The effect of ovalbumin and mannose-conjugated ovalbumin on the prevention of *Salmonella* adherence to the intestinal epithelium of chickens

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Summary

This investigation was designed to determine the effect of intact ovalbumin and mannose-conjugated ovalbumin on the prevention of *Salmonella typhimurium* adherence to the epithelium of small intestine of chickens. Mannose-conjugated ovalbumin was produced by Maillard-type reaction between chicken ovalbumin and D-mannose at 60°C. The results revealed that incubation up to 96 hrs caused the highest amount of covalent attachment of mannose to the ovalbumin. In order to determine the effect of native ovalbumin and mannose-conjugated ovalbumin on the prevention of *S. typhimurium* adherence to chicken small intestine, 60 one-day-old chicks were randomly assigned to 3 groups, with two replicates and ten birds per pen. Groups 1, 2 and 3 received normal diet, diet containing 0.5% native ovalbumin and diet containing 0.5% mannose-conjugated ovalbumin, respectively, for 12 days. On day 3, all groups received 1.3×10^6 CFU of *S. typhimurium* orally. On days 4, 7 and 10, two chicks from each group were killed and mean log 10 of CFU (colony forming unit) of *Salmonella* per 1 g tissues of cecum, liver and spleen was determined. Four chickens from each group were killed on day 12 and were examined as described above. The results showed that in group 3, number of viable *Salmonella* in cecum, liver and spleen was lower than groups 1 and 2. However, the difference was significant only in cecum on days 4 and 7 ($P<0.05$). These preliminary results suggest that mannose-conjugated ovalbumin might be effective in prevention of *Salmonella* colonization in the epithelium of small intestine if incorporated in the diet of chicks.

Key words: Ovalbumin, Mannose, Conjugation, Intestinal mannose receptors, Salmonellosis

Introduction

Salmonella has long been recognized as an important zoonotic pathogen of worldwide economic significance in humans and animals. Infection of animals with various species of *Salmonella* occasionally results in serious diseases and always constitutes a vast reservoir for the disease in human (Carlton and Charles, 1993). Moreover, infected poultry comprise one of the most important reservoirs of *Salmonella* transmitable to humans through food chain. In the past, the primary motivation for controlling *Salmonella* infections in poultry was to reduce disease losses. Today, public health concerns, political pressures, and consumer demands have increasingly made prevention of food-borne transmission of disease to humans an urgent priority for poultry producers (Daniel, 1995). *Salmonella* infection of food animals is therefore of concern to both the food industry and public health authorities. However, use of antibiotics to control the bacterial infection is a matter of concern because it may incite generation of drug resistant bacteria strains. Therefore, safe antimicrobial agents which are harmless to humans have been sought in the natural environment (Ogawa *et al*., 1999). Numerous studies have been conduced in an

effort to decrease contamination of broiler carcasses with *Salmonella* during production and processing. Some have focused on preventing attachment of *Salmonella* in the intestine and ceca or on the surface of carcass by various physical or chemical means (Izat *et al*., 1990). Invasion and growth inside gastrointestinal mucosal cells are important virulence factors for *Salmonella*. Most bacterial attachment appendages and receptor sites on animal tissues are carbohydrate in nature, and treatment with an appropriate carbohydrate may prevent effective attachment by obstructing the adhesion and attachment site (Abraham *et al*., 1983). *Salmonella* species produce surface-binding proteins that allow them to firmly attach to intestinal epithelial cells. Adherence, a key virulence factor for *Salmonella*, is governed by type 1 fimbriae. At the tip of each fimberium, there is a mannose-containing glycoprotein called lectin that is programmed to bind to the mannose receptors of intestine (Ofek *et al*., 1982). The result of studies suggested that adherence of *S. typhimurium* to the intestine of one-day-old chicks and to the ceca of one-week-old broilers is inhibited by D-mannose (Mchan *et al*., 1989). However since mannose is a monosaccharide, it is rapidly absorbed in the upper intestinal tract, thereby, rendering it unavailable to block the attachment sites. It has been reported that carbohydrates conjugated to proteins are resistant to digestion as compared with the unconjugated carbohydrates (Yang *et al*., 1998). The objective of this study was to prepare ovalbumin conjugated with mannose through Maillard-type reaction and to determine if native ovalbumin or mannose-conjugated ovalbumin added to the diet of one-day-old chicks could effectively prevent or reduce *S. typhimurium* colonization in the intestine of chicks.

Materials and Methods

Preparation of mannose-conjugated ovalbumin

Ovalbumin and mannose were mixed at the weight ratio of 1:5 (100 mg ovalbumin and 500 mg mannose) in 1 ml of 0.1 M phosphate buffer (pH = 7.4). After 96 hrs incubation at 60°C, the mixture was dialyzed against deionized water to remove unbound mannose from mannose-conjugated ovalbumin (Nakamura *et al*., 1991). Samples were lyophilized and the mannose content was determined by measuring the absorbance at 470 nm after colour development with the phenol-sulfuric acid reaction (Dubois *et al*., 1951).

Gel electrophoresis

SDS-polyacrylamide gel electrophoresis was done according to the method of Laemmli (1970), using a 10–20% gel gradient.

Experimental design

To determine the effect of native ovalbumin and mannose-conjugated ovalbumin on prevention of *S. typhimurium* adherence to chicken small intestine, 60 one-day-old, *Salmonella*-negative broiler chicks were randomly assigned into three groups; 10 birds per pen. Duplicate experiments were done. For sanitation of the diet, 120 ml formalin was added to 60 g of $KMnO_4$ in 100 ft^3 cabinet space for 20 min formaldehyde fumigation (Calnek *et al*., 1997). Groups 1, 2 and 3 received normal diet, diet containing 0.5% native ovalbumin and 0.5% mannose-conjugated ovalbumin, for 12 succeeding days, respectively. On the third day of experiment, all groups received 1 ml saline containing 1.3×10^6 CFU of *S. typhimurium*/ml orally. On days 4, 7 and 10, two chicks from each group were killed by cervical dislocation. Tissue samples aseptically collected from liver, spleen and cecum, weighed, ground and 10-fold serially diluted in sterile distilled water. Serial dilutions were streaked on brilliant green agar plates. Colonies were counted after 24 to 48 hrs of incubation at 37°C (Smith and Tucker, 1975).

The CFU of *Salmonella* per gram of different tissues (liver, spleen and cecum) was determined. The remaining four chicks from each group were killed on day 12 and were examined as described above.

Statistical analysis

Data were analysed by one-way ANOVA, using SPSS/PC software and

Duncan's test used to determine significant differences among different groups (Daniel, 1995).

Results

Table 1 shows that incubation up to 96 hrs resulted in the highest amount of covalent attachment of mannose to ovalbumin. After 120 hrs, a decrease in the conjugation was observed. This result was further confirmed by SDS-PAGE (Fig. 1). Glycosylation of protein with mannose resulted in appearance of a broad diffused band on the top of separating gel. After 96 hrs, bands corresponding to the conjugation of mannose with protein were decreased.

Table 1: Effect of incubation time at 60°C on the conjugation of ovalbumin with mannose

Incubation time (hr)	mg mannose per mg ovalbumin	Moles mannose per mole ovalbumin
0	0.013	4
24	0.032	9
48	0.024	7
72	0.020	6
96	0.045	12.5
120	0.022	6.5

Table 2 shows the effect of glycosylation on the colonization of *S. typhimurium* to liver, spleen and cecum. Results shows that mean log 10 of CFU of *Salmonella* in cecum, liver and spleen was lower than those of groups 1 and 2. However, the difference was significant only in cecum, on days 4 and 7 ($P<0.05$). The log 10 number of isolated bacteria from cecum in group 3 was 4 to 4.41. The mean log 10 number of isolated bacteria from cecum in group 1 was 4 to 5.85 (Table 2). No significant difference was observed when log 10 number of bacteria isolated from cecum in group 2 (received unconjugated ovalbumin) was compared with the control group.

Fig. 1: SDS-PAGE patterns of different ovalbumin samples. In each channel 50 µl saline containing 50 µl protein was loaded. Lane 1: molecular weight marker; Lane 2: native ovalbumin and Lane 3–7: samples in which ovalbumin-mannose mixture was incubated at 60°C for 24, 48, 72, 96 and 120 hrs, respectively

Discussion

Carbohydrate-protein conjugates can be produced by Maillard-type reaction by allowing the ε-amino group of lysine residues or N-terminal amino group of proteins to react with the carbonyl group of carbohydrates under controlled temperature, pH and relative humidity (Nakamura *et al*., 1992a, b; Nakamura *et al*., 1996). The effect

Table 2: Mean log 10 of CFU of *S. typhimurium*/g tissues of different groups

Days after inoculation	Organs								
	Liver			Spleen			Cecum		
	Group 1	Group 2	Group 3	Group 1	Group 2	Group 3	Group 1	Group 2	Group 3
1	4.38 ±0.44	4.41 ±1.00	3.51 ±0.59	4.43 ±0.33	4.64 ±0.97	3.57 ±0.85	5.62 ±1.13[a]	6.85 ±0.81[a]	4.00 ±0[b]
4	4.46 ±1.57	4.46 ±1.02	3.64 ±0.57	4.28 ±0.38	4.79 ±1.21	3.46 ±0.93	5.85 ±1.18[a]	4.91 ±0.83[a]	4.00 ±0[b]
7	3.61 ±0.44	3.36 ±0.62	3.53 ±0.29	4.03 ±0.88	4.04 ±0.99	3.49 ±0.99	4.00 ±0	4.92 ±0.83	4.41 ±0.64
9	3.92 ±0.60	3.80 ±0.48	4.09 ±1.49	4.07 ±0.95	4.36 ±0.71	4.63 ±0.71	5.46 ±1.15	5.27 ±1.21	4.37± 0.61

Results are expressed as mean ± SD log 10 of number of *S. typhimurium*/g tissue. Group 1: received normal diet (no egg protein added in the diet); Group 2: received 0.5% ovalbumin added to the diet; Group 3: received 0.5% mannose-conjugated ovalbumin to the diet. [a,b]Means in the same row with different superscripts differ significantly ($P<0.05$)

of experimental, conditions (pH, temperature, concentration of reactants, etc) on the Maillard reaction has been extensively determined (Hodge, 1953; Ellingson *et al*., 1954; Ashoor and Zent, 1984; Freidman, 1996; Bell, 1997; Feather and Mossine, 1998; Wedzinch and Leong, 1998; Ajandouz *et al*., 2001; Brands and Van Boekel, 2003). The present communication describes conjugation of mannose to ovalbumin under Maillard-type reaction. Degree of covalent attachment was followed by SDS-PAGE and by determination of mannose content of the products. Molar ratio of mannose attached to ovalbumin was calculated by considering the molecular weight of ovalbumin (45 kDa). The diffused bands observed in SDS-PAGE is an indication of the multiplicity of conjugated derivatives obtained during the course of the reaction of ovalbumin with mannose. These multiple forms probably originate from formation of molecules with different number of mannose attached to each molecule of proteins, extensive variability in conformation of different molecules, protein-protein interaction, isopeptide bond formation, or other unknown mechanisms, that might affect the electrophoretic mobility (Diftis and Kiosseoglou, 2003).

Under optimum conditions (96 hrs at 60°C), a maximum of 12.5 mole mannose was attached to one mole ovalbumin. Incubation beyond 96 hrs probably results in detachment of mannose residues from some ovalbumin molecules. This observation is confirmed by SDS-PAGE patterns. Samples incubated for 120 hrs are less diffused than those incubated for 96 hrs. Such phenomena may be related to the decomposition of Amadori compounds formed during the course of Maillard reaction (Brands and Van Boekel, 2003). Similar results were reported for the reaction of disaccharide-conjugated casein (Brands and Van Boekel, 2003) and dextran-conjugated lysozyme (Nakamura *et al*., 1991).

Results of this study indicated that inclusion of 0.5% mannose-conjugated ovalbumin in the diet of chicks on the first 12 days decreased the level of *Salmonella* in the ceca. These results suggest that the addition of mannose-conjugated ovalbumin to the diet may be a simple mean of significantly reducing *S. typhimurium* colonization in the intestine of chicks. Previous in vitro and in vivo studies have indicated that mannose was an effective sugar blocker of the intestinal mannose receptors for *S. typhimurium* (Lindquest *et al*., 1987; Oyofo *et al*., 1989; Otter *et al*., 1992). The reduction in the number of viable bacteria in the present research can be attributed to the role of mannose in the inhibiting of colonization of *Salmonella* to the intestinal mannose receptors.

The results of an assay for detecting *S. typhimurium* adherence to chicken intestine used by Oyofo *et al*., (1989) showed that the adherence of *Salmonella* could be blocked by addition of 2.5% mannose in the drinking water of chickens. However, in the present research, 0.5% mannose-conjugated ovalbumin in the diet was used and the results indicated that the log 10 number of isolated *Salmonella* from cecum on days 4 and 7 was lower than that in the control. It has been suggested that the absorption of monosaccharides occurs very rapidly from the upper intestine due to their high water solubility and small size. In addition, as the blocking action of D-mannose for bacterial adherence to epithelial cells is reversible (Duguid and Gillies, 1957), the effect of free mannose on the blocking mannose receptors is limited. On the other hand, it can be suggested that macromolecules such as glycoproteins (containing monosaccharides) can improve the blocking effect by decreasing the absorption of mannose from small intestine. The glycolytic action of α-amylase on the lysine and poly-lysine-conjugated starches was markedly decreased as compared with unconjugated starches (Yang *et al*., 1998). The indigestibility of the conjugates with α-amylase is thought to be for their low solubility and their inhibiting effect on amylase; similar to the effects of other Maillard-type reaction products on α-amylase and trypsin (Miura and Gomyo, 1994; Hirano *et al*., 1996).

It can be suggested that decrease in digestibility leads to decrease in absorption, so that Maillard-type reaction could improve the effect of mannose on the blocking of intestinal mannose receptors. However, the in vitro digestibility of the monosaccharide conjugation should further be investigated.

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