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EXPERIMENTAL ARTICLES

Degradation of Fucoidan by the Marine Proteobacterium *Pseudoalteromonas citrea*

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Abstract—It was found that *Pseudoalteromonas citrea* strains KMM 3296 and KMM 3298 isolated from the brown algae *Fucus evanescens* and *Chorda filum*, respectively, and strain 3297 isolated from the sea cucumber *Apostichopus japonicus* are able to degrade fucoidans. The fucoidanases of these strains efficiently degraded the fucoidan of brown algae at pH 6.5–7.0 and remained active at 40–50°C. The endo-type hydrolysis of fucoidan resulted in the formation of sulfated α-L-fucooligosaccharides. The other nine strains of *P. citrea* studied (including the type strain of this species), which were isolated from other habitats, were not able to degrade fucoidan.

Key words: fucoidan, fucoidanases, glycanases, glycosidases, marine bacterium *Pseudoalteromonas citrea.*

The sulfated water-soluble polysaccharides of brown algae, fucoidans, and their low-molecular-weight fragments, fucooligosaccharides, exhibit a wide range of biological activities and can be used to treat some human diseases [1–3]. The biological activity of fucoidans is evidently determined by their structure, which varies depending on the fucoidan source. The fucoidans of brown algae represent a family of homo- and heteropolysaccharides composed mainly of fucose residues sulfated at positions 2 and/or 4 (sometimes at position 3 [4]) and bound by α-1,2-, α-1,3-, or α-1,4-*O*-glycosidic bonds [5, 6]. In addition to fucose (Fuc), fucoidans may also contain minor amounts of mannose (Man), xylose (Xyl), galactose (Gal), glucose (Glc), uronic acids (Ua), and rhamnose (Rha) [1]. Recently, Sakai *et al.* reported on the structure of some fucooligosaccharides containing the Man, Gal, and GlcUa (glucuronic acid) residues, which were obtained through the hydrolysis of fucoidan by the fucoidanase isolated from the halophilic marine bacterium *Flavobacterium* sp. [7]. Data available in the literature on the fucoidanases of marine macro- and microorganisms are scarce [8–11]. To the best of our knowledge, terrestrial organisms do not contain fucoidanases.

Investigation of fucoidanases may provide insight into the structure and the mechanism of the biological activity of fucoidans. The fucooligosaccharides manufactured by the hydrolysis of fucoidan with fucoidanases may be used in medicine.

In our previous work [12], twenty-five strains of epiphytic marine bacteria isolated from the brown algae *Fucus evanescens* and *Chorda filum* and fifty-three bacterial strains isolated from the sea cucumber *Apostichopus* (*Stichopus*) *japonicus* were screened for fucoidanase activity using fucoidans prepared from the brown algae *F. evanescens* and *Laminaria cichorioides.* The bacterial epiphytes *Cytophaga* spp. and some bacterial isolates of the genera *Alteromonas* and *Pseudoalteromonas* from the sea cucumber were found to possess fucoidanase activity [12]. This activity was relatively low but comparable with the activity of the already known microbial fucoidanases [7, 10, 11].

The aim of the present work was to study the ability of some strains of the marine γ-proteobacterium *Pseudoalteromonas citrea* to degrade fucoidan and to investigate some properties of the partially purified fucoidanases of these strains.

MATERIALS AND METHODS

The strains of the aerobic marine bacterium *Pseudoalteromonas citrea* studied in the present work (see table) were isolated from the thallome of brown algae and from the homogenate or coelomic liquid of marine animals collected during scientific offshore expeditions aboard the research vessel *Academician Oparin* in the Sea of Okhotsk and the Bering Sea, as well as at the Marine Experimental Station of the Pacific Institute of Bioorganic Chemistry (PIBOC) in the Peter the Great Bay of the Sea of Japan. The type strain *P. citrea* ATCC 29719^T was isolated from a Mediterranean Sea water sample. The strains are stored in

the PIBOC Collection of Marine Microorganisms (KMM).

The strains were cultivated and cell-free bacterial extracts were prepared as described previously [12].

The activity of β-1,3-glucanases (laminarinases) was assayed using the laminarin isolated from the brown alga *L. cichorioides* [13]. The activity of fucoidanases was determined using the fucoidans isolated from the brown algae *F. evanescens* and *L. cichorioides* [14]. The activity of β-1,6-glucanases (pustulanases) was estimated using the pustulan isolated from a lichen [15]. The activity of amylases, agarases, alginases, and cellulases were determined using commercial amylose, agarose, alginic acid, and carboxymethylcellulose, respectively.

The reaction mixture was prepared by mixing 0.1 ml of bacterial extract (as the enzyme source) and 0.4 ml of a solution containing 4 mg substrate in 1 ml of 0.05 M phosphate buffer (pH 7.2). The mixture was incubated at 20°C for 1 to 20 h, depending on the enzymatic activity to be evaluated. Two control incubation mixtures were prepared by mixing (1) 0.1 ml of bacterial extract and 0.4 ml of buffer and (2) 0.1 ml of buffer and 0.4 ml of the substrate solution. After incubation, the reaction and control mixtures were analyzed for reducing sugars by the Somogyi–Nelson method [16] using the calibration curves that were constructed with the respective monosaccharides.

One unit of glycanase activity (U) was defined as the amount of enzyme (in mg protein) releasing 1 nmol reducing sugars per h at 20°C.

The protein concentration in bacterial extracts was determined by the method of Lowry *et al.*

The activity of glycosidases and sulfatases in bacterial extracts was determined with chromogenic substrates, i.e., the *p*-nitrophenyl derivatives of monosaccharides and sulfate [17].

One unit of glycosidase activity (U) was defined as the amount of enzyme (in mg protein) releasing 1 nmol *p*-nitrophenol per min at 20°C.

Fucoidanases were partially purified by passing bacterial extracts through a column (25 × 1.5 cm; 0.01 M phosphate buffer, pH 7.0) with Sephadex G-25. The fractions eluted from the column were tested for fucoidanase activity using the *L. cichorioides* fucoidan. The appropriate fractions were pooled and used in experiments.

To determine the optimum pH of fucoidanases, the reaction mixture containing 0.1 ml of crude fucoidanase partially purified by gel filtration on Sephadex G-25 and 0.2 ml of a substrate solution (4 mg/ml) was mixed with 0.2 ml of 0.2 M phosphate buffer (pH 4.7, 5.5, 6.1, 6.5, 6.9, and 7.7) or 0.2 M borate buffer (pH 8.0 and 8.8). After incubation for an appropriate time, the fucoidanase activity was determined as described above.

To estimate the thermoresistance of fucoidanases, their aliquots were incubated for 30 min at temperatures ranging from 20 to 60°C at 5°C-intervals and then rapidly cooled to room temperature. The thermoresistance was estimated by comparing the fucoidanase activities of the aliquots before and after heating.

To prepare and characterize the products of the enzymatic degradation of fucoidans, the reaction mixture containing fucoidan and bacterial extract was incubated for 48 h and then subjected to gel filtration on a column (100 × 1 cm; H_2O; 12 ml/h) with Toyopearl HW-40, which was calibrated using glucose (180 Da), melibiose (342 Da), raffinose (504 Da), and laminarin (5000 Da). The concentration of sugars in the reaction mixture and eluted fractions was determined colorimetrically with the phenol–sulfuric acid reagent [18].

^{1}H NMR spectra were recorded using a Bruker WM-250 NMR spectrometer (D_2O; 90°C; acetone with the signal $\delta = 2.22$ ppm as the internal standard).

The carbohydrate composition of the products of the enzymatic hydrolysis of fucoidans was determined after hydrolyzing these products in 4 N HCl at 100°C for 2 h. The hydrolysates were analyzed on a Biotronik carbohydrate analyzer (0.63 × 30 cm Durrum X4-20 column; 60°C; bicinchoninic acid reagent [19]; Shimadzu CR 2AX detector) using the authentic samples of fucose, glucose, galactose, mannose, xylose, raffinose, and rhamnose purchased from Serva (Germany) as reference standards.

RESULTS AND DISCUSSION

From a great number of marine bacteria tested earlier for fucoidanase activity [12], three strains, KMM 3296, KMM 3297, and KMM 3298 (see table), were chosen for the comparative study of the characteristics and specificity of their fucoidanases. All three strains were identified as belonging to the species *Pseudoalteromonas citrea* [20]. It should be noted that the content of fucoidanases in cell-free extracts of *P. citrea* is low and they readily lose their activity during purification. For this reason, investigations were performed using fucoidanase preparations partially purified on Sephadex G-25.

The partially purified fucoidanases of all three strains were able to degrade fucoidan in a broad range of pH values (Fig. 1). The pH dependence of the activity of two fucoidanases had two peaks, which can be interpreted as an indication of the existence of several molecular forms of fucoidanases. The maximum activity of the fucoidanases was observed at pH 6.5–7.0. The same pH optima were reported for the fucoidanases from the facultatively anaerobic marine bacterium *Vibrio* sp. 5 and aerobic *Flavobacterium* sp. [7, 10]. The similarity of the pH optima of fucoidanases from different sources may be due to a similarity of environmental pH in the habitats of the fucoidanase-producing bacteria.

Activity of O-glycohydrolases of various *P. citrea* strains isolated from different habitats

Habitat	Mediterranean Sea, France	Peter the Great Bay, the Sea of Japan, Russia								Kuril Islands, the Sea of Okhotsk, Russia		Komandor Islands, the Bering Sea, Russia
Strain source	Seawater	Mollusks				Ascidia			Sea cucumber	Brown algae		Sponge
		P. yessoensis			*C. grayanus*	*H. aurantium*		*A. translucidum*	*A. japonicus*	*F. evanescens*	*C. filum*	*Plocamia* sp.
Enzyme substrate / Strain	ATTC 29719^T	KMM 157	KMM 280	KMM 327	KMM 188	KMM 216	KMM 250	KMM 256	KMM 3297	KMM 3296	KMM 3298	504
Fucoidan from *F. evanescens*	0	0	0	0	0	0	0	0	350	1920	960	0
Fucoidan from *L. cichorioides*	0	0	0	0	0	0	0	0	470	1300	980	0
Alginic acid	1370	680	500	980	1190	3320	0	0	1700	1720	190	0
Amylose	350	1230	910	1640	400	6270	0	470	3340	3690	3210	0
Laminarin	5650	2140	1750	4220	2520	9170	3370	4140	6780	32190	5780	1820
Pustulan	1680	0	0	0	560	3950	0	80	0	670	610	0
Soluble agarose	770	970	810	580	210	3490	10	290	1700	950	2120	3700
CM-cellulose	1690	1880	520	1050	230	2620	0	50	1420	1890	2350	0
N*p*-α-D-galactopyranoside	0	0	0	0	35100	3800	20000	8270	2020	1570	1750	4130
N*p*-β-D-glucopyranoside	4	660	0	40	300	1560	150	1200	100	130	10	1560
N*p*-β-D-galactopyranoside	0	8	0	0	300	300	230	120	390	90	50	300
N*p*-β-D-N-acetylglucosamine	44000	2206	8650	2070	130	4	0	80	2	0	0	80
N*p*-α-D-mannopyranoside	3	0	0	0	0	0	0	0	0	0	0	0
N*p*-β-D-xylopyranoside	0	0	0	0	3	0	0	0	0	0	2	0
N*p*-α-fucopyranoside	0	50	3	0	0	0	0	0	0	0	0	0
N*p*-Nitrophenyl sulfate	0	90	1450	340	0	0	0	0	0	0	0	0

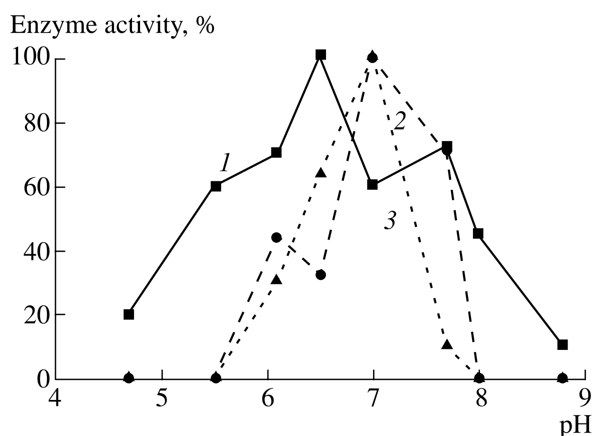

Fig. 1. The pH dependence of the fucoidanase activity of (*1*) *P. citrea* KMM 3296, (*2*) *P. citrea* KMM 3297, and (*3*) *P. citrea* KMM 3298.

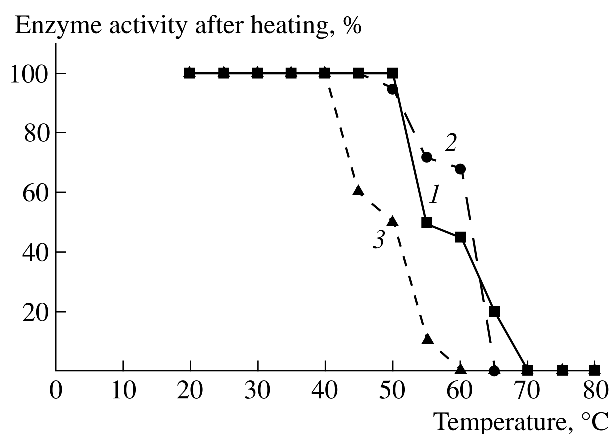

Fig. 2. The temperature dependence of the fucoidanase activity of (*1*) *P. citrea* KMM 3296, (*2*) *P. citrea* KMM 3297, and (*3*) *P. citrea* KMM 3298.

Figure 2 shows the temperature resistance of the fucoidanases studied. It can be seen that the thermostability of the fucoidanases from strains KMM 3296 and KMM 3297 is better than that of the fucoidanase from strain KMM 3298.

The substrate specificity of fucoidanases was studied using two structurally different fucoidans, one isolated from the brown alga *L. cichorioides* collected in the Sea of Japan and the other isolated from the brown alga *F. evanescens* collected in the Sea of Okhotsk near the Kuril Islands. The first fucoidan has a molecular mass of 20–40 kDa and the degree of sulfation Fuc : SO_4 = 1 : 1.6 and the second fucoidan has a molecular mass of 150–500 kDa and the degree of sulfation Fuc : SO_4 = 1 : 0.8. In the first fucoidan, sulfate groups are bound to C-2 and C-4 atoms, whereas they are bound predominantly to C-2 atoms in the second fucoidan. The ratio Fuc : Glc : Gal : Man : Xyl : Rha is 90 : 6 : 1.2 : 0 : 2.8 : 0 for the first fucoidan and 81 : 3 : 4 : 2 : 8 : 2 for the second fucoidan.

Experiments showed that the degree of hydrolysis of the first and second fucoidans by fucoidanases was 10 and 2%, respectively. For this reason, further investigations of fucoidanases were carried out using the fucoidan of *L. cichorioides.*

Figure 3 presents the results of the gel filtration of this fucoidan before (elution profile a) and after hydrolysis by fucoidanases from strains KMM 3296 (profile b), KMM 3297 (profile c), and KMM 3298 (profile d). As follows from this figure, the fucoidanase from strain KMM 3297 (Fig. 3c) degrade fucoidan less intense (the molecular masses of hydrolysis products is from 2.2 to 5.0 kDa) than the fucoidanases from strains KMM 3296 and KMM 3298 (Fig. 3b and 3d), which give hydrolysis products with molecular masses of 1.7–5.0 kDa and 1.3–5.0 kDa, respectively. None of the fucoidanases studied produced mono- or disaccharides in noticeable amounts. The hydrolysis products were found to be mainly composed of fucose (98%).

The ^{1}H NMR spectra of the low-molecular-weight fraction (1.3–1.7 kDa) of the hydrolysis products of fucoidan produced by the KMM 3298 fucoidanase had resonance signals with δ = 5.42, 4.36, 4.51, and 1.33 ppm, which are typical of fucose sulfated at positions 2 and 4 [6]. Therefore, these products represent sulfated fucooligosaccharides. Presumably, the fucoidanases studied catalyze the endo-type hydrolysis of the *O*-glycosidic bonds of fucoidan with the formation of relatively large fragments. The exo-type fucoidanases are most likely absent, since the products of their action, monosaccharides, were not detected. The fucoidanases did not exhibit sulfatase activity. This fact agrees with the observation that the hydrolysis products of fucoidan are sulfated fucooligosaccharides.

As was mentioned above, the *P. citrea* strains under study were isolated from the thallome of brown algae, which contain fucoidan in the cell wall, and from the coelomic liquid of sea cucumbers, which feed on detritus and have fucoidan in their epidermis [6]. It was of interest to clear up whether or not other strains of this species isolated from other habitats contain fucoidanases. For this reason, we analyzed, along with KMM 3296, KMM 3297, and KMM 3298, the other eight *P. citrea* strains obtained from the KMM and the type strain of this species, *P. citrea* ATCC 29719^T.

As was mentioned above, the fucoidans of *L. cichorioides* and *F. evanescens* contain, in addition to fucose, some minor monosaccharides, such as mannose, galactose, xylose, glucose, and uronic acids, whose position and type of bonding to the main polysaccharide chain are as yet unknown [14]. Therefore, there is a probability that fucoidans may be degraded by alginases, laminarinases, galactosidases, and other hydrolytic enzymes, whose substrates are widely spread in marine environments. The evaluation of the activity of different glycanases and glycosidases in the strains under study is also important for their more complete characterization.

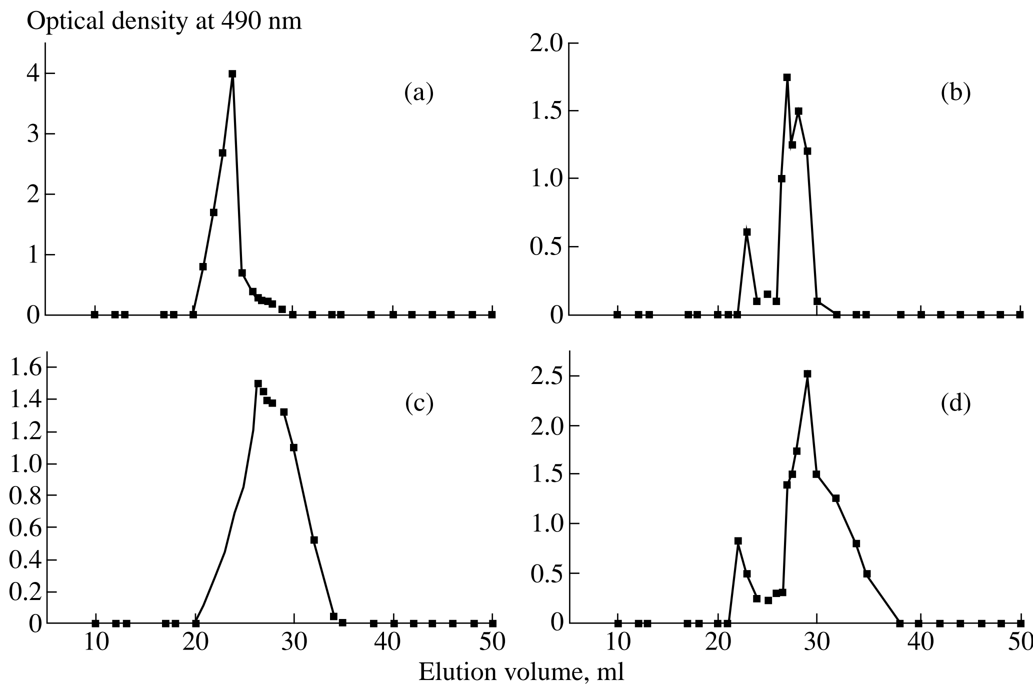

Fig. 3. The gel filtration on Toyopearl HW-40 (100 × 1 cm; 12 ml/h) of (a) fucoidan from *L. cichorioides* and the products of its hydrolysis under the action of fucoidanases from (b) *P. citrea* KMM 3296, (c) *P. citrea* KMM 3297, and (d) *P. citrea* KMM 3298.

The results of this study are summarized in the table. It can be seen that the extracts of all strains, irrespective of their habitats, can efficiently hydrolyze laminarin. This agrees with the observation that laminarinases (β-1,3-glucanases) are present in many marine macro- and microorganisms [21]. Alginic acid, soluble agarose, and carboxymethylcellulose were hydrolyzed less efficiently. In general, the ranges of the glycanases of all twelve *P. citrea* strains studied are similar, except that only three strains, KMM 3296, KMM 3297, and KMM 3298, which were isolated from the fucoidan-rich brown algae and sea cucumbers, have fucoidanases. The other nine strains, which were isolated from habitats lacking fucoidans, did not possess fucoidanase activity. The fucoidanases of strains KMM 3296, KMM 3297, and KMM 3298 can be used for the manufacture of biologically active fucooligosaccharides from the fucoidans of *L. cichorioides* and *F. evanescens*

With respect to glycosidases, whose involvement in the degradation of fucoidan cannot be excluded (table), the strains fell into two groups. The first groups, which comprised the type strain ATCC 29719^T isolated from seawater and strains KMM 157, KMM 280, and KMM 327 isolated from the mollusk *Patinopecten yessoensis* was distinguished by the production of an active β-*N*-acetylglucosaminidase. The second group, which contained strains KMM 3296 and KMM 3298 isolated from the brown algae *F. evanescens* and *Chorda filum*, strain KMM 3297 isolated from the sea cucumber *A. japonicus*, strain KMM 188 isolated form the mussel *Crenomytilus grayanus*, strains KMM 216, KMM 250, and KMM 256 isolated from the ascidia *Halocynthia aurantium* and *Amaroucium translucidum*, and strain KMM 504 isolated from the sponge *Plocamia* sp., was distinguished by the synthesis of an active α-galactosidase. Most strains of both groups produced no α-L-*fucosidases,* α-*N-acetyl*-D-*galactosaminidases,* α-D-mannosidases, and β-D-xylosidases.

Virtually all strains synthesized, in addition to laminarinase, β-D-glucosidase. Strains KMM 216, KMM 250, KMM 256, KMM 504, and KMM 3296 through KMM 3298 [20, 22] with agarolytic activity almost did not hydrolyze agarose but synthesized α- and β-D-galactosidase. The three fucoidanase-producing strains KMM 3296, KMM 3297, and KMM 3298 did not possess α-fucosidase activity. Therefore, these strains seem to violate the rule that the polysaccharide hydrolyzing enzymatic systems involve not only glycanases but also the respective glycosidases. It is likely that the fucooligosaccharides produced from fucoidan by the three strains are further degraded by other microorganisms of the microbial community with the involvement of exofucoidanases, fucosidases, sulfatases, and other enzymes.

The epigenetic, or phenotypic, characteristics of the *P. citrea* strains isolated from the Sea of Japan are very diverse and greatly differ from those of the type strain. The difference between various strains of this species is so great that Ivanova *et al.* proposed to call them ecosubspecies [22]. The results of our present investigation allow the suggestion to be made that the synthesis of fucoidanases, β-*N*-acetylglucosaminidases, and α-galac-

tosidases is a distinguishing intraspecies characteristic of *P. citrea* which depends on the degree of manifestation of some genomic features (which by themselves are insufficient from the standpoint of molecular taxonomy) in the habitat of a particular strain and is determined by its physiological role in the community of organisms and by the environmental pressure (i.e., by the ecophysiology of this strain). This suggestion is confirmed by the observation that the fucoidanase activity of a strain is not related to its taxonomic position. For instance, fucoidan can be hydrolyzed not only by the *P. citrea* strains but also by *Vibrio* sp. strain 2-40, which is close to *Microbulbifer hydrolyticus* and some cellulolytic nitrogen-fixing bacteria, as well as by the bacteria of another phylogenetic cluster of the domain *Bacteria*, namely, by the bacteria *Cytophaga* sp. and *Flavobacterium* sp. of the cluster *Cytophaga–Bacteroides–Flavobacterium–Flexibacter* [7, 10–12].

Thus, the *P. citrea* strains KMM 3296 and KMM 3298 isolated from the brown algae *Fucus evanescens* and *Chorda filum*, respectively, and strain 3297 isolated from the sea cucumber *Apostichopus japonicus* are able to degrade fucoidans. This property makes them biotechnologically promising. The fucoidanases of these strains can efficiently degrade the fucoidan of brown algae at pH 6.5–7.0 and remain active at 40–50°C. The endo-type hydrolysis of fucoidan resulted in the formation of sulfated α-L-fucooligosaccharides. The other nine strains of *P. citrea*, including the type strain of this species, which were isolated from the fucoidan-lacking habitats, are not able to degrade fucoidan. At the same time, some of these strains possess high β-*N*-acetylglucosaminidase and α-galactosidase activities.

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