Efficiency and Protective Effect of Encapsulation of Milk Immunoglobulin G in Multiple Emulsion

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Milk immunoglobulin G (IgG), separated with protein G affinity chromatography, and IgG in colostral whey were encapsulated by 0.5% (w/v) of Tween 80, sucrose stearate, or soy protein, which were used as secondary emulsifiers in the water in oil in water type multiple emulsion. The residual contents of separated IgG and IgG in colostral whey, ranging from 58.7 to 49.7% and from 13.2 to 21.3%, respectively, in the inner water phase (water phase surrounded by oil phase) with emulsifiers were determined by ELISA. However, the emulsion stability decreased after 24 h, and the residual IgG content in the inner water phase was lowered. Encapsulation of IgG in the multiple emulsion increased the stability of separated IgG against acid (pH 2.0) and alkali (pH 12.0) by 21−56% and 33−62%, respectively, depending on the emulsifier used. Moreover, multiple emulsion also provided a remarkable protective effect on separated IgG stability against proteases. The residual contents of separated IgG in multiple emulsion, using Tween 80 as secondary emulsifier, incubated for 2 h with pepsin (pH 2.0) and trypsin and chymotrypsin (pH 7.6) (enzyme/substrate = 1/20) were 35.4, 72.5, and 82.3%, whereas those of separated IgG in enzyme solution were only 7.2, 33.1, and 35.2%, respectively. However, the separated IgG loss during the preparation of multiple emulsion was almost 41−50%.

Keywords: *Milk IgG; multiple emulsion; protection; encapsulation*

INTRODUCTION

Milk contains some factors that prevent the growth of infectious agents (Hanson et al., 1988; Pickering and Marrow, 1993). Among these, immunoglobulins are the most effective factor against pathogens, which can bind to specific sites of the most infectious agents or their products and result in the inactivation of these agents or compounds. Therefore, passive immunization using bovine immunoglobulins have been proved to be effective in human clinical trials against specific enteropathogenic (Mietens et al., 1979) and enterotoxigenic (Tacket et al., 1988) *Escherichia coli*, rotavirus (Hilpert et al., 1987), and S*higella flexneri* (Tacket et al., 1992), and suggestions have been made to fortify the immunity of young children by adding the immunoglobulins to infant formula and other foods (Goodman, 1989; Facon et al., 1993).

Bovine immunoglobulin G (IgG) in colostrum is estimated to be ∼50 mg/mL and is composed of 80% IgG_1 (Jenness, 1988). However, it is limited in quantity. Li-Chan et al. (1994) have reported surveys on the levels of IgG antibody in cow's milk and indicated that the IgG content in normal milk was 0.03−0.71 mg/mL from 254 samples in Canada from 1990 to 1991. Musher et al. (1990) have pointed out that 50 ng of specific antibody is sufficient to protect a mouse against a lethal dose of *Streptococcus pneumoniae*. On the basis of body weight, an estimated 50 μg of a specific antibody is required for a child weighing 20 kg to fight against the same bacterial infection.

However, immunoglobulins are liable to denaturation under pH values <3 or >10 (Shimizu et al., 1988; Cheng and Chang, 1998). To preserve the immunoprophylatic or therapeutic potential of immunoglobulins, it was important to consider the stability of immunoglobulin during storage and processing and upon ingestion. Ou-Yang (1997) has reported that microencapsulation using 5% gum arabic as filming material was effective in protecting immunoglobulin in chicken yolk (IgY) from enzymatic hydrolysis. Multiple emulsions, such as water in oil in water type (W/O/W), have been employed to encapsulate and protect substances, such as vitamins and minerals, from inactivation during storage in the pharmaceutical field (Owusu et al., 1992; Dickinson et al., 1994). Recently, Shimizu and Nakane (1995) have prepared W/O/W multiple emulsions in which IgY, rabbit serum IgG, α-amylase, and lysozyme were entrapped in the inner water phase, and the rate of protein inactivation after encapsulation was investigated.

In an attempt to study the protective effect of emulsion on milk IgG stability against acid, alkali, and proteases, multiple emulsions (W/O/W type) were prepared using different proteins and emulsifiers as secondary emulsifiers. The encapsulation efficiency of IgG in the inner water phase as well as the protective effects against acid (pH 2.0), alkali (pH 12.0), and proteases, such as pepsin, trypsin, and chymotrypsin, were compared, and the changes in the entrapped IgG after incubation at room temperature were also investigated.

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10.1021/jf9809075 CCC: $18.00
Published on Web 01/29/1999

MATERIALS AND METHODS

Materials. Bovine colostrum was collected, immediately after milking, within 6 days of postpartum from the Taiwan University dairy herd and was kept frozen at −20 °C until required. The frozen colostrum was thawed in flowing tap water and was subsequently centrifuged (10000*g*, 30 min, 4 °C) to remove the upper cream layer; thereafter, the pH of the colostrum was adjusted with 1 N HCl to pH 4.6. Thus, the colostrum obtained was kept at 40 °C for 30 min in a water bath to facilitate curd formation. Centrifugation was performed again to collect the clear whey. The pH of the obtained whey was soon raised to 7.0 by 1 N NaOH to protect the immunoglobulins from denaturation. Subsequent centrifugation was repeated to remove the suspended precipitants formed during the pH neutralization, and the supernatant (4.8 mg of IgG/mL) finally collected was used as the experimental material. IgG concentration was determined with ELISA method.

The whole milk used was from a commercial product (Klim Co., Taiwan). Rabbit anti-bovine IgG whole serum, bovine serum IgG, *p*-nitrophenyl phosphate, agarose type IV, and alkaline phosphatase conjugated rabbit anti-chicken IgG were purchased from Sigma Chemical Co. (St. Louis, MO). Radial immunodiffusion plates were purchased from Fisher Scientific (Ottawa, ON, Canada). All other reagents were of analytical grade.

Separation of IgG from the Colostrum. IgG was prepared according to the method described by Akerstrom et al. (1985) and Fredriksson and Nilsson (1987). Protein G Sepharose 4 Fast Flow (Pharmacia, Uppsala, Sweden), packed in the column (10 ×1.0 cm inner diameter) and equilibrated with binding buffer (0.02 M phosphate buffer, pH 7.0), was applied with 4 mL (∼19 mg of IgG/mL) of colostral whey and was subsequently eluted with binding buffer until its absorbance at 280 nm was ∼0. Glycine buffer (0.1 M glycine, pH 2.8) was then applied to the column, and the collected effluent was neutralized with 1 M Tris-HCl (pH 9.0), previously added in the test tubes, to a pH of ∼7.0, to protect IgG from denaturation. The IgG thus obtained was dialyzed against 100 volumes of PBS (0.15 M NaCl/0.01 M phosphate buffer, pH 7.0) at 4 °C for 24 h and was then freeze-dried. The IgG content and the total protein concentration in PBS was determined by ELISA (Kummer et al., 1992) and Bio-Rad protein assay dye reagent (Bradford, 1976), respectively. The IgG solution thus obtained, with a recovery and purity of 97.1 and 86.6%, respectively, was freeze-dried and frozen (−20 °C). The separated IgG powder was dissolved to a final concentration of 0.5 mg/mL in PBS (pH 7.0), determined by ELISA, before being applied to the following stability studies.

Preparation of W/O/W Multiple Emulsion. Multiple emulsion was prepared according to the method described by Shimizu and Nakane (1995). Five milliliters of separated IgG in PBS (pH 7.0) (0.5 mg/mL) or colostral whey (0.82 mg of IgG/mL; 12.7 mg of protein/mL) was added slowly into 5 mL of soybean oil (containing 0.5% Span 80, Sigma Chemical Co.) in a test tube (30 × 100 mm) and was homogenized at 250000 rpm for 5 min by a Polytron (PT 1200, Kinematic AG, Switzerland), using a shaft of 5 mm i.d., in an iced bath to prepare W/O emulsion. The emulsionthus obtained was then poured slowly into 10 mL of 0.5% protein (whey, egg yolk, soy protein, and casein, Sigma Chemical Co.) or emulsifier (Tween 80 and sucrose stearate, Sigma Chemical Co.) solutions to form W/O/W emulsions while being homogenized at 10000 rpm for 3 min in an iced bath.

Stability of IgG in Multiple Emulsion. Multiple emulsions were diluted 10-fold with distilled water, and then the pH was adjusted to 2 and 12 with adequate volumes of 0.1 N HCl and NaOH, respectively. The diluted emulsions thus obtained were incubated in a water bath at 37 °C for 2 h with shaking. Finally, the pH values were neutralized to 7.0 with 2 M Tris (Sigma Chemical Co.), and the residual IgG content was determined by ELISA.

The enzymatic stability of IgG in the inner water phase of multiple emulsion was determined by adding 1 mL of multiple emulsion to 9 mL of pepsin (pH 2.0) or trypsin or chymotrypsin (Sigma Chemical Co.) (0.05 M phosphate buffer, pH 7.6) solution (enzyme/substrate = 1/20) to react at 37 °C for 2 h. Solid Tris base was then added to stop the pepsin reaction by raising the pH to 8.0, whereas 1/10 volume of 1.25% (w/v) *N*-ethylmaleimide was used to stop the trypsin and chymotrypsin reaction (Weir et al., 1986; Hilpert et al., 1987).

Determination of Protein. The protein content was determined according to the method described by Bradford (1976), using the Bio-Rad protein assay dye reagent. Various concentrations (100−500 μg/mL) of bovine serum IgG (Sigma Chemical Co.) were used to obtain the calibration curve.

Encapsulation Efficiency of W/O/W Emulsion. To select adequate emulsifiers for multiple emulsion, glycine (Sigma Chemical Co.) was previously added to the inner aqueous phase as a marker to estimate the encapsulation efficiency. The multiple emulsion obtained was 26-fold diluted with 0.5% of NaCl solution and filtered by a membrane filter (0.45 μm) to remove the oil droplets. Fifty microliters of filtrates wss mixed with 1.85 mL of 50 mM borate buffer (pH 8.5) and 150 μL of 1.14 mM fluorescamine (Sigma Chemical Co.)/acetone solution. The mixture was vigorously stirred for 20 s, and the fluorescene derived from the reaction between glycine and fluorescamine was measured at the excitation wavelength at 390 nm and the emission wavelength at 475 nm using a fluorospectrophotometer (F-4500, Hitachi Ltd., Japan). Encapsulation efficiency was calculated by subtracting the glycine present in the outer aqueous phase from the total amount of glycine (Shimizu and Nakane, 1995).

Residual Content Percentage of Encapsulated IgG. Samples (0.35 mL) of W/O/W multiple emulsion were mixed well with 9.65 mL of 50 mM phosphate buffer (containing 0.5% sodium deoxycholate and 0.01% lipase, pH 8.0), and then the mixtures were incubated at 37 °C for 1 h before centrifugation (2000*g*, 3 min) to remove the oil phase. Residual IgG content in the aqueous phase was measured by ELISA. The IgG content was not affected by the presence of sodium deoxycholate or lipase (Shimizu and Nakane, 1995) from the results of the pretest. Residual IgG content (%) = (content of IgG in inner water phase/total IgG content in starting material) × 100%.

Determination of IgG Concentration. Enzyme-linked immunosorbent assay (ELISA) (Kummer et al., 1992) was carried out to quantify the IgG contents in IgG solution and colostral whey samples during encapsulation after 24 h of incubation. To analyze the residual content of IgG, standard curves for ELISA were constructed using bovine serum IgG (Sigma Chemical Co.) of known concentration as determined by Bio-Rad protein assay dye reagent (Bradford, 1976), to prepare a standard solution of IgG in PBS and colostral whey. Fresh raw colostral whey, the IgG concentration of which was previously assayed by single radial immunodiffusion assay (SRID) (Fukumoto et al., 1994), was used for subsequent ELISA analysis of IgG in the inner water phase of multiple-emulsion samples. Triplicate samples of each encapsulating preparation were twice analyzed for IgG by ELISA.

Statistical Analysis. The data were analyzed by analysis of variance (ANOVA). The mean difference was determined using the least significant difference (LSD) multiple-range test, whereas significance of difference was established at $p < 0.05$.

RESULTS AND DISCUSSION

Encapsulation Efficiency. Various emulsifiers and proteins were used as secondary emulsifiers to determine the encapsulation efficiency of glycine in the W/O/W multiple emulsion. The results (Table 1) showed that Tween 80 exhibited highest efficiency (65.8%) followed by sucrose stearate (62.6%) and soy protein (61.2%) at the time the multiple emulsions were prepared (0 h). However, glycine entrapped in the inner water layers declined by ∼10−12% as the holding time increased to 24 h, displaying the trend that emulsion stability decreased with increasing holding time.

In the IgG multiple-emulsion test, the highest residual IgG content percentage was in the emulsion

Table 1. Encapsulation Efficiency of Glycine in W/O/W Multiple Emulsion Using Proteins and Emulsifiers as Secondary Emulsifiers ($n = 3$)

	glycine entrapped[a] (%)	
emulsifier	0 h	24 h
whey	54.3 ± 2.5[bc]	42.5 ± 3.1[c]
casein	49.7 ± 1.8[bc]	37.8 ± 1.1[d]
Tween 80	65.8 ± 2.1[a]	57.4 ± 1.7[ab]
soy protein	61.2 ±1.0[ab]	49.8 ± 3.4[bc]
egg yolk	42.9 ± 1.6[c]	33.7 ± 2.5[d]
sucrose stearate	62.6 ± 1.4[a]	50.6 ± 2.3[bc]

[a] Calculated by subtracting the amount of glycine present in the aqueous phase from total glycine amount. Means ± standard deviations with different letters are significantly different ($p < 0.05$).

Table 2. Residual Content Percentage of Separated IgG (0.5 mg of IgG/mL) in PBS[a] and IgG (0.82 mg of IgG/mL) in Colostral Whey in W/O/W Multiple Emulsion Using Proteins and Emulsifiers as Secondary Emulsifiers ($n = 3$)

	residual IgG content[b] (%)		
	separated IgG in PBS[a]		IgG in colostral whey
emulsifier	0 h	24 h	0 h
Tween 80	58.7 ± 1.7[a]	49.6 ± 2.6[ab]	13.2 ± 0.4[c]
soy protein	49.7 ± 0.8[ab]	38.3 ± 1.1[b]	14.6 ± 0.9[c]
sucrose stearate	56.1 ± 1.8[a]	43.9 ± 2.2[ab]	21.3 ± 0.8[b]

[a] 0.15 M NaCl/0.01 M phosphate (pH 7.0). [b] Calculated by subtracting the content of IgG present in the aqueous phase from total IgG content. Means ± standard deviations with different letters were significantly different ($p < 0.05$).

prepared by Tween 80 followed by sucrose and soy protein (Table 2). The IgG contents in the inner water phase were observed to be 50−59% soon after the emulsion preparationn; however, a decrease of 38−50% was observed after 24 h of incubation at ambient temperature (Table 2). The loss of IgG content by encapsulation could be partly due to the adsorption of IgG in the oil/water interfaces, which resulted in the denaturation of proteins (Shimizu and Nakane, 1995), and partly due to the shearing force during the emulsion preparation (Cheng and Chang, 1998). The emulsifying stability could be improved by adding thickening agents such as vegetables gums, gelatin, or viscous agents to strengthen the oil/water interfaces (Dickinson et al., 1994). IgY and rabbit serum IgG were entrapped in the inner water phases of the W/O/W emulsions, using polyglycerol condensed ricinolate as the secondary emulsifier, and the residual content was found to be as low as 20 and 30−40%, respectively (Shimizu and Nakane, 1995). The difference between the above two encapsulation results was considered to be due to the difference in the molecular stability and hydrophobicity of the protein surface (Shimizu and Nakane, 1995). The residual IgG contents in colostral whey were all observed to be lower than those in PBS (Table 2). A high protein content in colostral whey was considered to cause possible protein−protein interactions, such as disulfide exchange, between IgG and other protein components during emulsion preparation (Deng et al., 1976; Peng et al., 1982).

Stability of IgG in Multiple Emulsion. The pH of the multiple emulsion prepared with separated IgG was adjusted to 2.0 and 12.0, and the residual IgG content was determined by ELISA after 2 h of incubation. As shown in Table 3, sucrose stearate showed the most satisfactory residual IgG content percentages, 68.2 and

Table 3. Residual Content Percentage of Separated IgG (0.5 mg of IgG/mL) in PBS[a] in W/O/W Multiple Emulsion at pH 2 and 12[b] for 2 h ($n = 3$)

	residual IgG content[c] (%)	
emulsifier	pH 2.0	pH 12.0
Tween 80	42.8 ± 1.3[b]	44.3 ± 3.1[b]
	(25.1 ±1.1)	(26.0 ± 1.2)
soy protein	33.2 ± 2.1[b]	54.8 ± 0.6[a]
	(16.5 ± 1.0)	(27.2 ± 0.5)
sucrose stearate	68.2 ± 2.4[a]	73.4 ± 1.7[a]
	(38.3 ± 1.5)	(41.2 ± 1.2)
control[d]	12.3 ± 0.7	11.5 ± 0.3

[a] See footnote *a* in Table 2. [b] pH was adjusted with 0.1 N HCl or NaOH. [c] Residual IgG content was the same as in Table 2. Values in parentheses indicate the residual IgG content percentage calculated from the starting materials, and those not in the parentheses are the relative residual IgG content percentage calculated from the residual IgG content shown in Table 2. Means ± standard deviations with different letters were significantly different ($p < 0.05$). [d] IgG was treated with acid or alkali without emulsification.

Table 4. Residual Content Percentage of Separated IgG (0.5 mg of IgG/mL) in PBS[a] in W/O/W Multiple Emulsion Hydrolyzed with Proteases (E/S = 1/20) for 2 h ($n = 3$)

	residual IgG content[b] (%)		
emulsifier	pepsin (pH 2.0)	trypsin (pH 7.6)	chymotrypsin (pH 7.6)
Tween 80	35.4 ± 0.9[ab]	72.5 ± 3.4[a]	82.3 ± 2.5[a]
	(20.8 ± 0.6)	(42.6 ± 1.2)	(48.3 ± 1.2)
soy protein	26.8 ± 1.3[b]	74.2 ± 2.9[a]	80.3 ± 2.1[a]
	15.7 ± 0.8)	(36.9 ± 1.2)	(39.9 ± 1.4)
sucrose stearate	43.8 ± 3.0[a]	69.4 ± 3.4[a]	78.4 ±1.6[a]
	(24.6 ± 0.5)	(38.9 ± 1.3)	(44.0 ±1.5)
control[c]	7.2 ± 0.4	33.1 ± 0.9	35.2 ± 0.7

[a] See footnote *a* in Table 2. [b] Residual IgG content was the same as in Table 2. Values in parentheses indicate the relative IgG content percentage calculated from the starting materials, and those not in parentheses are the relative residual IgG content percentage calculated from the residual IgG content shown in Table 2. Means ± standard deviations with different letters were significantly different ($p < 0.05$). [c] IgG in PBS was treated with enzyme without emulsification.

73.4%, after incubation at pH 2.0 and 12.0, respectively. However, only 38.3% (pH 2.0) and 41.2% (pH 12.0) of the original IgG content in the starting material were detected. The loss of IgG could be due to the denaturation that resulted from the shearing force during emulsion preparation, the adsorption of IgG to the oil/water interfaces, and the pH instability (Chen and Chang, 1998). The residual IgG content was only 12% when the separated IgG was incubated in solution at pH 2.0 or 12.0 for 2 h (Table 3). It was thus obvious that the oil phase in the multiple emulsion was remarkably effective in preventing contact between IgG and acid or alkali. Tween 80 displayed high encapsulation efficiency (Tables 1 and 2) but did not show better protective effect against acid or alkali than sucrose stearate. The protective effect of the multiple emulsion was remarkable because it increased the residual IgG content to 3 times as high as control.

Multiple emulsion also exhibited a marked protection effect against proteases. As seen in Table 4, residual content of IgG in pepsin solution (pH 2.0) was observed to be 7.2% after 2 h of incubation at room temperature, whereas that of IgG in the multiple emulsion was as high as 35.4%. However, ∼15% of IgG was denatured during the emulsion preparation and, therefore, the net residual IgG content (20.8%) was ∼3 times as high as control. In the case of trypsin and chymotrypsin, mul-

tiple emulsion also provided a protective effect for IgG against these two proteases, but significant decreases in IgG contents were observed during the multiple-emulsion preparations.

CONCLUSION

Encapsulation efficiencies of glycine in the inner water phase of W/O/W multiple emulsions were determined in the present study. The leakage of glycine was estimated to be 43−50% using Tween 80, soy protein, and sucrose stearate as secondary emulsifiers after 24 h of incubation. The separated IgG in PBS and IgG in colostral whey were then encapsulated in the multiple emulsions, and the residual IgG content percentages were determined by ELISA. It is interesting to note that the protective effects of multiple emulsion on IgG stability against acid, alkali, and proteases were remarkable, suggesting its possible use in oral administration of immunoglobulins. IgY was reported to be more liable than serum IgG to conformational changes and denaturation induced by acid (Cheng and Chang, 1998). Thus, the application of the multiple emulsion to the IgY from eggs laid by specifically immunized hens is greatly expected to protect against acidic denaturation upon ingestion. However, the emulsion stability and, especially, the destruction of IgG during the encapsulation steps still needd to be improved. Besides, experimental conditions for multiple emulsification need modification. The addition of thickening agents will probably be effective in the prevention of the leakage of IgG during incubation and seems worthy of investigation.

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Received for review August 13, 1998. Revised manuscript received November 16, 1998. Accepted November 16, 1998.

JF9809075