Productivity and Some Properties of Immunoglobulin Specific against *Streptococcus mutans* Serotype c in Chicken Egg Yolk (IgY)

Hung Min Chang,*,† Ray Feng Ou-Yang,† Yu Tang Chen,† and Chao Cheng Chen‡

Graduate Institute of Food Science and Technology, National Taiwan University, Taipei 106, Taiwan, and Department of Food Health, Chia-Nan College of Pharmacy, Tainan 717, Taiwan

Hens were immunized on thighs by using whole cells of *Streptococcus mutans* MT8148 serotype c strain as antigen through intramuscular (im) and subcutaneous (sc) routes to investigate the difference of immunization reactions and the changes in yolk antibody activities against antigen after initial immunization. Several properties of crude IgY were examined to evaluate the stability during food processing. Results showed that the specificity of IgY of im treated hens was nearly 10 times as high as those of sc treated antibody. IgY from the hens immunized with the serotype c strain showed significant cross-reactions against serotypes e and f, while minor reactions against serotypes a, b, d, and g were observed. In thermal stability tests, IgY activity in both yolk and crude IgY decreased with the increasing temperature, from 70 to 80 °C, but the thermal denaturation rates between those two samples were not significantly different. The addition of high levels sucrose, maltose, glycerol, or 2% glycine displayed effective protection against thermal denaturation of IgY. Lyophilized yolk−5% gum arabic powder showed better stability against proteases.

Keywords: *Immunoglobulin in yolk (IgY); immunization; cross-reaction; thermal stability; gum arabic*

INTRODUCTION

Bovine milk immunoglobulins have immunoprophylatic or therapeutic potential which can be used as immunological supplements in infant formulas and other foods (Goldman, 1989; Facon et al., 1993). The ingestion of specific antibodies from the colostrum of immunized cows was proven to be effective in the prevention of *Escherichia coli* (Mietens et al., 1979; Tacket et al., 1988), rotavirus (Ebina et al., 1985; Hilpert et al., 1987), cryptosporidum (Tzipori et al., 1986), and *Shegella flexneri* (Tacket et al., 1992). Although colostral milk contains approximately 50 mg/mL of immunoglobulins, it is not available in large amounts, which makes it an unreliable source of immunoglobulins. Thus finding other sources, such as egg yolk, would be very beneficial (Shimizu et al., 1988).

The high immunoglobulin content (approximately 10 mg/mL) in the yolk (Otake et al., 1991), the ease of its collection, as well as the large quantities of chicken eggs available make IgY an potential source for immunological supplementation of foods. Many researchers (Bartz et al., 1980; Hamada et al., 1991; Hatta et al., 1993) have already developed chicken eggs with specific antibodies from immunized hens.

Dental caries is perhaps the most prevalent disease affecting humans today (Scherp, 1971), and it is mainly caused by the *Streptococcus mutans* which forms dental plaque (Gibbons and Van Houte, 1975). Preventive methods of active and passive immunizations have been suggested by many investigators (Lehner et al., 1978, 1981; Michalek and Mcghee, 1977). Due to the excessive side effects of active immunization induced by the causative bacterium (Hughes et al., 1980), passive immunization with egg yolk antibody (immunoglobulin in yolk, IgY) is preferred.

In this study, eggs from hens im or sc immunized with *S. mutans* serotype c were collected, and the productivity of this antibody against *S. mutans* c using different routes of immunization was compared. Furthermore, to preserve the immunoprophylactic or therapeutic potential of IgY for human clinical trials against dental caries, it is important to consider the IgY stability during storage or following processing methods involving thermal treatments such as pasteurization, sterilization, or spray drying. Although studies investigating the thermal stability of IgY have been reported (Shimizu et al., 1988; Otani et al., 1991), there is little information available on the specific antibody activity for IgY.

MATERIALS AND METHODS

Preparation of Antigen. The *S. mutans* MT8148 c strain used as antigen was cultured for 18 h in brain−heart infusion broth (Difco Laboratories, Detroit, MI) containing 5% sucrose at 37 °C under aerobic condition. The microorganisms were treated with 0.5% formalin for 24 h and then collected by centrifugation (10000*g*, 15 min). The pellets were washed three times with sterile saline containing 0.5% formalin and resuspended in sterile saline using a Vortex mixer. The microrganism samples were kept frozen at −20 °C until required after the concentration adjustment to 1×10^9 CFU/mL.

S. mutans a, b, d, e, f, and g were cultured in the same way and used as antigens to react with antibody against *S. mutans* c. The cross-reaction tests were conducted using the ELISA method as described below.

Immunization of Hens. *S. mutans* c samples, mixed homogeneously with an equal volume of Freund's incomplete adjuvant, were used as antigen and were intramuscularly or subcutaneously injected into both legs of 10 hens (Shimizu et

* Author to whom correspondence should be addressed (phone, +886-2-2363-0231 ext 2776; fax, +886-2-2362-0849).
† National Taiwan University.
‡ Chia-Nan College.

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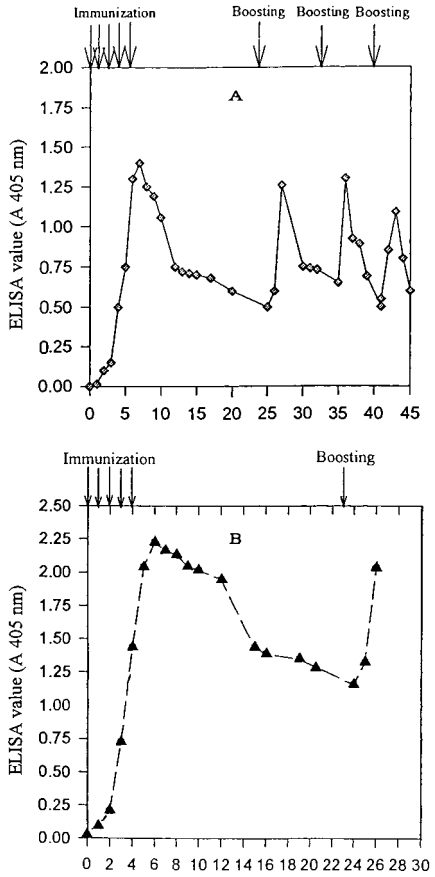

Figure 1. Changes of egg yolk antibody activities against *S. mutans* during the immunization period. Ten hens were im (A) and sc (B) immunized, respectively, with *S. mutans* MT8148 serotype c once a week for 4 weeks after initial immunization. Boostings were performed at 24, 35, and 41 weeks (A) and 24 weeks (B). Egg yolks from im and sc immunized hens were diluted 50 000- and 5000-fold, respectively, prior to ELISA assay. Each value is the average of three determinations.

al., 1988). After initial immunization, the immunization was repeated once a week for 4 weeks (as indicated in Figure 1). The immunization treatment was repeated (boosting) when the antibody titer declined. Eggs were collected before the immunization and over a 4 and 10 week period after initial immunization and stored at 5 °C prior to antibody purification and yolk lyophilization (DVE-200D, EYELA, Tokyo Rikakikai Co., Japan).

Partial Purification of Yolk Antibody (IgY). Eggs were collected from 10 im or iv immunized hens, and antibodies were purified by high methoxy pectin method (HMP, DE = 64) (Sigma Chemical Co.). In short, a 2-fold diluted yolk solution was mixed well with 4 volumes of 0.225% HMP, and the homogeneous solutions were allowed to stand for approximately 30 min after the pH was adjusted to 5.0 with 1 N HCl. The aggregated lipoproteins were then removed by centrifugation (10000*g*, 15 min), and the collected supernatants (crude IgY) were filtered through a No. 2 filter paper (Advantec Tokyo, Japan) with an antibody recovery and purity of up to 70% (Lu, 1995), determined by ELISA and single radial immunodiffusion (SRID) as described below. The crude IgY whose pH was adjusted to 7.0 with 1 N NaOH was lyophilized (Eyela FD-5N, Rikakikai Co., Japan) and refrigerated at 4−5 °C for use in thermal and hydrolysis stability tests after adjustment to the desired IgY concentration with distilled water.

The homogeneous yolk (mixed with a Vortex mixer) from eggs of immunized hens collected between 4 and 10 weeks after initial immunization was lyophilized and used as immunized yolk powder. The IgY concentration was adjusted with an adequate amount of distilled water prior to the thermal stability tests. In the stability test against proteases, 5% gum arabic was added to the above yolks and then lyophilized. Proteases such as trypsin, chymotrypsin (Sigma Chemical Co.) (both in a 0.05 M phosphate buffer, pH 7.6), and pepsin (Sigma Chemical Co.) (pH 2.0 and 4.0, adjusted with 0.1 N HCl) were reacted with lyophilized yolk−5% gum arabic powder in an *E*/*S* (enzyme/substrate) ratio of 1/40 (w/w) at 37 °C for 1−8 h. The degree of hydrolysis was determined according to the method described by Adler-Nissen (1979).

Gel Filtration. Sephacryl S-300 gel, packed in a column (1.6 cm × 95 cm), was rinsed thoroughly and equilibrated with two column volumes of 0.85% NaCl/0.01 M phosphate buffer (PBS, pH 7.2) prior to use. Crude IgY, prepared by HMP, was applied to the column and then was eluted with PBS at a flow rate of 12.0 mL/h. The absorbance at 280 nm was monitored, and the effluents were collected (2.0 mL/fraction). Fractions of each peak in chromatographic profiles were pooled, and their binding activity to *S. mutans* c was determined by ELISA.

Thermal Stability of IgY. Yolk solution and crude IgY (0.5 mg of IgY/mL) in paraffin-sealed test tubes were thermally treated at 70, 74, 78, and 80 °C in a temperature-controlled water bath for various periods of time. Maltose (50, 30%) and glycerol (20, 10%) (Sigma Chemical Co.) were added to the diluted yolk (1.39 mg of IgY/mL) to observe their protective effect at low temperature (70 °C). For the ease in evaluating the effect of thermal protectants (sucrose, maltose, glycerol, and glycine) and protein levels (7.57, 1.71 mg protein /mL) on IgY at high temperatures, yolk solutions were heated to 80 °C in a water bath. The residual binding activity of IgY with *S. mutans* MT8148 c antigen was determined by the ELISA method after the IgY concentration adjustment to 0.5 mg/mL.

Protein Determination. The total protein concentrations in yolk powder and crude IgY were assayed by the Bradford (1976) method. Microtiter plate protocol of Bio-Rad protein assay was conducted, and chicken serum IgG (Sigma Chemical Co.) was used as the standard solution (0.1−0.6 mg of IgG/mL) to prepare the standard curve (McCannel and Nakai, 1990).

RID. The relative amount of IgY in yolk powder and crude IgY was determined with RID plates containing rabbit anti-chicken IgG (Sigma Chemical Co.) (Hudsan and Hay, 1989). RID plates were incubated at room temperature in a moist chamber until there was no further increase in precipitin ring diameter (about 48 h). Chicken serum IgG was used as the standard solution (0.1−2.0 mg of IgG/mL) to prepare the standard curve which was plotted with the square of the diameter of precipitin ring versus IgY concentration.

Enzyme-Linked Immunosorbent Assay (ELISA). ELISA was conducted according to Otake et al. (1991). In short, *S. mutans* MT8148 c antigen was suspended in a carbonate buffer (0.1 M, pH 9.6), and the suspension (A660 nm = 1.0) at 100 μL/well was used to coat an ELISA plate. After being blocked with ovalbumin (1 mg/100 μL) (Sigma Chemical Co.), each well was washed three times with 200 μL of PBS (0.85% NaCl−0.01 M phosphate buffer, pH 7.2)-Tween (containing 0.05% Tween 20), and egg yolk or crude IgY from immunized hens at different time intervals was applied to the well in triplicate for reaction with the antigen for 2 h at 37 °C. Egg yolks from im and sc immunized hens were diluted 50 000-fold and 5 000-fold, respectively, with PBS-Tween prior to reaction with antigen. After each well was washed again with 200 μL of PBS-Tween, 100 μL of alkaline-phosphatase-conjugated rabbit anti-chicken IgG (Sigma Chemical Co.) diluted 2000-fold with PBS-Tween was added to each well, and the plate was incubated at 37 °C for 2 h. Each well was washed again with 200 μL of PBS-Tween, and then 100 μL of 0.1% *p*-nitrophenyl phosphate/10% diethanolamine buffer (pH 8.9) (Sigma Chemical Co.) was added. After being incubated at 25 °C for 30 min, 50 μL of 5 N NaOH was applied to each well to stop the reaction, and the color developed was read at 405 nm with an ELISA reader (Emax, Molecular Devices Co. USA). Yolks and crude IgY from nonimmunized hens were used as control. The ELISA results were confirmed for some samples using RID.

In cross-reaction tests, *S. mutans* c was replaced by other serotypes of *S. mutans*, and ELISA was conducted in the same procedure as described above.

RESULTS AND DISCUSSION

Effect of Immunization Route on Antibody Activity. The routes of immunization can affect the antibody levels in experiment animals (Kuby, 1994). Antibody levels in egg yolks from both im and sc immunized hens were investigated by ELISA for 45 and 26 weeks, respectively, after initial immunization (Figure 1). When immunization was conducted once a week for 5 weeks, regardless of the immunization route, the levels of antibody against *S. mutans* c began to climb beginning in the third week in each hen, reaching a peak at approximately the sixth week. Subsequently the levels declined sharply from the 6th week to the 16th week and then decreased gradually to the 23rd week (Figure 1A). However, when boostings were conducted at the 23rd week for both im and sc immunizations, the antibody levels recovered quickly again. The curve of antibody level was repeated until the 45th week, at which point the boosting was terminated (Figure 1A). The shape of the curves in Figure 1 was similar to that reported by Otake et al. (1991); however, the immunization period was much longer in the present study.

Otake et al. (1991) stated that both hen serum and yolk were diluted 1000-fold to get the ELISA values, conducted by the same ELISA method, close to those in Figure 1, when hens were also im immunized on thighs using *S. mutans* MT8148 c as antigen. In this experiment, egg yolks from im immunized hens were diluted 50 000-fold prior to ELISA analysis, and a linear relationship between the yolk dilution level and the ELISA values was observed under this condition. The dilution level for ELISA analysis was dependent on the antibody level in the sample, thus, the level of specific antibody obtained in this experiment was reasonably higher than those reported by Otake et al. (1991). Similarly, the dilution fold of yolk in Figure 1B was only 5000, and the antibody level in yolks from eggs laid by im immunized hens were much higher than those from sc immunized hens.

Cross-Reaction of Antibody against *S. mutans* c with Other Serotypes of *S. mutans*. Mcghee and Michalek (1981) reported that the immunodominant sugars in the cell walls of *S. mutans* a, c, e, and f are glucose−glucose, while those in the cell walls of *S. mutans* b, d, and g are galactose. Thus, from the results presented in Figure 2, it was obvious that the ELISA values against *S. mutans* c, e, and f were closely grouped at a higher level, while values against *S. mutans* a, b, d, and g were all much lower.

The human dental caries is mainly (over 70%) caused by *S. mutans* c followed by *S. mutans* d (Loesche et al., 1975; Loesche and Straffon, 1979), which suggests that the antibody against serotypes c and d must be applied simultaneously in clinical trials to effectively prevent the human dental caries by passive immunization.

Thermal Stability of IgY in Yolk and Crude IgY. To understand the stability of IgY to thermal treatment and processing for the possible immunoprophylatic potential of IgY for human clinical trials against dental caries, IgY with specific binding activity with *S. mutans* c was prepared, and the change of the activity during the thermal processing was investigated. Antibodies of eggs, collected from im immunized hens, were partially

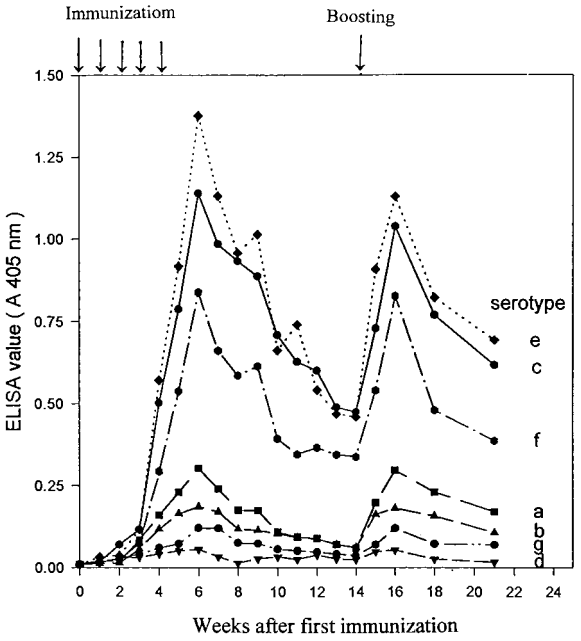

Figure 2. Egg yolk ELISA values against *S. mutans* serotypes a, b, c, d, e, f, and g. Egg yolks were collected from hens im immunized with *S. mutans* serotype c. Each value is the average of three determinations.

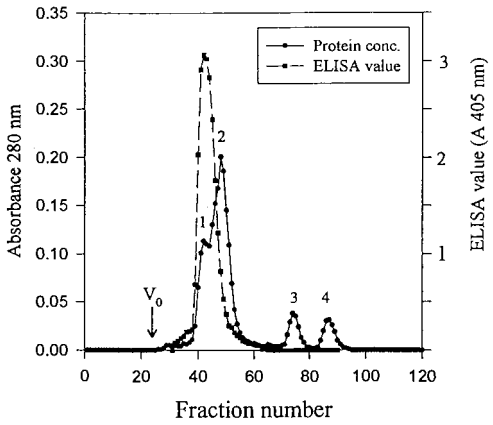

Figure 3. Elution profile of crude IgY from Sephacryl S-300 and ELISA values against *S. mutans* c. Crude IgY was prepared with high methoxy pectin from the eggs laid by the hens im immunized with *S. mutans* c.

purified by HMP and applied to Sephacryl S-300 gel chromatography. The elution profile and ELISA values (A 405 nm) of crude IgY were determined as shown in Figure 3.

Yolk and crude IgY (0.5 mg IgY/mL) were thermally treated at various temperatures (70, 74, 78, and 80 °C) for different periods of time (Figure 4). The residual binding activity of IgY, in diluted yolk or in crude solution, with antigen decreased with increasing temperature and heating time. The components other than IgY in the diluted yolk appeared to be insignificantly effective in protecting the IgY from thermal denaturation. The changes of thermal stability of immunoglobulin G in bovine skim milk, with or without the addition of 2% cream, showed almost the same decreasing trend (Li-Chan et al., 1995) after HTST treatment. However, when the IgG-containing samples were thermally treated at 75 °C and the treatment lasted for 600 s, the residual IgG content in PBS was decreased by 44%, compared to around 20% loss in colostrum and whey (Chen and Chang, 1998). Results in Figure 4 are

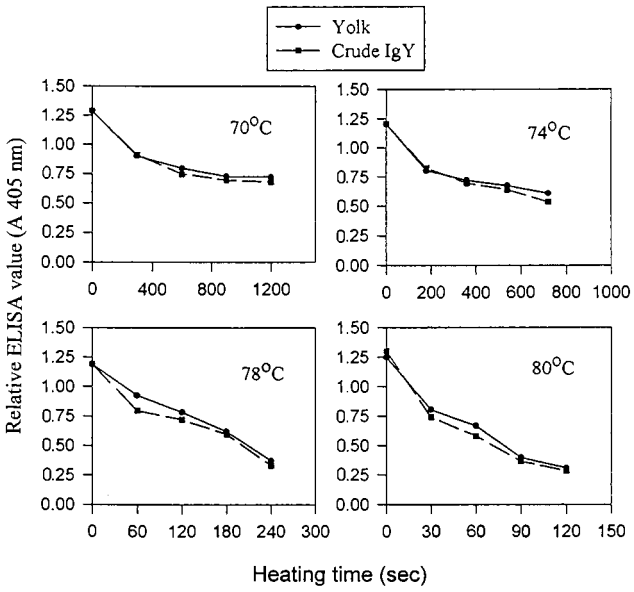

Figure 4. Changes of relative ELISA values of yolk solution and crude IgY (0.5 mg of IgY/mL) against *S. mutans* c after various thermal treatments. Each value is the average of three determinations.

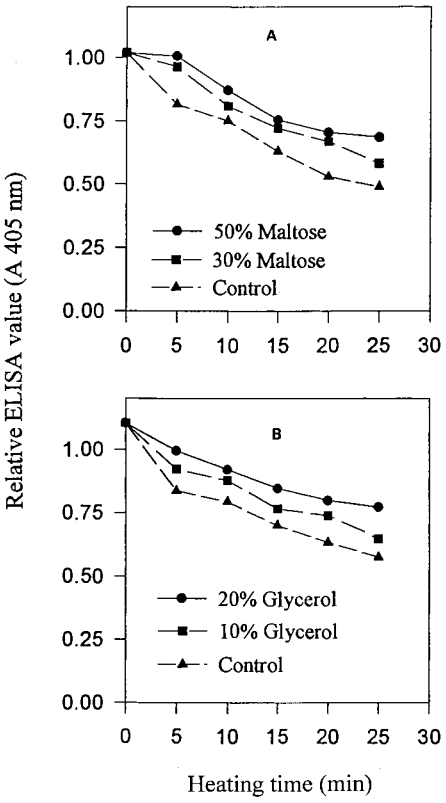

Figure 5. Effect of the addition of maltose (A) and glycerol (B) on the relative ELISA values of yolk (1.39 mg of IgY/mL) thermally treated at 70 °C for various periods of time. IgY concentration was adjusted to 0.5 mg/mL prior to ELISA assay. Each value is the average of three determinations.

similar to those reported by Shimizu et al. (1988) that IgY denatured seriously when thermally treated at temperatures higher than 75 °C. The IgY used in those thermal tests had a high specificity against *S. mutans* c (Figure 1). Thus, it was evident that the thermal property of IgY in eggs from immunized hens was similar to that in eggs from nonspecially immunized hens.

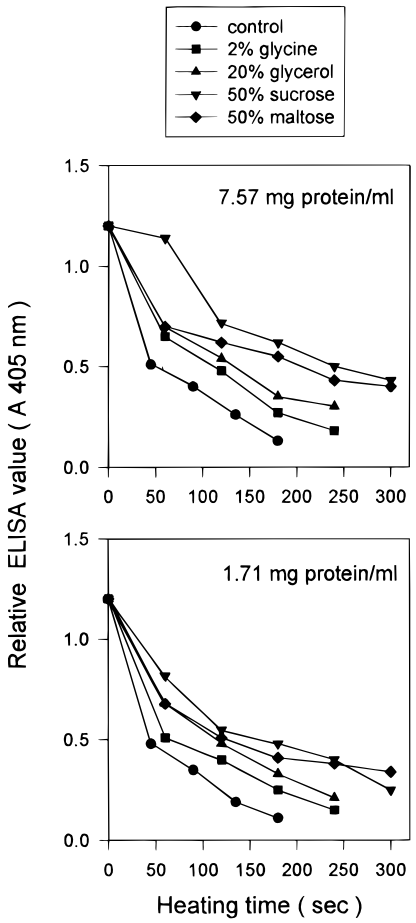

Figure 6. Changes of the relative ELISA values of yolk (7.57, 1.71 mg of protein/mL) added with various protectants thermally treated at 80 °C for various periods of time. IgY concentration in each sample was adjusted to 0.5 mg/mL prior to ELISA assay. Each value is the average of three determinations.

Sugars and certain kinds of amino acids have been found effective in preventing the denaturation and destruction of proteins, such as actomyosin and myosin, during thermal and freezing treatment. Chen and Chang also reported (1998) that 5% of maltose or fructose and 2% of glycine displayed a remarkable protection effect on the thermal denaturation of bovine milk IgG when IgG in PBS was heated at 95 °C for 15 s. Hence disaccharides (sucrose and maltose), sugar alcohol (glycerol), and glycine were used to investigate their influence on the heat denaturation of IgY. As shown in Figure 5, addition of 50 and 30% maltose (Figure 5A) or 20 or 10% glycerol (Figure 5B) to the diluted yolk solution (0.5 mg of IgY/mL) effectively prevented the heat denaturation of IgY during the thermal treatment of 70 °C.

Protein concentration in the solution affects the denaturation rate of protein itself. Iametti et al. (1995) reported that a lower level of β-lactoglobulin solution was liable to heat denaturation owing to the lower protection from the intramolecular disulfide bonds and from the formation of higher amounts of hydrophobic interaction between proteins. In Figure 6, yolk samples (7.57 and 1.71 mg of protein/mL) were heated at 80 °C to observe the possible variation of IgY denaturation rate and effects of thermal protectants on IgY. The results suggest that samples with a higher IgY level (7.57 mg/mL) appear to be much more resistant to heat

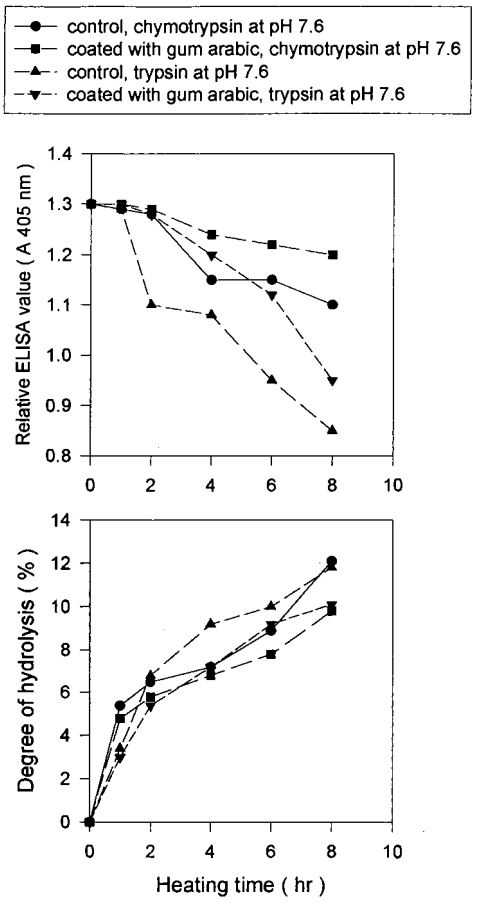

Figure 7. Relationship between degree of hydrolysis and relative ELISA values of freeze-dried yolk powder coated with gum arabic and hydrolyzed with chymotrypsin and trypsin at 37 °C (E/S = 1/40). Each value is the average of three determinations.

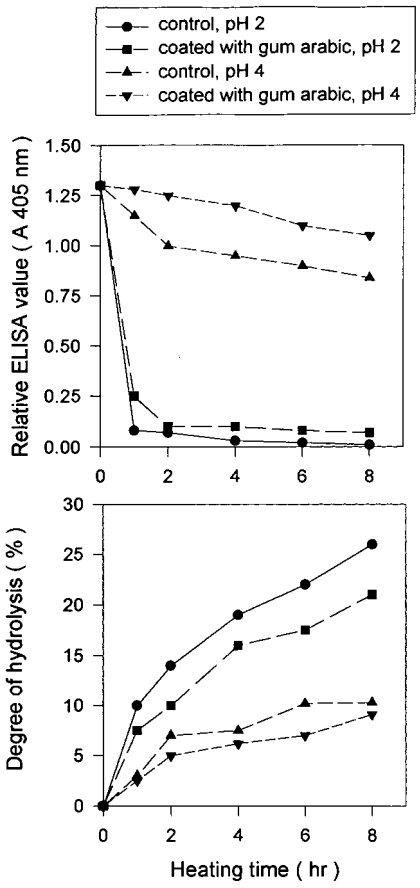

Figure 8. Relationship between degree of hydrolysis and relative ELISA values of freeze-dried yolk powder coated with gum arabic and hydrolyzed with pepsin at 37 °C (E/S = 1/40). Each value is the average of three determinations.

denaturation, especially for those samples with 50% sucrose or maltose. Addition of 2% glycine also revealed a certain level of protection for thermal denaturation of IgY, although detailed investigation is still needed. However, much better protection effect of glycine on milk IgG was observed. Addition of 2% glycine to the bovine milk separated IgG in PBS when heated at 95 °C for 15 s was found effective in increasing the residual IgG content to 26.7%, compared to about 0% of the control heated under the same conditions (Chen and Chang, 1998).

The protective effect of sugars on thermal denaturation of proteins has been pointed out to be due to the enhancement of hydrophobic interactions inside the protein molecule (Shimizu et al., 1994) and the changes in preferential solvation of protein molecules which facilitates the stabilization of proteins during thermal treatment (Timasheff, 1993).

Stability against Proteases. Figures 7 and 8 represent the degrees of hydrolysis and relative ELISA values of yolk powders, coated or uncoated with 5% gum arabic, hydrolyzed with trypsin and chymotrypsin and pepsin, respectively. Results show that yolk powder coated with gum arabic appeared to have a lower degree of hydrolysis and a relatively higher ELISA value during the hydrolysis. Thus, the addition of gum arabic to coat the IgY during lyoplilization was proven to be effective in enhancing the antibody against proteases.

CONCLUSION

Hens were im and sc immunized with the whole cells of *S. mutans* c, and the response and productivity of the specific antibody against *S. mutans* c through the former route were almost 10 times higher than that of the latter route. *S. mutans* e and f significantly bound with the antibody against c, thus suggesting such antibodies could be effective in preventing the human dental caries caused by *S. mutans* e and f. This indicates that antibodies against *S. mutans* c and d should be applied simultaneously to effectively prevent human dental caries.

Since there was severe thermal destruction of IgY at temperatures higher than 75 °C, protectin of IgY appeared to be important. Addition of sugars, glycerol, or glycine to immunoglobulin solutions were found to be effective. However, sugars which are good mediums for *S. mutans* are not suitable additives for use in prevention of dental caries. Film coating with gum arabic was proven to be effective in reducing the degree of hydrolysis while maintaining the ability of IgY to bind with *S. mutans* c.

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