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Acid Stability of Anti-*Helicobacter pyroli* IgY in Aqueous Polyol Solution

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IgY was separated from a hen's egg yolk that was immunized with *Helicobacter pyroli*. The anti-*H. pyroli* IgY activity at acidic pH and the suppressive effect of polyol on acid-induced inactivation of IgY were investigated. Sorbitol and xylitol were used as polyols. IgY was quite stable at pH 5~7. Irreversible inactivation of IgY was observed at pH below 4, and proceeded rapidly at pH below 3. The acid stability of IgY was enhanced in the presence of 30% sorbitol or above. In a 50% aqueous sorbitol solution, an acid-induced inactivation was almost completely suppressed at pH 3. However, the improvement of IgY activity was not observed in the aqueous xylitol solution. IgY showed almost the same activity as native IgY when sucrose was substituted for sorbitol. On the other hand, the xylitol replacement with sucrose did not enhance the acid stability of IgY. The acid-induced inactivation of IgY was related to tryptophyl fluorescence. Fluorescence emission spectra suggested that structural changes near the tryptophan residues may occur under acidic conditions. An increase in sorbitol concentration induced a blue shift. The fluorescence emission of IgY in a 50% sorbitol solution had a peak at 330 nm, which was the same emission peak that was exhibited by native IgY. Sorbitol could, therefore, be used as a good stabilizer of IgY under acidic conditions.

Keywords: Acid stability, *Helicobacter pyroli*, IgY, Sorbitol, Xylitol

Introduction

Helicobacter pyroli (*H. pyroli*) is a key pathogen of chronic gastritis, duodenal ulcer, and gastric ulcer. Infection with *H. pyroli* generally occurs in children before the age of 10 years (Ernst and Gold, 2000). In developed countries, 25~50% of the population carry *H. pyroli*. The prevalence of infection in developing country is higher, ranging from 70 to 90% (Dunn *et al*, 1997). Although *H. pyroli* is sensitive to various antibiotics, antibacterial agents alone have mostly failed to eradicate *H. pyroli* (Chiba *et al*., 1992). Moreover, increased occurrences of antibiotics-resistant strains of *H. pyroli* have become a problem (Ling *et al*., 1996). An antibody could confer protection against *H. pyroli*. Therefore, oral passive immunization by an antibody may be useful in preventing *H. pyroli* infection.

Polyclonal antibodies are usually produced by mammalian sera. However, collecting sera requires a lot of care, and these antibodies are very expensive and limited so that another antibody source should be investigated. A hen's egg can be an effective antibody supplier. Only one class of immunoglobulins, IgG, among the immunoglobulins in the hen's sera can be selectively transported in large quantities and efficiently accumulated in an egg yolk (Rose *et al*., 1974; Rose and Orlands, 1981). Chicken housing is inexpensive and egg collection is noninvasive. Moreover, isolation of IgG-type immunoglobulins is comparatively simple. An egg yolk contains about 1% IgG-type immunoglobulins (Lösch *et al*, 1988); therefore, it is a rich antibody source. The yolk immunoglobulins are slightly different from mammalian IgG in their molecular size, isoelectric point, binding ability with protein A or G, activation ability of the mammalian complement, and domain structure (Higgins, 1975; Benedict, 1979; Parvari *at al.*, 1989; Reynaud *et al.*, 1989; Kwack, 2000). Therefore, the immunoglobulins in yolk are now called IgY (named by Leslie and Clem, 1969).

Recent investigations, concerning the passive immunity by oral administration of IgY, have increased interest in IgY. IgY decreased the incidence of diarrhea, due to the rotavirus or *E. coli* (Yoelken *et al*., 1988; OFarrelly *et al*., 1992; Hatta *et al*., 1993). IgY also conferred protection against dental caries that are formed by *Streptococcus mutans* (Otake *et al*., 1991; Hatta *et al*., 1997; Smith *et al*., 2001). Yang *et al*. (1997) showed that IgY could specifically recognize digestive system cancer,

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which indicates its potential use in cancer prevention. Such protective effects have increased interest in applying IgY to pharmaceutical and food products. However, the reduced stability of IgY under acidic conditions are limited for expanded use in food products. Therefore, the stability of IgY at low pH should be improved in order to enhance its use.

This research was performed to investigate the production and separation of anti-*H. pyroli* IgY, and possible stabilizing effects of polyols on IgY under acidic conditions.

Materials and Methods

Antigen preparation The *H. pyroli* strain ATCC 43504 was grown in broth culture at 37°C for 36 h, then the cells were harvested. One gram of the harvested cells was suspended in 50 ml of phosphate-buffered saline (PBS, pH 7.2). After sonication, the supernatant was separated by centrifugation at 10,000 × *g* for 15 min. The supernatant that was obtained by the additional centrifugation at 100,000 × *g* for 30 min was then used as an antigen.

Immunization of hens Hens (Hy-Line Brown), 21-wk-old, were used for immunization and egg production. One hundred μl of a 0.1% antigen solution in PBS was emulsified with an equal volume of complete Freund's adjuvant (Difco Co., Detroit, USA), then intramuscularly injected into the hens. Two booster injections, each with 100 μg of antigen and incomplete Freund's adjuvant (Difco Co., Detroit, USA), were intramuscularly given at 2 and 4 weeks after the first injection. The eggs were collected for IgY separation after a second booster injection.

Crude IgY preparation Crude IgY was prepared from an egg yolk by three separation methods. (1) The water dilution method: IgY was separated by the method presented by Akita and Nakai (1993). Ten ml of egg yolk was diluted 60 ml with deionized water. The supernatant, crude IgY solution, was isolated by centrifugation. (2) The λ-carrageenan method: IgY was isolated by the procedure that is described by Hatta *et al.* (1990). Ten ml of egg yolk was diluted to 100 ml with 0.15% λ-carrageenan (Sigma Chemical Co., St. Louis, USA). The resulting solution was held at room temperature for 30 min. The supernatant, crude IgY solution, was separated by centrifugation. (3) The polyethylene glycol method: IgY was isolated by the method of Polson *et al.* (1980). Briefly, 10 ml of egg yolk was diluted to 50 ml with PBS. Polyethylene glycol 6000 (PEG 6000) was added to a concentration of 3.5% and centrifuged. PEG 6000 was added to the supernatant to give 12%. The pellet that was obtained by centrifugation was redissolved in 5 ml of PBS, and ammonium sulfate was added to a concentration of 50%. The protein precipitate, IgY, was collected by centrifugation and resuspended in 1 ml of PBS. The crude IgY solution was extensively dialyzed against PBS.

The crude IgY solution that was prepared by the three separation methods was stored at 4°C for further analysis.

Acid treatment The pH of the 0.2% IgY solution in saline in the presence of 0~50% polyol was adjusted to pH 2~7 with HCl. The resulting solution was incubated at 37°C. After incubation at each pH, the solution was neutralized with PBS that contained 0.05% tween 20 (PBS-T). ELISA examined the antibody activity.

Protein determination The protein concentration of antigen or the crude IgY solution was determined with a protein assay kit (Bio-Rad Co., Hercules, USA). Bovine serum albumin was used as the standard.

IgY determination The crude IgY concentration was determined by measuring the absorbance at 280 nm, based on 13.6 as extinction coefficient at 280 nm of a 1% IgY solution (Tenenhouse and Deutsch, 1966).

The specific IgY concentration in crude IgY was determined by immunoprecipitation (Japanese Biochemical Society, 1986). Then 0.5 ml of the crude IgY solution and 1 ml of a 0.01% antigen solution in PBS were added to the same tube and incubated overnight at 37°C. The supernatant was separated by centrifugation (3,000 × g, 30 min), and the absorbance was measured at 280 nm. The specific IgY concentration was calculated as follows:

$$\text{Specific IgY}(\%) = \frac{\text{absorbance without antigen} - \text{absorbance with antigen}}{\text{absorbance without antigen}} \times 100$$

ELISA Noncompetitive ELISA assessed the IgY antibody activity. A polystyrene plate (Nunc Co., Roskilde, Denmark) was coated with 0.1 ml of the 0.01% antigen solution in PBS for 2 h at 37°C. The plate was then washed with PBS-T, then appropriately-diluted native or acid-treated IgY was added. After incubating for 2 h, the wells were washed with PBS-T, and 0.1 ml of an alkaline phosphatase-conjugated rabbit anti-chicken IgG (Sigma Chemical Co., St. Louis, USA) was added. After incubating for an additional 2 h, the plate was washed with PBS-T, then 0.1 ml of the substrate solution (0.01% p-nitrophenyl phosphate in diethanolamine buffer at pH 9.8) was added. After further incubation for 30 min, 5 N NaOH was added to stop the reaction. The absorbance was measured at 405 mn on a microplate reader (Molecular Device Co., Sunnyvale, USA). Relative IgY activity (B/Bo) was calculated as absorbance at 405 nm of the acid-treated IgY ÷ absorbance at 405 nm of native IgY.

SDS-PAGE SDS-PAGE was performed by the method of Weber and Osborn (1969) using 10% acrylamide gel. The protein was stained with Coomassie blue R-250 and destained with 10% acetic acid.

Fluorescence measurement The fluorescence emission spectra of IgY were measured by a spectrofluorometer (Jasco, Tokyo, Japan). The 0.02% IgY solutions in saline were excited at 296 nm, then the emission peak was measured.

Results

Separation of IgY IgY was separated by the water dilution, λ-carrageenan, or polyethylene glycol methods. SDS-PAGE showed a 64-kDa band (II) that corresponded to the heavy chain of IgY and a 25-kDa band (IV) that corresponded to the light chain of IgY (Fig. 1). Water-soluble protein that was

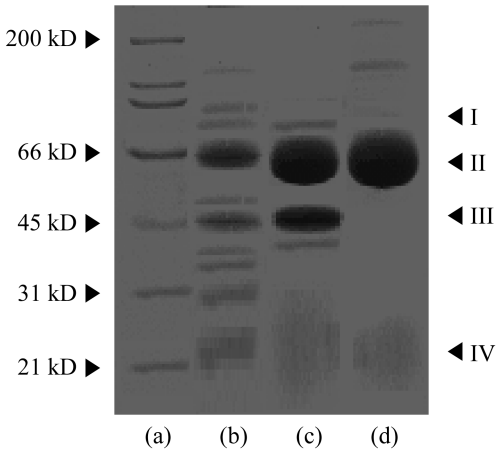

Fig. 1. SDS-PAGE patterns of separated IgY. IgY was separated with water dilution method (WD), λ-carrageenan method (λC) and polyethylene glycol method (PEG). (a) marker, (b) WD, (c) λC, (d) PEG

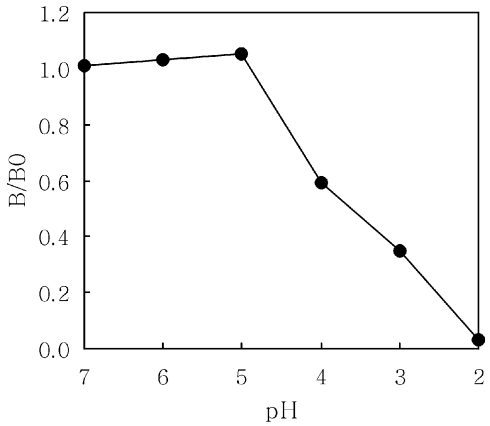

Fig. 2. Antibody activity of IgY after incubating at different pH. IgY was incubated at pH 7~2 for 24 h. B/Bo was calculated as absorbance of acid-treated IgY ÷ absorbance of native IgY. Absorbance was measured at 405 nm.

separated by the λ-carrageenan method contained α-(I) and β-livetin (III), as well as γ-livetin, known as IgY (Fig. 1). SDS-PAGE indicated that comparatively pure IgY was isolated by the polyethylene glycol method. Yield, purity, and specific IgY content of the three separation methods are compared in Table 1. The polyethylene glycol method gave the highest yield and purity, followed by λ-carrageenan method; the water dilution method showed the lowest yield and purity. IgY that was isolated by the polyethylene glycol method also contained more specific IgY than other methods. These results indicate that the polyethylene glycol method (among the three separation methods) was the most efficient in IgY separation. Therefore, IgY that was prepared by the polyethylene glycol method was used for stability determination.

IgY activity under acidic conditions The stability of IgY at each pH is shown in Fig. 2. IgY showed good stability at pH 5~7 after incubating 24 h at each respective pH. IgY was inactivated irreversibly at pH below 4. IgY inactivation was irreversible. The time-dependent changes of IgY activity at acidic pH are presented in Fig. 3. When incubating at each pH for 4 h, IgY lost antibody activity at pH 4 or below. The antibody activity was significantly reduced (up to 50%) at pH below 3. IgY almost completely lost its activity by incubating for 4 h at pH 2. The most noticeable loss in antibody activity was observed during the first 30 min.

To examine the structural changes of IgY after acid treatment, the fluorescence emission peak was measured. The tryptophan residues of IgY were excited at 296 nm; this reflected the structural changes near the tryptophan residues. Figure 4 shows that the acid treatment of IgY caused a red shift of the fluorescence emission peak, due to the change of the local environment around the tryptophan residues. After incubating at pH 7 for 4 h, IgY had an emission peak at 330 nm, which was the same peak as native IgY. The emission peak for IgY changed from 330 nm to 337 nm or 338 nm after incubating at pH 3 or pH 2 for 4 h.

Suppressive effects of polyols on acid-induced inactivation The IgY activity at pH 3 in the presence of polyol is shown in Fig. 5. Sorbitol and xylitol were used as polyols. Sorbitol suppressed IgY inactivation in a concentration-dependent manner. The IgY activity was significantly increased in the presence of 30% sorbitol or above. Fifty percent sorbitol showed almost complete suppression of acid-induced

Table 1. Separation efficiency of three isolation methods

separation method	protein (mg/mL)	IgY[e] (mg/mL)	purity[d] (%)	specific IgY (%)
WD[a]	5.5	1.1	20.0	10.7
C[b]	5.8	1.4	24.1	14.1
PEG[c]	6.9	2.6	37.1	23.5

[a]: water dilution method
[b]: λ-carrageenan method
[c]: polyethylene glycol method
[d]: purity was calculated as conc. of IgY ÷ conc. of protein × 100
[e]: crude IgY

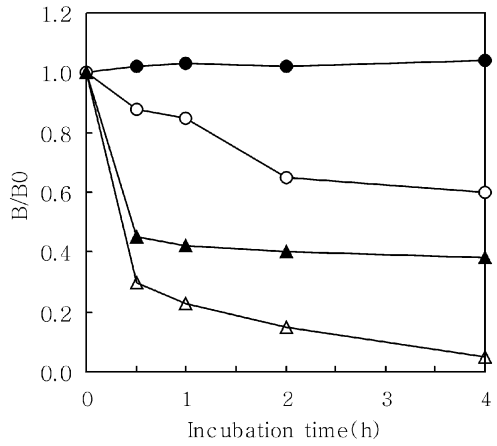

Fig. 3. Time-dependent changes of IgY activity under acidic conditions. IgY was incubated at pH 7 (●), pH 4 (○), pH 3 (▲) or pH 2 (△). B/Bo was calculated as absorbance of acid-treated IgY ÷ absorbance of native IgY. Absorbance was measured at 405 nm.

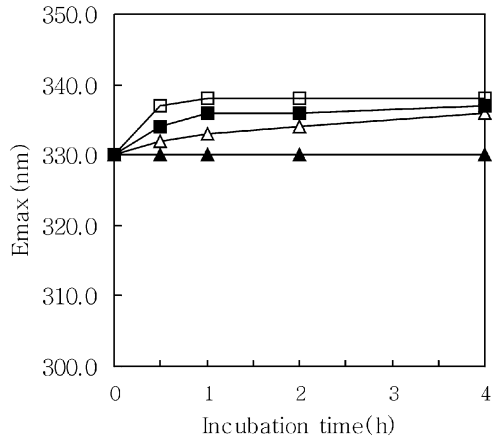

Fig. 4. Fluorescence emission spectra of IgY. IgY was incubated at pH 7 (▲), pH 4 (△), pH 3 (■) or pH 2 (□) for 4 h. E*max* was the emission peak, when excited at 296 nm.

inactivation at pH 3; however, xylitol caused no increase in IgY activity.

At larger intakes, polyols generally have a laxative effect and are usually used in limited amounts, or in combination with sucrose. Therefore, the effect of sucrose substitution for sorbitol, or xylitol on IgY activity at pH 3, was investigated (Fig. 6). IgY showed almost the same activity as native IgY when sorbitol was replaced by sucrose. However, the acid-induced inactivation of IgY was not enhanced by xylitol replacement with sucrose. Because the acid-induced inactivation was related to the tryptophyl fluorescence (Fig. 4), the fluorescence emission spectral changes by polyol or sucrose substitution for polyol were also examined (Fig. 7). The emission peak for IgY showed a blue shift with an increase in the sorbitol concentration. The emission peak for IgY in the 50% sorbitol solution was 330 nm, which was the

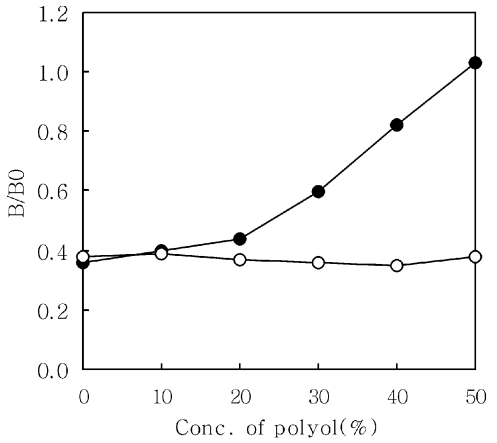

Fig. 5. Antibody activity of IgY in aqueous polyol solution. IgY was incubated with sorbitol (●) or xylitol (○) at pH 3. B/Bo was calculated as absorbance of IgY at pH 3 ÷ absorbance of native IgY. Absorbance was measured at 405 nm.

same peak that was exhibited by native IgY. Sorbitol replacement with sucrose had no effect on the emission peak. However, xylitol or xylitol replacement with sucrose did not cause a blue shift. These results suggest that sorbitol may inhibit structural changes near tryptophan residues by acidification, which results in the improvement of IgY stability.

Discussion

H. pyroli is a significant human pathogen. Up to 50% of the world's population is infected with *H. pyroli* (Goodwin *et al.*, 1997). Several investigators reported the potential use of IgY in immunotherapy (OFarrelly *et al.*, 1992; Yang *et al.*, 1997; Smith *et al.*, 2001); therefore, passive immunity by oral administration of IgY may be helpful to protect against *H. pyroli*. The reduced acid stability of IgY hindered its use for protection from *H. pyroli*. Therefore, the possible improvement of IgY stability at pH 3 in a polyol solution was examined.

IgY showed an irreversible inactivation under acidic conditions (Fig. 2). A remarkable inactivation of IgY occurred at pH below 3 during the first 30 min (Fig. 3). Palmer *et al.* (1963) showed that the noncovalent bonds of rabbit IgG were disrupted under acidic conditions. According to the increase in positively-charged groups by acidification, the electrostatic interactions (including electrostatic repulsion) may be changed. These changes are attributable to the destruction of the noncovalent bonds.

The acid-induced inactivation of IgY suggests a decrease in the antigen-binding ability that is probably due to structural changes. Fluorescence emission spectrum is a useful tool for the investigation of structural changes (Cho and Song, 2000). Therefore, IgY fluorescence emission spectra, when excited at 296 nm, were observed. The quenching effect on tryptophan

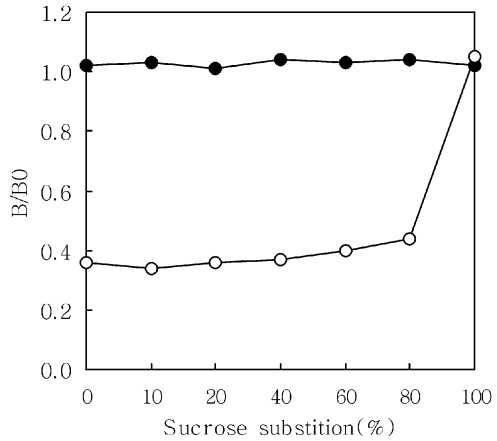

Fig. 6. Antibody activity of IgY in the presence of polyol in combination with 0~50% sucrose at pH 3. Sorbitol (●) or xylitol (○) was used as polyol. Total sugar concentration was 50%. B/Bo was calculated as absorbance of acid-treated IgY ÷ absorbance of native IgY. Absorbance was measured at 405 nm.

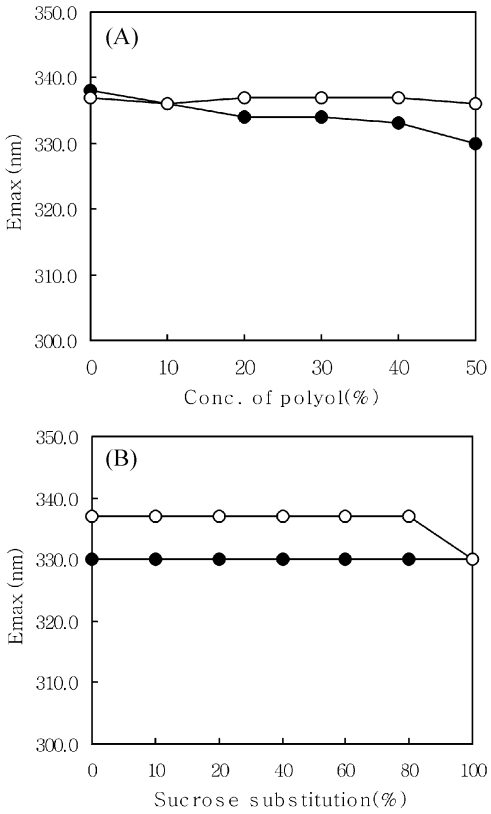

Fig. 7. Fluorescence emission spectral changes of IgY by polyol (a), or sucrose substitution for polyol (b) at pH 3. Sorbitol (●) or xylitol (○) was used as polyol. Total sugar concentration was 50%. E*max* was the emission peak, when excited at 296 nm.

residues was gradually removed by acidification (Fig. 4). Disulfide bonds quench tryptophyl fluorescence of native immunoglobulin. The IgY primary sequence data indicates that IgY possesses intra- and intermolecular disulfide bonds, which confer quenching effects on tryptophan residues, such as mammalian IgG (Thompson *et al*., 1987; Parvari *et al*., 1988; Reynaud *et al.*, 1989). This result, therefore, shows that the quenching effect is irreversibly removed by partial exposure of the tryptophan residue at low pH. In contrast to mammalian IgG, IgY has a heavy chain with four constant domains and no hinge region, which gives considerable flexibility to antigen binding. There are regions in constant domains that contain proline and glycine residues, which act like a switching hinge and confer a limited flexibility on antigen-binding of IgY (Warr *et al*., 1995). This limited flexibility may decrease the antigen-binding activity of IgY, even when small structural changes are evident.

Polyols are often used for stabilizing protein. Sorbitol and xylitol (among polyols) are widely-used sugar substitutes, due to their usefulness for diabetics, reduced cariogenecity, and inhibition of bone resorption (Giese, 1993; Matilla *et al*., 1996). IgY activity at pH 3 was enhanced in 30% sorbitol or above, but xylitol did not increase the acid stability of IgY (Fig. 5). An increase in sorbitol concentration induced a gradual blue shift in the emission peak for IgY. The acid-induced inactivation of IgY was suppressed almost completely in a 50% sorbitol solution (Fig. 6). As indicated by several researchers (Gekko, 1981; Gekko and Timasheff, 1981), the preferential hydration of IgY may occur in an aqueous sorbitol solution; therefore, hydrophobic interactions around tryptophan residues would be strengthened. These changes might protect the partial exposure of tryptophan residues, which would result in stabilization of the IgY structure. However, xylitol could not effectively stabilize hydrophobic interactions. Gekko *et al.* (1999) reported that the stabilizing effects of polyols would increase if the polyol chain length was increased. Xylitol shows less viscosity than sorbitol at the same concentration (Marie and Piggott, 1991). For these reasons, xylitol may not suppress the acid-induced inactivation of IgY.

In conclusion, the IgY stability at pH 3 was considerably improved using high concentrations of sorbitol in solution. Sorbitol prevented the partial exposure of tryptophan residues, which resulted in the enhancement of IgY stability. However, xylitol could not suppress the acid-induced inactivation. These results suggest that sorbitol may be useful in stabilizing IgY at low pH. Therefore, IgY could potentially be applied in high-acid food products and extensively used for protection against *H. pyroli* infection.

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