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Purification of the Anorectic Agent Satietin From Bovine Serum

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NAGY, J. *Purification of the anorectic agent satietin from bovine serum.* PHARMACOL BIOCHEM BEHAV 48(1) 17–22, 1994. – Purification and properties of an endogenous anorexigenic substance extracted from the bovine serum has recently been reported. The semipurified and HPLC-purified substances have shown similar biological effect in ad lib-fed or fasted rats, as was found in the case of human serum satietin. This substance purified from bovine serum or plasma suppressed food intake following intracerebroventricular (ICV) or peripheral administration in rats. A similar purification procedure as was used for the preparation of human serum satietin resulted in semipurified bovine material. This preparation was further purified by HPLC reversed-phase column and yielded two peaks (peak 1 and peak 2). The retention time of peak 2 revealed by HPLC (peak addition method) and the molecular weight measurements carried out by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) and high performance size-exclusion chromatography (SE-HPLC) proved that peak 2 contains bovine serum albumin mostly. Rats were injected with 20 μg/rat of peak 1 and peak 2. Peak 1 can be considered as a putative satiety agent. Preliminary chemical and biochemical studies showed that the semipurified active agent contained 20–22% protein, 28–30% lipids, and an undetermined amount of carbohydrate. The molecular size of anorectic bovine serum preparation was 38–40 kDa, determined by means of SE-HPLC and SDS-PAGE. Based upon the similarity of chemical and biological nature to human serum satietin we named this new material bovine serum satietin (BS-SAT).

Food intake Endogenous bovine satiety agent HPLC purification

OBESITY is a major problem to be resolved in physiological regulation. An endogenous substance with long-lasting regulatory effects on food intake has been the target of a long search. Various known peptides as a tripeptide (pGlu-His-GlyOH) isolated by Reichelt (19), cholecystokinin, calcitonin, bombesin-like peptides (6,10), and a few hydroxy-fatty acids extracted from human blood and sugar acid derivatives described by Oomura (17,18), although these effects have each lasted less than 24 h.

Presumably, a substance should exist in the blood that can regulate food intake and satiety and it may function as a messenger between stomach and the unspecified brain site to terminate feeding. Therefore, preliminary studies were done in our institution that resulted in extraction of a preparation from human serum that behaved as a satiety agent. It was named satietin. Human serum satietin has shown significance and dose dependence with long-lasting anorectic activity in 96-h fasted rats following intracerebroventricular and/or peripheral administration (7). Later on, this substance was further purified by chromatography and electrophoresis and biochemically characterized by Nagy et al. (11,12). According to these chemical studies, the molecular size of human satietin estimated 50–70 kDa and consisted of high carbohydrate and low protein content behaving as a proteoglycan. Meanwhile, the presence of these substances suppressing food intake have been successfully detected in various sera of other mammals.

In 1984 the satietin preparation was thought to be homogenous, but Mendel and Palieschekey (9) demonstrated it could be separated into two peaks by HPLC. One peak was found to contain a biologically active molecule that could be satietin. The other peak was biologically inactive and contains albumin and alpha-1-glycoprotein.

The original semipurified human satietin preparation was found to be highly aversive when tested using the two-bottle taste aversive method (1). However, the biologically active molecule obtained by HPLC purification of semipurified human satietin preparation was found not to be aversive (4). Thus, an impurity in the preparation was most likely producing the aversion response (3). Rat satietin was shown not to have aversive properties (2).

In the present study, satietin was isolated from bovine serum according to the original method of Nagy et al. (12) and then further purified by HPLC. Satietin was isolated from bovine serum for a variety of reasons, including the high cost

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of rat plasma, concerns with using large volumes of human plasma, and the need for a large quantity of material for basic research.

After detecting a satietin-like effect in the bovine serum the procedure was processed by following the method described previously (12,14), which resulted in a substantial amount of crude material. Fractions obtained after this purification process have shown similar biological effects by ICV or intravenous administration (IV) and IP in fasted rats as was found in the case of human satietin. Therefore, we named the preparations bovine serum satietin (BS-SAT).

Accordingly, in the present study BS-SAT was further purified by HPLC and the resultant fractions were tested for biological activity by infusing them ICV into rats. Detailed behavioral studies and statistical analyses of the BS-SAT preparations have been done by Bellinger et al. (5). The molecular size, chemical analyses of the peptede portion, and determination of lipids are also described.

METHOD

Bovine serum was purchased from the Veterinary Biologicals Company, Budapest. Chemicals used for this study were of analytical grade. Solvents in HPLC quality were obtained from Aldrich Chemical Co., Inc., Milwaukee, WI, Sephadex G-15, and Bio-Gel P-2, as gel filtration media were from standard sources. Low molecular weight standard protein mixture and the reagents for SDS-PAGE were purchased from Bio-Rad Laboratories, Richmond, CA.

Amicon cell, Model 2000 C and YM-10 flat membranes were applied for flat membrane filtration. HPLC columns, Ultrasphere ODS columns were from Beckman Instruments Co., PROTEIN-PAK 300 SW columns were obtained from Waters Division of Millipore, Milford, MA. Reversed-phase HPLC separations were performed by using a Waters system, Model 600 equipped with a UV-VIS detector, Model 481, and U6K injector. Size exclusion high-performance chromatography was carried out by isocratic mode on a Beckman HPLC system consisted of a 114B solvent delivery pump, a controller, Model 421A, and a variable wave length, UV-VIS detector, Model 163. The solvents were continuously degassed by helium exchange.

SDS-PAGE: the electrophoresis experiments were carried out on slab gel as described by Laemmli (8) by using an LKB 2050-001 Midget Electrophoresis Unit. The total acrylamide concentration was 13%. 50, 100 μg loads of BS-SAT-SP and BS-SAT-HPLC purified materials were used for electrophoresis and gels were stained with Coomassie Brilliant blue R-250, and silver staining, respectively.

Silica-gel HPTLC (10 × 10 cm) plates were purchased from E. Merck, Darmstadt, Germany. Camag Nanomat Scanner was from Camag Inc., Switzerland. The starting material used in the experiments was semipurified bovine serum satietin (BS-SAT-SP) obtained by using published procedures (11, 12,15). Briefly, this purification involves ultrafiltration of the serum on a 10 kDa molecular size flat membrane (YM-10), 10% trichloracetic acid (TCA) precipitation, and Sephadex G-15 chromatography of the TCA soluble fraction. This was followed by further gel filtration on Bio-Gel P-2 column of the G-15 active fractions. The fractions eluted at the void volume of the gel column were pooled, lyophilized, and bioassayed. As a result of these manipulations we gained salt-free, bioactive yellowish-white powder. The anorectic efficiency of BS-SAT-SP was checked by ICV administration in fasted rats as was earlier reported.

RESULTS

Further Separation of BS-SAT-SP by Reversed-Phase High Performance Chromatography (RP-HPLC)

Further separation of the BS-SAT-SP preparation was processed by RP-HPLC technique on two Ultrashere ODS columns, in series mode, with a linear gradient of 0 to 80%

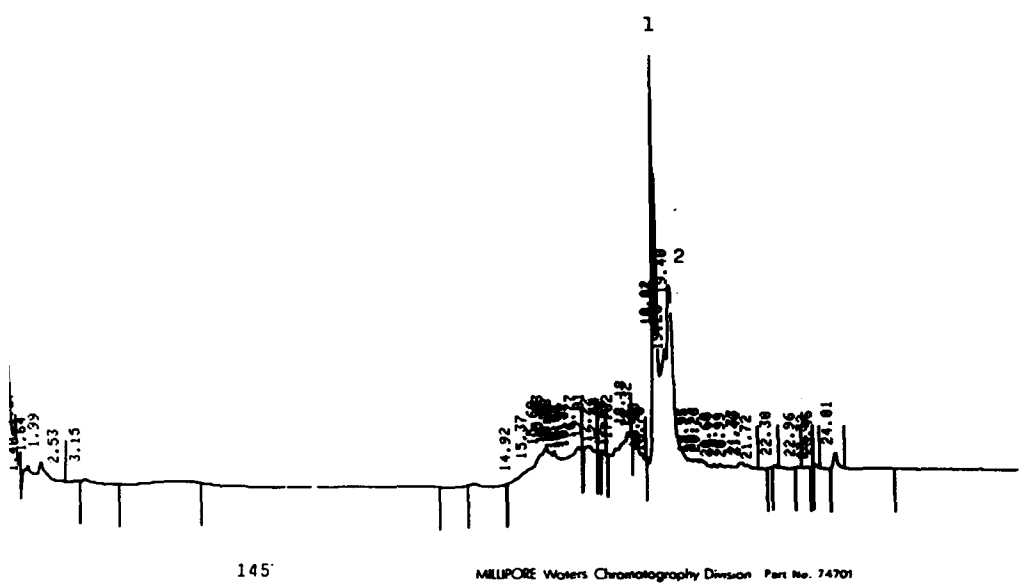

FIG. 1. Separation of BS-SAT-SP by reversed-phase HPLC. Column: two Ultrasphere ODS, 5 μm (4.6 mm × 4.5 cm) in series mode. Mobile phase, A: water/TFA 0.1% B: acetonitrile/water 80:20/TFA 0.1%, linear. Flow rate: 1.5 ml/min. Detection: 280 nm. Aufs.: 0.05. Sample: 60 μg BS-SAT-SP.

elution by using an acetonitrile-water-trifluoroacetic acid solvent system. This separation is depicted in Fig. 1. Peak 1 and peak 2 were collected, freeze-dried, and subjected to feeding bioassay (Table 1). As it was turned out from the ICV bioassay, peak 1 carries the anorectic activity and is considered BS-SAT-HPLC. Peak 2 was evidently just contaminated with the active material. Based upon the retention behavior on HPLC, peak 2 was identified as bovine serum albumin (BSA). This finding was in good agreement with the Coomassie-stained band on the slab gel following SDS-PAGE, and the band was identified as BSA.

It was found that ca. 60% of the recovered starting BS-SAT-SP was in peak 1 and the rest in peak 2. The total recovery of the starting material was about 65–70%. Peak 1 (BS-SAT-HPLC) appeared as a 38–40 kDa band on Coomassie-stained SDS-PAGE. This fraction was also used for amino acid composition analysis in comparison of the BS-SAT-SP preparation (Tables 2, 3).

Molecular Weight Determination of BS-SAT-SP by Means of Size-Exclusion High-Performance Chromatography

Waters PROTEIN-PAK 300 SW columns were used as media for the separation to determine the actual molecular weight of the anorectic BS-SAT-SP. The distribution coefficient (K_d) of standard proteins of known molecular weight were employed for the determination of the size of BS-SAT. In this study K_d was plotted against the molecular weight (M). A typical elution profile can be seen in Fig. 2. After checking the anorectic activity of peak 1, 2, 3, 4, and 5 by ICV administration, it was found that peak 4 carried the anorexigenic activity. Slight effect was detected in fractions of peak 1 that eluted at the void volume, indicating that some amount of nonsoluble active material coeluted with other high molecular weight serum proteins. At the region of peak 3 BSA was detected. Peak 2 proved to be the dimer form of BSA, while peak 5 contains inactive fractions, indicating a mixture of small molecules that appear at the total permeation volume of the high-performance gel column.

On the basis of this measurement, a calibration graph was constructed of K_d vs. M values as described previously (13). When K_d value of BS-SAT was located on the calibration graph, the molecular weight was found to be 38–40 kDa. The results demonstrate the usefulness of the employment of 1% SDS in the mobile phase, which makes BS-SAT more soluble and also prevents the aggregation of other serum proteins.

TABLE 2

AMINO ACID COMPOSITION OF SEMIPURIFIED BOVINE SERUM SATIETIN (BS-SAT-SP)

Amino Acid	Concentration (pmol)	Nearest Integer
Asp	350.000	7
Glu	553.33	11
Ser	325.93	6
Gly	295.45	6
His	52.08	6
Arg	64.81	1
Thr	173.61	3
Ala	327.08	6
Pro	212.60	4
Tyr	61.11	1
Val	162.00	3
Met	28.26	1
Cys	44.78	1
Ile	78.43	2
Leu	247.57	5
Phe	139.20	3
Lys	206.42	4

Calculating for reference standard of Leu.
Total MW: 8,114.

Molecular Weight Determination by Means of SDS-PAGE

Denaturing polyacrylamide gel electrophoresis using sodium dedecyl sulfate was performed essentially as described by Laemmli (8). Prior to loading, samples were placed in boiling water for 3 min in sample buffer containing 10% of 2-mercaptoethanol as sulfhydryl reductant. The electrophoresis was carried out by using a Midget Electrophoresis Unit at a constant current of 25 mA. Commercially available standard proteins were used for calibration. A curve was constructed, where the relationship expressed between molecular weight and the logarithm of mobility (Fig. 3). It can be concluded that the molecular weight of bovine serum satietin proved to be 38–40 kDa.

Lipid Analysis

Extraction of lipids: a modified microextraction method was applied for the lipid separation which is outlined in Fig.

TABLE 1

THE ANORECTIC EFFICIENCY OF HPLC PURIFIED MATERIAL (PEAK 1 AND PEAK 2)

Treatment	Dose (μg)	No. of Animals	Food Intake Means ± SE	
			g/1 h	g/24 h
Saline	–	9	9.17 ± 0.65	24.67 ± 2.20
Peak 1	20	9	1.71* ± 0.78	7.29* ± 1.44
Peak 2	20	9	3.89* ± 0.95	16.33† ± 2.44

Intracerebroventricular administration in rats.
*$p < 0.001$.
†$p < 0.05$.

TABLE 3

AMINO ACID COMPOSITION OF HPLC PURIFIED BOVINE SERUM SATIETIN (BS-SAT-HPLC)

Amino Acid	Concentration (pmol)	Nearest Integer
Asp	81.65	2
Glu	170.27	5
Ser	76.82	2
Gly	193.98	6
His	120.83	3
Arg	50.00	1
Thr	59.86	2
Ala	149.36	4
Pro	167.68	5
Tyr	28.38	1
Val	144.60	4
Met	183.63	5
Cys	85.77	2
Ile	35.15	1
Leu	150.25	4
Phe	91.76	2
Lys	128.01	4

Calculating for reference standard of Ile.
Total MW: 8,012.

4. Concentration solution (2.5 mg/ml) was made from the lyophilized BS-SAT-SP sample. Aliquots were extracted with methanol-chloroform (1 : 2 v/v) solution, which can be basically followed on the flow-sheet presented in Fig. 4. The final sample solutions dissolved in chloroform-methanol (1 : 1 v/v) were subjected to chromatographic analyses. These analyses have been accomplished by means of high performance thin-layer chromatography (HPTLC). The determination of cholesterol ester subfractions, neutral, and polar lipids were performed on Silica-gel 60 HPTLC (10 × 10 cm) plates carried out the separation by using different solvent mixtures as developing systems. Reagent for visualization of the separated spots was Copper sulfate 10% w/v in phosphoric acid of 8% v/v. The content of lipids were determined by densitometry by using a Camag Nanomat instrument. The results of lipid determination will be published elsewhere. On the basis of the analyses, the total lipid content was found to be amounts of 28–30%.

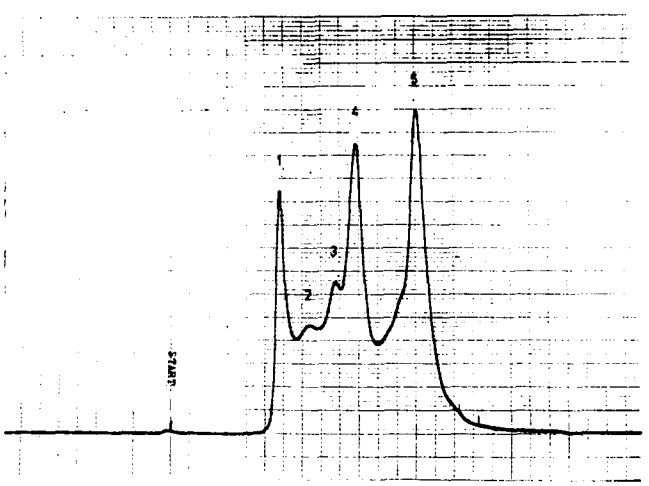

FIG. 2. Separation and molecular weight measurement of BS-SAT-SP by SE-HPLC. Column: two PROTEIN-PAK 300 SW (7.6 mm × 30 cm) in series mode. Mobile phase: 25 mM Triethylamine-Acetic acid buffer, pH 5.0, containing 1% sodium dodecyl sulfate. Detection: 280 nm, 1.0 Aufs. Sample: 500 μg BS-SAT-SP.

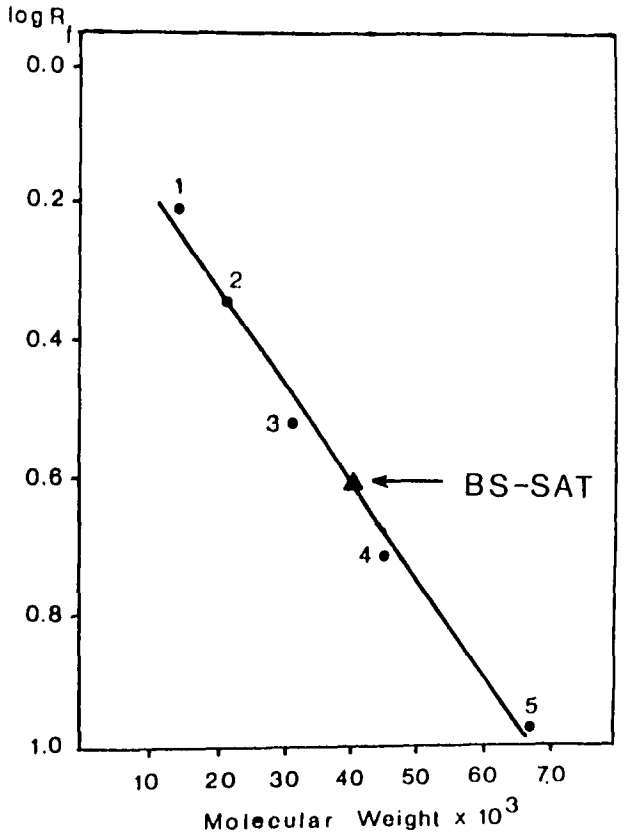

FIG. 3. The relationship between molecular weight and the logarithm of mobility in SDS-PAGE. Mobility is expressed relative to bromophenol-blue. Gels contained 13.0% w/v acrylamide. Each value is the mean of from three measurements. Correlation coefficient was 0.995. Symbols: 1 = lysozyme, 2 = soybean trypsin inhibitor, 3 = carbonic anhydrase, 4 = ovalbumin, 5 = bovine serum albumin.

Analysis of the Peptide Portion

Amino acid analyses of BS-SAT preparations were performed by the PICO-TAG method by using the Waters PICO-TAG Amino Acid Analysis System. The samples were hydrolyzed in 6 N HCl to yield free amino acids. Treatment with phenylisothiocyanate has provided phenylthiocarbamyl (PTC) amino acids. The PTC amino acids liberated from the peptide bone of BS-SAT were separated by reversed-phase HPLC on Waters PICO-TAG C_{18} column and detected in the UV at 254. nm.

The quantity of free amino acids obtained on hydrolysis of the BS-SAT-SP is seen in Table 2. Similarly, the HPLC purified material was also analyzed and the amino acid composition and quantity are listed in Table 3.

According to these results, the size of the peptide part of

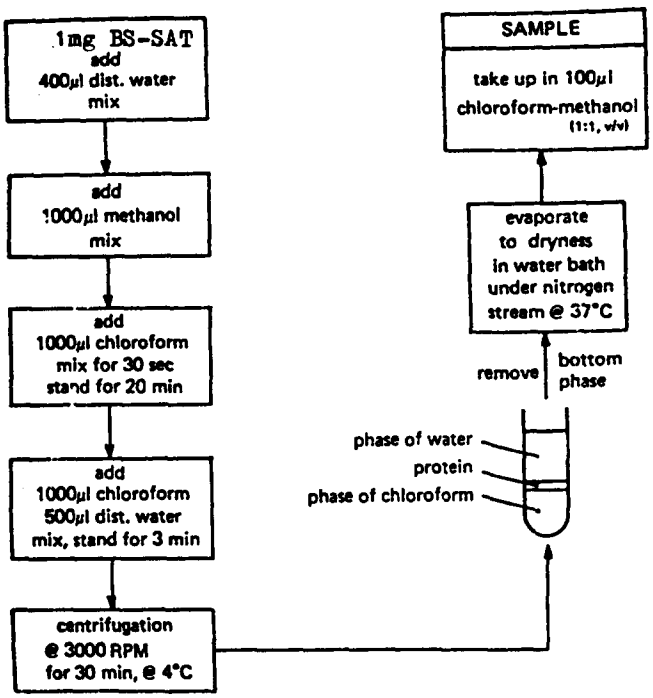

FIG. 4. Flow sheet of microextraction method for the lipid separation.

BS-SAT-SP and BS-SAT-HPLC were found to be comparable. Thus, the molecular weight of the peptide portion of BS-SAT is around 8,000–8,200 Dalton, which means 20–22% peptide content of the 38–40 kDa molecular weight active material.

The carbohydrate part of the BS-SAT molecule has been further subjected to analysis. This effort should reveal the sugar composition and content as well as the concentration of amino sugars. The latter would deliver additional evidence for the glycoprotein nature of BS-SAT.

DISCUSSION

We succeeded to detect the existence of a new potent satiety agent in the bovine serum. The procedure of extracting the endogenous satiety agent from the bovine serum was basically the same as described previously in the course of the purification of semipurified human serum satietin. The BS-SAT-SP preparation has already shown significant activity (5) by ICV infusion in rats. Based on this preliminary observation it can be expected that structural and biological properties will be similar to those preparations which were earlier purified from the human serum. To prove this hypothesis, more data will need to be collected.

First of all, the BS-SAT-SP must be subjected to further separation, because detailed studies on the chemical and biochemical nature of the material can be done only by using highly purified samples. In addition, the bioassay of the purified fractions mean a feed-back for controlling the purification level. Therefore, BS-SAT-SP was subjected to HPLC studies to find a proper method for effective purification. Finally, the HPLC separation succeeded on Ultrasphere C18 column, particle size of 5 µm, and the resultant major peaks were peak 1 and peak 2. Typical HPLC patterns are shown in Fig. 1, which represents routine separation of the BS-SAT-SP and makes possible to collect HPLC purified bioactive fractions. Notable, the overall yield for peak 1 and peak 2 was calculated around 60–65% in comparison to the amount of starting material. The ratio in amounts of peak 1 and peak 2 was found to be 70 : 30.

The RP-HPLC separation resulted in that peak 1 was found to be more active than the starting material (BS-SAT-SP) by ICV infusion in fasted rats. Peak 2, as it was chemically verified as BSA, only showed slight nonsignificant activity, indicating that this peak contains a small amount of active material (Table 1). It is, nevertheless, imperative to do further detailed analyses to learn the properties of BS-SAT-HPLC as taste aversion, tolerance, etc., and to prove that this satiety agent is working through physiological mechanism, as it was basically studied by Bellinger and Mendel (3,4), Bellinger et al. (5).

The size-exclusion chromatographic separation as shown in Fig. 2 can also be utilized for purification of BS-SAT-SP in micropreparative scale, producing larger quantity of highly purified material. The SDS content present in the solvent can be removed from the active fractions by well-known techniques. The SDS-free preparations have not incurred any troubles doing the bioassay.

The electrophoretic mobilities of BS-SAT-HPLC calculated by the method of Neville (16) found the value to be 38–40 kDa, and the calculated correlation coefficient was 0.995 (Fig. 3). It should be noted that the appearance of a single band of BS-SAT-HPLC under the conditions of SDS-PAGE described above does not necessarily prove homogeneity.

The amino acid compositions of BS-SAT-SP and BS-SAT-HPLC (Tables 2 and 3) indicate that the low peptide content is very comparable to those purified preparations obtained from human serum. Surprisingly, the major difference between the two satiety agents is the unexpectedly high lipid content in the BS-SAT preparations.

It can be concluded from these findings that both types of separation method as SE-HPLC and SDS-PAGE are useful for determination of molecular size of BS-SAT because the two measurements delivered the same results. The RP-HPLC as well as the SE-HPLC chromatographic techniques also proved suitable producing samples for further investigations. The existence of these two satiety agents obtained from human and bovine serum presumably belong to the same endogenous family of anorectic agents circulating in the blood which can play a role in the physiological regulation of food intake and body weight.

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