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USE OF GLUCAN FROM *SACCHAROMYCES CEREVISIAE* AS AN IMMUNOSTIMULANT IN CARP: IMPACT ON HEMATOLOGY, PHAGOCYTE FUNCTION, AND INFECTION WITH *AEROMONAS HYDROPHILA*

V. Selvaraj[1]*, K. Sampath[2] and V. Sekar[3]*

[1] *Spic Research and Development, Spic Limited, Tuticorin 628 005, Tamilnadu, India*

[2] *Department of Zoology, V.O.C. College, Tuticorin 628 008, India*

[3] *Department of Molecular Microbiology, School of Biotechnology, Madurai Kamaraj University, Madurai 625021, India*

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Key words: carp, leukocytes, macrophage, monocytes, NBT assay, neutrophils, opsonin, phagocytes

Abstract

Glucan was extracted from yeast *(Saccharomyces cerevisiae)* and subjected to chemical analyses. Thin layer chromatography (TLC) revealed that glucan is primarily composed of glucose units and contains 97.2% glucose. Infrared spectrum showed a major band at 883/cm, suggesting that glucan is linked to β-glycosidic. Intraperitoneal administration of different doses of glucan enhanced the survival rate of carp *(Cyprinus carpio)* infected by *Aeromonas hydrophila*. When a concentration above 500 µg glucan per fish was administered, survival relative to the uninfected control was 100%. The total leukocyte count and neutrophil and monocyte populations increased after administration of the glucan. The maximum effect occurred on day 6 after injection. Macrophages of compound-administrated test animals significantly increased production of superoxide anions, efficiently killing the bacterial pathogen. This study showed that glucan can be effective in inducing non-specific cellular defense mechanism in carp.

Introduction

Infectious diseases are a major concern in aquaculture. To prevent financial losses, fish farmers must take sufficient disease-preventing measures. Immunostimulants have recently been used as prophylactics to control infectious disease. Fish depend more heavily on non-specific defense mechanisms than mammals (Anderson, 1992). Immunostimulants

* Corresponding authors. V. Selvaraj, SGS Life Science, SGS India Pvt. Ltd. TICEL Bio Park Ltd, Taramani Road, Taramani, Chennai-600113, Tamilnadu, India; e-mail: sselvarajv@yahoo.co.in; V. Sekar, tel.: 91-452-2458684; e-mail: sekarpsb2001@yahoo.com

elevate non-specific defense mechanisms of animals. Unlike vaccines, which are costly and specific to particular pathogens, immunostimulants are inexpensive and effective against a broad spectrum of pathogens.

There are at least 20 different compounds, including levamisole, lipopolysaccharide, glucan, etc., that are used as immunostimulants, adjuvants, and vaccine carriers in fish (Anderson, 1992). Among these compounds, glucan is one of the best stimulators of non-specific defense mechanisms in fish. This compound can be extracted from various sources such as yeast, barley and algae (Yano et al., 1991; Jeney and Anderson, 1993; Dalmo and Seljelid, 1995) and used as immunomodulators in animals. Glucan has been reported to enhance resistance against bacterial pathogens such as *Vibrio anguillarum, Salmonicida*, and *Yersiniaruker* in several species of fish such as the carp, *Cyprinus carpio* (Yano et al, 1989), Atlantic salmon, *Salmo salar* (Robertson et al., 1990), rainbow trout, *Oncorhynchus mykiss* (Jeney and Anderson, 1993), yellowtail, *Seriola quinquradiala* (Matsuyama et al., 1992) and African catfish, *Claris gariepinus* (Yoshida et al, 1995).

Application of β-(1,3)D-glucan enhances the non-specific cellular defense mechanisms of animals by increasing the number of phagocytes and the bacterial killing activity of macrophages in rainbow trout, Atlantic salmon, and catfish (Nikl et al., 1991; Yano et al., 1991; Chen and Ainsworth, 1992; Jeney and Anderson, 1993; Jorgensen et al., 1993b; Dalmo and Seljelid, 1995; Jorgensen and Robertsen, 1995; Solem et al., 1995) and through the production of superoxide anions by macrophages (Jorgensen et al., 1993a; Dalmo and Seljelid,1995; Solem et al., 1995). The impact of glucan on control of infection caused by *Aeromonas hydrophila* on fish, particularly *C. carpio*, has not been studied extensively. The present investigation reports the effects of administrating β-glucan (isolated from the baker's yeast, *Saccharomyces cerevisiae*) on survival, phagocytosis, NBT assay, and hematology of *C. carpio* infected by *A. hydrophila*.

Materials and Methods

Experimental animals. *C. carpio* were purchased from Manimutharu Dam, Manimutharu, Tirunelveli District, Tamilnadu, and transported to the laboratory in aerated plastic bags. The animals were allowed to acclimatize to laboratory conditions for 15 days before being used in the experiment. The weight of the animals ranged 25-30 g. The experiments were carried out in 250-l glass tanks containing tap water at 30±2°C. Water was changed on alternate days. The fish were fed a pelleted diet containing 35% crude protein that was prepared in our laboratory.

Experimental compound. β-glucan was isolated from the yeast *S. cerevisiae* and characterized following the methods of Northcote and Horne (1951) and Bacon et al. (1969). Purified β-glucan was suspended in phosphate buffered saline (PBS; pH 7.2) and subjected to sonication to reduce its particle size.

Experimental pathogen. Infected fishes were collected from a fish farm at Manimutharu Dam, Manimutharu, Tirunelveli District, Tamilnadu, India. The infected fish had characteristic symptoms of dropsy in the abdomen, blisters, abscesses, and hemorrhagic septicemia, particularly in the gills, vent, and abdomen. Experimental pathogens from the infected fish were isolated according to Shome and Shome (1999) by taking swabs from the gills, liver, heart, kidney, and abdominal fluid, streaking them on Tryptone soya agar, and incubating them at 37°C for 24 h.

Predominately cream-colored, mucoid, round, elevated colonies were chosen and inoculated into LB broth for further biochemical characterization using standard procedures. Based on the comparative biochemical test, the isolated bacterium was identified as *A. hydrophila* (Table 1). To check for the Koch postulate, a pathogenecity test was conducted by intraperitoneal inoculation of live *A. hydrophila* at a concentration of 2.11 x10^7 CFU/ml into uninfected fish using PBS-injected fish as a control. The same clinical signs were observed two days after fish were inoculated with the bacteria.

Table 1. Comparison of biochemical characteristics of a known culture and bacterium isolated from infected *Cyprinus carpio*, according to Bergey's Manual.

Biochemical characteristic	*Known culture*	*Bacterium isolated from infected carp*
Motility	+	+
Indole production	+	+
MR	-	-
VP	+	+
Citrate utilization	D	D
Nitrate reduction	+	+
Casein hydrolysis	D	+
Starch hydrolysis	+	D
Gelatin liquification	+	+
Urea hydrolysis	-	-
Acid from		
Adonitol	-	-
Cellobiose	D	V
Dulcitol	-	+
Fructose	-	ND
Galactose	+	-
Glucose	+	+
Inositol	-	+
Lactose	V	+
Maltose	+	+
Mannitol	+	ND
Raffinose	-	+
Salicin	+	ND
Sucrose	+	ND
Trehalose	+	+
Xylose	-	-
Sorbitol	-	-

V = variable result, D = doubtful, ND = not detected

The bioassay method of Saeed and Plumb (1986) was used to determine the LD_{50} concentration of *A. hydrophila*. Thoroughly acclimated experimental animals were divided into five groups, each containing 10 animals. Five concentrations of the pathogen in 0.1 ml of PBS were administered intraperitoneally to the animals and mortality was observed for 72 h. Bacterial density in each concentration was measured colorimetrically at 620 nm and the number of bacteria present in each concentration was determined by the pour plate method.

Intraperitoneal injection. Fish were taken from the stock tank and divided into four groups. Each group contained 24 fish. Three groups were used for treatment and one served as a control. The fish received β-glucan by intraperitoneal (ip) injection at one of three concentrations - 100, 500, 1000 µg/fish - on days 1, 3, and 5. The control animals received an injection of only 0.1 ml PBS.

Challenge study. The test groups and control were challenged with an ip injection of LD_{50} concentration of *A. hydrophila* on day 7. Mortality was recorded daily up to seven days and the relative percent survival (RPS) was calculated by the method of Baulny et al. (1996): RPS = (mortality of the control – mortality of the treated fish/mortality of the control) x 100.

Functional assay determination. Blood samples were taken from glucan treated and control fish for hematology tests. The kidneys were removed to study bacterial killing by NBT assay on day 7.

Total leukocyte count (TLC). Total leukocyte counts were made in a Neubauer counting chamber by the method of Schaperclaus et al. (1991).

Differential count. Blood smears were prepared with May-Grunwald/Giemsa. One hundred leukocytes were counted under the microscope and the percentage of each type of leukocytes was calculated. The cell types - granulocytes (basophils, neutrophils, and eosinophils), monocytes, and lymphocytes - were identified by shape, color, and size according to the method of Schaperclaus et al. (1991).

Isolation of anterior kidney macrophages. Macrophages were isolated from the kidneys as described by Braun-Nesje et al. (1981). The kidneys were dissected and transferred to 7 ml of L-15 medium (HI Media, India) supplemented with 10% fetal calf serum (FCS). A cell suspension was prepared by pressing the kidney with a glass rod through a stainless steel mesh (0.3 mm diameter) in a plastic petri dish on ice.

Cells were suspended in L-15 medium supplemented with 0.33 g/ml glucose, 100 IU/ ml penicillin-streptomycin, and 10% FCS. The medium was adjusted to pH 7.6 and sterilized by syringe filtration. 10 IU/ ml heparin (sterile) was added to the medium.

Cell suspensions were loaded onto a discontinuous (densities 1.08 and 1.07) Percoll gradient (Sigma) and centrifuged at 4°C for 40 min at 400 g (Hitachi). The macrophage enriched cell fraction was collected and the number of cells counted in a hemocytometer. The cells were washed in L-15 medium twice by centrifugation, the supernatant was discarded, and the pellet was resuspended in L-15 medium containing 10% FCS at a concentration of 1 x 10^6 cells/ml.

Bacterial killing assay. This experiment was performed according to Chen and Ainsworth (1992). A bacterial concentration of approximately 1 x 10^7 CFU/ml *A. hydrophila* was used as the stock. From this, 0.1 ml was mixed with 0.1 ml of the macrophage suspension (1 x 10^6 cell/ml) and 0.04 ml of heat-inactivated pooled fresh carp serum was added. The mixture was mixed well and incubated for 2 h with occasional shaking in a water bath at 27°C. After 2 h, 0.1 ml of the mixture was diluted with 9.9 ml sterile distilled water to release living bacteria from the phagocytes. This was serially diluted, plated on an LB agar plate, and incubated overnight at 37°C. The number of colonies was counted.

Zymosan A activation. This experiment was performed following the method of Jorgensen and Robertsen (1995). Zymosan A (Sigma) was suspended in 0.9% NaCl (10 mg/ml), boiled in a water bath for 30 min, washed twice in PBS, and opsonized by incubation with fresh carp serum (5 mg/ml) for about 1 h at 14°C. The coated zymosan was then washed twice in PBS and diluted to 10 mg/ml in PBS and stored in small aliquots at -20°C.

Oxygen burst activity assay. This experiment was performed as described by Chung and Secombs (1988) and Dalmo et al. (1996). From the macrophage suspension, 100 µl was placed into a 96 well microtiter polystyrene plate and allowed to adhere for 2 h at 18°C. Non-adhered cells were removed by three washes with 100 µl/well of L-15 medium supplemented with 10% FCS. To the

macrophage monolayer, 100 µl/well of NBT solution (1 mg/ml L-15, 10% FCS) containing activated opsonized Zymosan A at 500 µg/ml was added. After 30 min incubation at 12ºC, the medium was removed and the culture was washed twice with isotonic PBS, fixed with 100 µl/well of 100% methanol for 3 min, and washed twice with 70% methanol. The cells were air-dried. Formazan was solubilized in 120 µl KOH (2M) and 120 µl DMSO (100%) and the absorbance was read spectrophotometrically (Hitachi) at 620 nm using KOH/DMSO as a blank.

Results

Infrared spectrum. The alkaline insoluble glucan infrared absorption spectrum, with peaks at 885, 1035, 1070, 1105, 1150, 1200, 1240, 1300, and 1450/cm, clearly exhibited an absorption band at 885/cm indicating β-glycosidic linkage; there were no absorption bands at 845 and 820/cm (Fig. 1).

Thin layer chromatography (TLC). Acid hydrolysis of alkaline insoluble glucan had a similar chromatography pattern as that of glucose and no other bands appeared in the chromatogram (Fig. 2).

Experimental pathogen. The organism was a gram-negative, motile, non-spore forming bacterium. Table 1 shows the comparison of the bacterium isolated from the infected animals with known culture characteristics according to Bergey's Manual.

Total leukocyte counts. There were no significant changes in the total number of leukocytes in the control group during the experiment. The number of leukocytes in glucan treated fish gradually increased in direct proportion to the concentration (Fig. 3).

Differential count. There was no significant change with time in the distribution of leukocyte types in the control group (Table 2). Neutrophils were dominant, followed by monocytes in treated fish. On the second day, there was a statistically significant difference (t =13.96, n = 6; p<0.01) in neutrophils between the control and the group exposed to the 100 µg concentration. Monocytes in the 100-µg concentration significantly differed (t = 3.68, n = 6; p<0.05) from the fourth day. Eosinophils and basophils declined considerably in relation to the exposure period and concentration. The lymphocyte count did not vary much between the control and experimental groups.

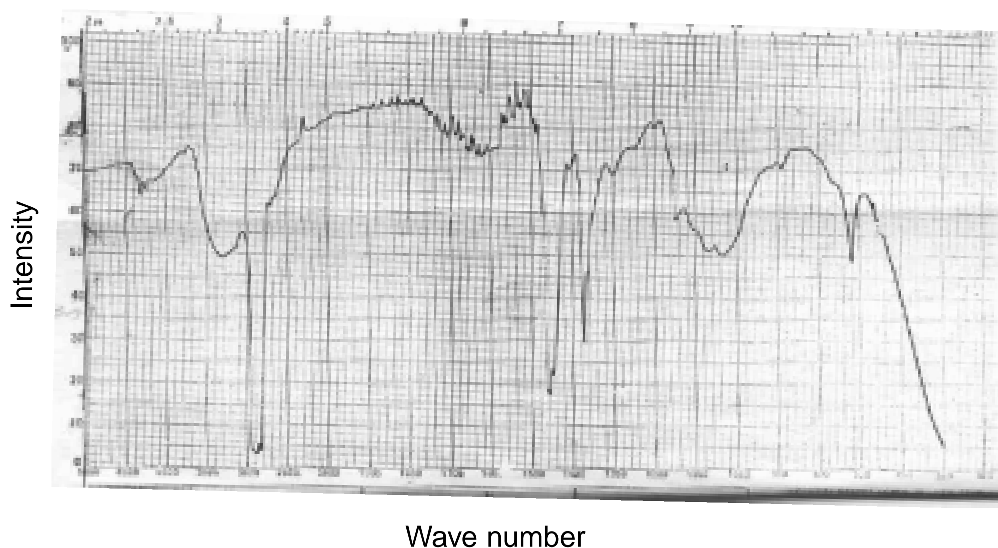

Fig. 1. Infrared spectrum of β-glucan isolated from yeast *(Saccharomyces cerevisiae).*

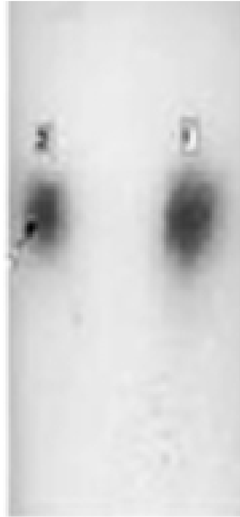

Fig. 2. Thin layer chromatography of acid hydrolysis of β-glucan: 1 = standard glucose, 2 = hydrolyzed product of β-glucan.

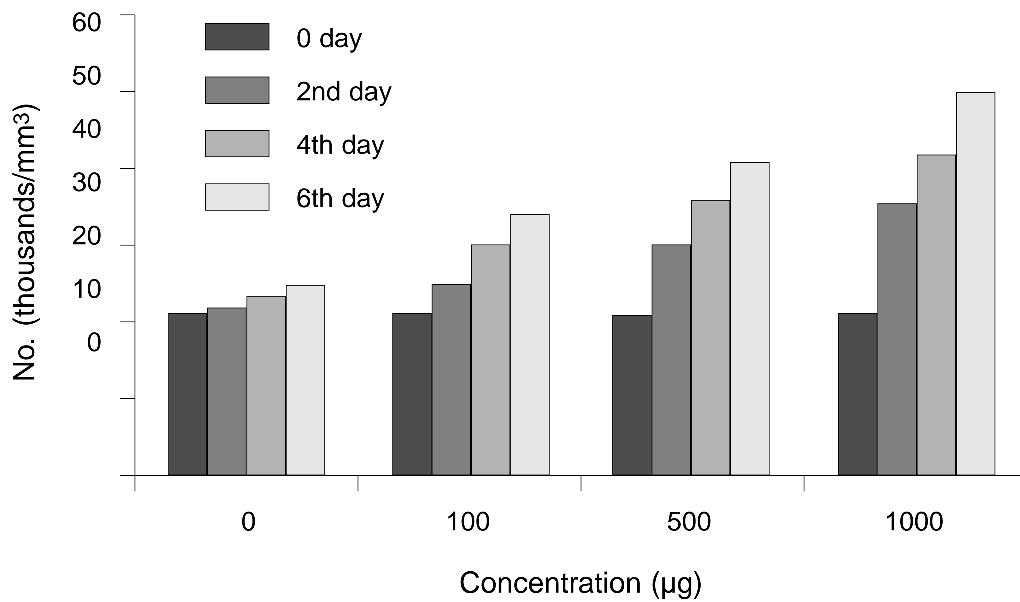

Fig. 3. Effect of intraperitoneal injection of glucan on leukocyte count in experimental *Cyprinus carpio.* Each value is the average of three observations.

Table 2. Effect of intraperitoneal injection of β-(1,3)D-glucan on cell counts (%) in *Cyprinus carpio* infected by *Aeromonas hydrophila* (means±SD of three observations).

Concentration (µg)	*Day of sampling*	*Type of leukocyte*				
		Neutrophilic granulocytes	*Basophilic granulocytes*	*Eosinophilic granulocytes*	*Monocytes*	*Lymphocytes*
Control	0	21.0 ± 0.816	18.33 ± 0.471	17.0 ± 0.816	20.33 ± 0.471	21.66 ± 1.247
	2	24.0 ± 0.816	17.0 ± 0.816	16.33 ± 0.471	20.66 ± 0.471	22.8 ± 0.816
	4	26.0 ± 0.816	14.66 ± 0.471	14.0 ± 0.816	23.0 ± 0.816	22.33 ± 0.942
	6	28.0 ± 0.816	20.0 ± 0.816	12.66 ± 0.471	23.66 ± 0.471	23.66 ± 0.942
100	2	33.3 ± 0.471*	9.0 ± 0.816	12.0 ± 0.816	22.0 ± 0.816	24.33 ± 0.942
	4	36.33 ± 0.33*	5.0 ± 0.816	8.66 ± 0.471	26.0 ± 0.816**	24.0 ± 0.816
	6	35.0 ± 0.18*	3.0 ± 0.816	5.66 ± 0.942	30.33 ± 0.471*	26.0 ± 0.816
500	2	40.0 ± 0.816*	3.66 ± 0.471	5.66 ± 0.471	26.0 ± 0.816*	24.66 ± 0.471
	4	43.6 ± 0.471*	1.66 ± 0.471	2.0 ± 0.816	28.0 ± 0.471*	24.33 ± 0.471
	6	44.6 ± 1.247*	-	1.0 ± 0.816	31.0 ± 0.816*	23.33 ± 0.471
1000	2	42.4 ± 0.471*	-	1.33 ± 0.471	30.0 ± 0.816*	25.0 ± 0.816
	4	45.0 ± 0.816*	-	0.33 ± 0.471	30.0 ± 0.816*	24.66 ± 0.471
	6	46.3 ± 0.471*	-	-	30.6 ± 0.816*	23.0 ± 0.816

Relative survival of carp challenged with A. hydrophilla. The treated fish had a higher relative survival than the control fish. Relative survival was significantly higher in fish treated with 500 or 1000 µg (Table 3).

Bacteria killing assay. A. hydrophila was killed more efficiently by macrophages in treated fish than in control fish and the bacterial count was significantly lower (t = 26.6, n = 6; p<0.01) even at the lowest concentration (100 µg/fish).

Superoxide anion production/NBT assay. The ability of yeast glucan to trigger superoxide anion production in macrophages was measured by NBT reduction assay. Fish treated with 500 µg/fish significantly differed (t = 2.168 n = 6; p<0.05) in superoxide anion production compared to the control.

Discussion

When subjected to infrared absorption spectrum, the substance extracted from the yeast (*S. cerevisiae*) showed a predominant band at 883/cm, suggesting that this compound is made up of β-glycosidic linkage. Absorption bands were absent at 845 and 820/cm, indicating the absence of α-glycosidic linkage. Alkaline insoluble glucan of the M-form was identified as β-glucan, which included a β-(1-3) glycosidic linkage (Kanetsuna et al., 1969; Kanetsuna and Carborell, 1970). Robertson et al. (1990) reported that they extracted an insoluble polysaccharide from the cell wall of *S. cerevisiae* and that it was M-glucan containing only glucose. Glucan obtained from *S. cerevisiae* contained over 95% glucose linked through β-(1-3) and β-(1-6) glycosidic linkage

Table 3. Effect of β-(1,3)D-glucan administration on relative survival, NBT assay, and bacteria killing in carp subjected to a LD_{50} concentration (2.94 x 10^7 CFU/ml) of *Aeromonas hydrophila* (means±SD of three observations).

Assay	*Injection* *PBS*	*100*	*500*	*1000*
Relative survival (%)	0	71.4	100	100
NBT assay (OD value)	0.20±0.015	0.30±0.005*	0.38±0.005*	0.37±0.015*
Bacterial killing assay (CFU)	268×10^3±10.066	65×10^2±7.435**	48×10^2±3.511**	17×10^2±4.163**

* Significant at 5% level
** Significant at 1% level

(Engstad and Robertsen, 1993). TLC studies revealed that the alkaline insoluble glucan prepared in the present study was made up of only glucose units (~97.2%).

In the present study, the purified form of glucan given to the animals by intraperitoneal injection resulted in enhanced resistance against a virulent strain of *A. hydrophila.* The effective dose was above 500 µg/fish. Administration of glucan at 500 and 1000 µg/fish provided complete (100%) protection to animals challenged with an LD_{50} concentration of the pathogen. Chen and Anisworth (1992) reported that an intraperitoneal injection of β-(1-3) glucan in channel catfish (*Ictalurus punctatus*) greatly reduced mortality from infection with *Edwardisella ictaluri* and that an intraperitoneal injection of an insoluble polysaccharide (M-glucan) obtained from the cell wall of *S. cervisiae* enhanced the non-specific disease resistance of Atlantic salmon, *Salmo salar*.

The total leukocyte number increased on the second day after injection of the primary dose. The highest number of leukocytes on the sixth day after injection was found in fish treated with 1000 µg/fish. The significant increase in total leukocytes was proportionate to the dose of the compound. Monocytes and neurophils increased in number proportionately to the glucan concentration and correlated with relative survival. This may be attributed to the fact that monocytes and neutrophils are in the first line of defense and involved in antigen processing and presenting. The number of esosinophils and basophils considerably declined in relation to concentration. The present results concur with reports of Jeney and Anderson (1993), Jorgensen et al. (1993b), Siwicki et al. (1994), and Logambal and Dinakaran Michael (2000).

The ability of macrophages to kill pathogenic microbes is probably one of the most important protection mechanisms. The present study showed that macrophages obtained from fish treated with glucan by intraperitoneal injection enhanced bacteria killing activity, produced more superoxide anions, and is involved in the destruction of the pathogen *A. hydrophila*. The NBT assay was significantly enhanced in respect to all concentrations compared to the control animals, as observed by many earlier studies. Bacteria killing activity was enhanced in glucan-treated rainbow trout (Anderson, 1992), Atlantic salmon (Jorgensen and Robertsen, 1995) and catfish (Yoshida et al., 1995).

The present study indicated that a dose of 500 µg glucan/fish or more increased the relative survival of *C. carpio.* Protection was provided by the enhancement of the total number of leukocytes, the significant increase in number of neutrophils and monocytes, and significantly increased bacteria killing and NBT.

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