damage in the environmental pH of the rumen and that it should pass through the rumen without loss of activity.

In addition, cultures of ruminal microorganisms were performed to determine whether the AS-HF/E100 microcapsules and shellac/AS-HF/E100 microcapsules were resistant to ruminal degradation. Figure 3 shows the variations in residual amounts of microcapsules after treatment with rumen contents. In AS-HF/E100 microcapsules, about 94% of AS-HF/E100 microcapsules were degraded during a 48 h treatment. By contrast, although

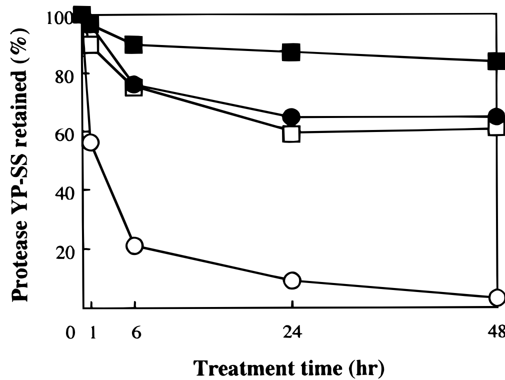

Figure 4. Protease YP-SS activity retained after digestion by ruminal microorganisms. AS-HF/E100 microcapsules: activity per gram (□); total activity (○). Shellac/AS-HF/E100 microcapsules: activity per gram (■); total activity (●).

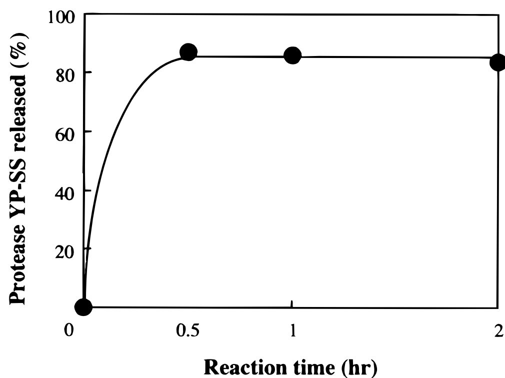

Figure 5. Release of protease YP-SS from shellac/AS-HF/E100 microcapsules under conditions that mimic the abomasal environment (38 °C and pH 3.0).

the residual amount of shellac/AS-HF/E100 microcapsules decreased linearly down to 35% in a 24 h treatment, the reduction then held constant. Consequently, we concluded that shellac used as coating agent might be useful and acceptable for protection from microbial degradation in the rumen.

To evaluate the resistance of the shellac/AS-HF/E100 microcapsules to digestion in the rumen, we next assayed the protease YP-SS activity that was retained after treatment with rumen fluid. Figure 4 shows the time course of enzyme activity per gram and total enzyme activity of the shellac/AS-HF/E100 microcapsules. As a result, these microcapsules retained 84% protease YP-SS activity during 48 h treatment with rumen fluids. In terms of total activity, the retention in the microcapsules was 20 times that of AS-HF/E100 microcapsules. Accordingly, it appears that shellac/AS-HF/E100 microcapsules had the potential to pass through the rumen without negative effects of the environmental pH of rumen and rumen microorganisms within its residence time of a few days.

Release Efficiency of Rumen-Bypass Microcapsule after Treatment with Ruminal Microorganisms. After passing through the rumen, which was tested by the culture experiments of ruminal microorganisms, these microcapsules must be released in an abomasal environment. With the goal of developing the rumen-bypass microcapsules for use in diet of ruminants, we examined the release of protease YP-SS from the microcapsules in an acidic solution (pH 3.0, citrate buffer) that mimicked the abomasal environment. As shown in Figure 5, about 85% of encapsulated protease YP-SS was released within 30 min in acidic fluid, and then the reaction proceeded only slowly. These findings suggest that these microcapsules permit passage of the inclusion substances through the rumen with little degradation and enable its subsequent release in the abomasum.

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