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Large-scale purification of prosomes from calf's liver

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ABSTRACT

Prosomes, cytoplasmatic ribonucleoprotein complexes containing small ribonucleic acid (19S small cytoplasmic RNPs), are ubiquitous in eukaryotic organisms. A new method for the preparation of prosomes in large amounts, starting with *ca.* 2 kg of calf's liver, is described. A combination of centrifugation and low- and high-pressure chromatography was used to purify intact particles. An alternative purification of prosomes with *Solanum tuberosum* agglutinin bound to divinyl sulphone-activated agarose is discussed. Calf's liver prosomes have a similar protein composition and RNA content to prosomes isolated from other tissues.

INTRODUCTION

Prosomes were first identified as cytoplasmic ribonucleoprotein complexes (19S scRNPs) in mouse and duck erythroblasts [1]. In the meantime they have been found in a great variety of eukaryotic cells ranging from plants to man [1–7]. The cellular function of prosomes is still unknown. Our group has recently demonstrated that prosomes are involved in the repression of translation of some viral mRNAs *in vitro* [8], and other workers found that they have specific proteinase or peptidase activity [9,10]. The fact that they exist in all eukaryotic organisms investigated so far suggests an important physiological function for the cell.

Prosomes consist of a specific set of proteins which band in Laemmli polyacrylamide gels in the range 19 000–35 000 dalton. Some of them, *e.g.*, the 27 000-dalton protein, were highly conserved during evolution, whereas others vary from species to species [2]. Some of the prosomal proteins are glycosylated [11]. Similarly, the content of small RNA of prosomes seems to be related but not identical among species. RNA is an intrinsic part of prosomes. We have recently shown that

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prosomal proteins protect RNA fragments of *ca.* 76–80 nucleotides against nuclease digestion [12].

Most interestingly, prosomes resist the strong detergent 1% sarcosyl and high ionic strengths. These properties were used for the purification of prosomes by a series of sucrose gradient centrifugations [1]. Here, we present a more effective method for isolating prosomes, using a combination of ultracentrifugation and low- and high-pressure chromatography, which leads to a high yield of prosomes. This enormously facilitates the study of the structure and function of these particles.

EXPERIMENTAL

Cell fractionation procedure

Calf's liver was removed from a freshly slaughtered calf, cut into small pieces and washed several times with cold buffer containing 20 m*M* Tris–HCl (pH 7.4)–5 m*M* $MgCl_2$–2 m*M* 2-mercaptoethanol–0.1 m*M* EDTA (pH 7.4)–20 m*M* KCl (wash buffer). All connective tissue was removed from the liver fragments, which were again washed twice with wash buffer and quickly dried on filter-paper.

Portions of 100–200 g were homogenized for 1 min in a Waring blender with 3 volumes of buffer H [20 m*M* Tris–HCl (pH 7.4)–5 m*M* $MgCl_2$–2 m*M* 2-mercaptoethanol–0.1 m*M* EDTA (pH 7.4)–20 m*M* KCl–200 m*M* sucrose]. Homogenates (5.8 l) were filtered through Miracloth and portions of 20 ml were then further homogenized by 20 strokes in a Dounce homogenizer and centrifuged for 15 min at 700 *g* at 4°C to sediment nuclei and unbroken cells. The postnuclear supernatant was centrifuged for 20 min at 3500 rpm at 4°C (GSA rotor, Sorvall RC 2-B) and the remaining supernatant was centrifuged again for 30 min at 10 000 rpm at 4°C (SS 34 rotor, Sorvall RC 2-B). The supernatant from this sedimentation (postmitochondrial supernatant) was transferred to Beckman Quickseal tubes (Ti 45 rotor) containing a cushion of 10 ml of 20% sucrose in wash buffer and centrifuged 2 h at 42 000 rpm at 4°C to sediment polysomes and other particles larger than 80S. The postribosomal supernatant (PRS) was centrifuged again in Beckman Quickseal tubes (Ti 45 rotor) containing a cushion of 10 ml of 30% sucrose in wash buffer (19 h, 42 000 rpm, 4°C).

The sediments containing postribosomal particles (PRPs) were resuspended in TBK 300 [20 m*M* Tris–HCl (pH 7.4)–5 m*M* $MgCl_2$–2 m*M* 2-mercaptoethanol–300 m*M* KCl], frozen at −80°C or immediately subjected to different chromatographic procedures.

One- and two-dimensional protein gel electrophoresis

One-dimensional sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) of proteins was performed according to ref. 13 and two-dimensional SDS-PAGE as described in ref. 14. Molecular weight markers were phosphorylase *b* (94 000 dalton), bovine serum albumin (68 000), ovalbumin (43 000), carboanhydrase (29 000), soybean trypsin inhibitor (20 000) and lactalbumin (14 000).

RNA extraction and RNA gel electrophoresis

Prosomes were treated with proteinase K (1 mg/ml) for 30 min at 37°C, then the RNA was extracted with chloroform–phenol according to ref. 15. RNA fragments were labelled at their 3'-ends with [^{32}P]pCp (Amersham, 3000 Ci/mmol) in a reaction

catalysed by T4-RNA-ligase [16]. RNA was analysed on 300-mm long 15% polyacrylamide gels containing 7 *M* urea–50 m*M* Tris–borate (pH 8.3)–2 m*M* EDTA. Electrophoresis was performed until the bromophenol blue reached the bottom of the gel.

Probing of protein blots with Solanum tuberosum agglutinin

Solanum tuberosum agglutinin (STA) was obtained from Kem-En-Tec. Streptavidin horseradish peroxidase complexes from Amersham were used as probes for the biotinylated lectin.

After separation on gels, prosomal proteins were transferred to nitrocellulose (Schleicher & Schüll BA 85) according to ref. 17. After the transfer, the remaining binding sites were blocked by incubation in 20 m*M* Tris–HCl (pH 7.4)–500 m*M* NaCl–2% Tween 20 for 2 min [11]. The nitrocellulose was then washed twice in Tris-buffered saline (TBS) [20 m*M* Tris–HCl (pH 7.4)–500 m*M* NaCl] for 5 min. After a short wash in lectin buffer [10 m*M* Tris–HCl (pH 7.4)–1 *M* NaCl–1 m*M* $CaCl_2$–1 m*M* $MgCl_2$–1 m*M* $MnCl_2$–2 m*M* NaN_3], the blots were incubated overnight with biotinylated STA (2 μg/ml) in the same buffer. The nitrocellulose sheets were again washed twice with TTBS (TBS containing 0.05% of Tween 20) for 5 min and incubated with streptavidin-horseradish peroxidase complexes diluted with TTBS for 1 h. After washing three times in TBS, the blots were assayed for peroxidase activity by incubation with peroxidase substrate buffer [50 m*M* sodium citrate (pH 5.0)–0.25 mg/ml carbazole–0.5% hydrogen peroxide). The reaction was stopped by rinsing with tap water and the nitrocellulose was dried between Whatman filter-papers.

Affinity chromatography with immobilized Solanum tuberosum agglutinin

STA was covalently bound to divinyl sulphone-activated agarose according to ref. 18. The activated agarose was washed three times with 1.4 *M* potassium phosphate (pH 8.6). A 1-ml volume of the gel was shaken gently with 1 mg of STA for 16 h at room temperature. The gel mixture was packed into an HR 5/5 column (Pharmacia LKB) and washed thoroughly with lectin buffer.

Prosomes were applied to the column in the same buffer. Bound material was eluted with lectin buffer containing 1 *M* N-acetylglucosamine.

RESULTS AND DISCUSSION

To accelerate the investigations on the structure and function of the prosomes, we developed a rapid and effective method for the purification of these RNP particles. We chose calf's liver, because it is easy to dissect and contains well defined cells, mostly hepatocytes. We started the purification with 2030 g of calf's liver, using about 6 l of wash buffer during dissection and preparation of the liver fragments and 5.8 l of buffer H for the homogenization of the fragments. The 100 000 *g* sediments containing ribosomal subunits, free mRNPs, small cytoplasmic RNPs and proteins larger than 10S (PRPs) were further purified by anion-exchange chromatography and gel filtration.

Approximately 50 ml of PRP suspension in TBK 300 (1500 A_{280} units) were applied to 100 ml of Q-Sepharose Fast Flow anion exchanger in a C 26/40 column (Pharmacia–LKB) equilibrated with TBK 300. Particles and unbound proteins were washed off with the same buffer until the absorbance at 280 nm reached a constant

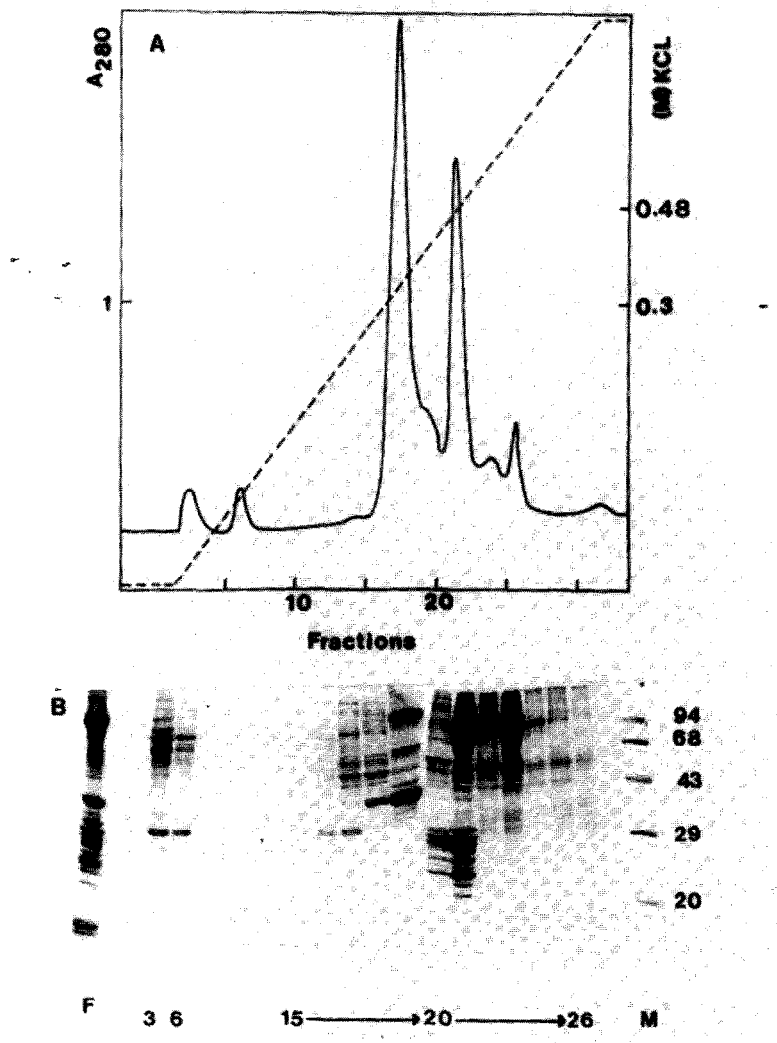

Fig. 1. Chromatogram of particles eluted from the Q-Sepharose Fast Flow applied to an FPLC Mono Q HR 5/5 column. (A) Bound particles were eluted with a linear salt gradient ranging from 0 to 600 m*M* KCl in Tris–HCl buffer (pH 7.4). Dashed line, KCl concentration; solid line, absorbance at 280 nm (A_{280}). (B) Protein composition of the fractions obtained by Mono Q chromatography. Fractions 20 and 21 contain prosomes. The proteins were analysed by one-dimensional SDS-PAGE in 12.5% polyacrylamide gels and stained with Coomassie brilliant blue. F = Protein composition of the suspension applied to the column; M = molecular weight marker proteins in kilodaltons.

value near the baseline. Fractions containing prosomes were eluted by a step gradient to 600 m*M* KCl and detected by SDS–PAGE (data not shown). To concentrate the eluate, containing prosomes, the particles were sedimented by ultracentrifugation (19 h, 235 000 *g*). The sediments were resuspended in FPLC buffer A [20 m*M* Tris–HCl (pH 7.4)–5 m*M* $MgCl_2$–7 m*M* 2-mercaptoethanol] and 20–30 A_{280} units were applied to a fast protein liquid chromatography (FPLC) Mono Q column (HR 5/5, Pharmacia–LKB). A linear salt gradient up to 600 m*M* KCl was formed. Prosomes were eluted at 480 m*M* KCl with a pronounced peak of absorbance in fractions 20 and 21, while other particles and proteins were eluted in fractions 16–19 (Fig. 1A and B).

To obtain highly concentrated amounts of prosomes, fractions 20 and 21 from five Mono Q runs were collected, diluted to a final concentration of 300 m*M* KCl and again loaded on a Mono Q column. After a short wash with TBK 300, a step gradient to 500 m*M* KCl was applied. Under these conditions, all particles bound on the exchanger were recovered in 1 ml, the void volume of the column. In the final step, prosomes were purified to homogeneity by gel filtration, using an FPLC Superose 6 column (HR 10/30, Pharmacia–LKB) equilibrated with Tris–HCl (pH 7.4) buffer containing 480 m*M* KCl. As demonstrated in Fig. 2, almost 90% of the injected

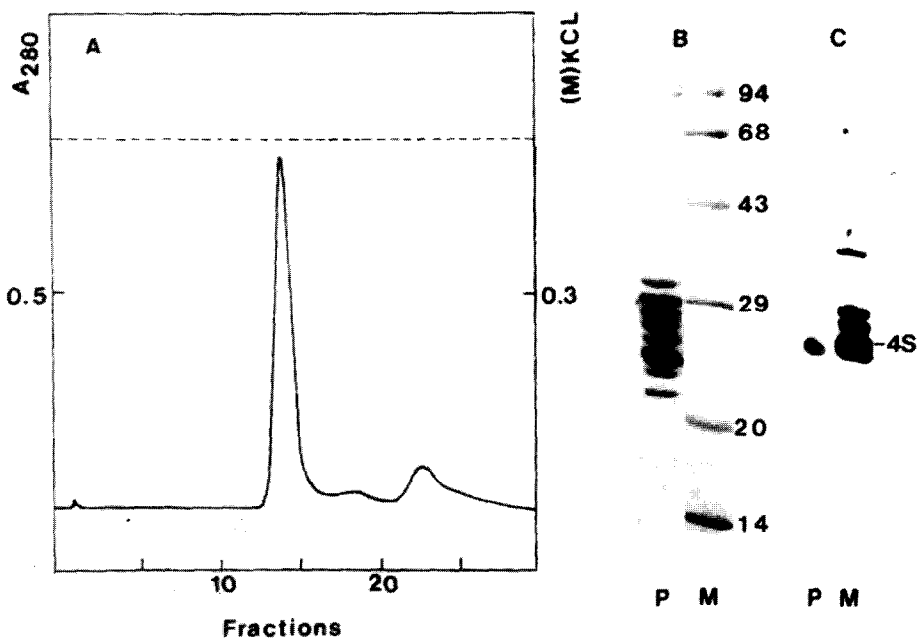

Fig. 2. Purification of prosomes by gel filtration with FPLC Superose 6. (A) Prosomal fractions (2 A_{280} units) isolated and concentrated by Mono Q chromatography were passed through a Superose 6 column equilibrated with Tris–HCl buffer (pH 7.4) containing 480 m*M* KCl. Dashed line, KCl concentration; solid line, absorbance at 280 nm (A_{280}). (B) Eluted particles of fractions 13 and 14 (prosomes) were analysed in 12.5% polyacrylamide gels [13] and proteins were stained with Coomassie brilliant blue (P). M = Molecular weight markers in kilodaltons. (C) RNA was extracted from prosomes as described under Experimental and analysed by urea–SDS-PAGE. RNA bands were rendered visible by autoradiography. M = t-RNA from brewers yeast; P = prosomal RNA.

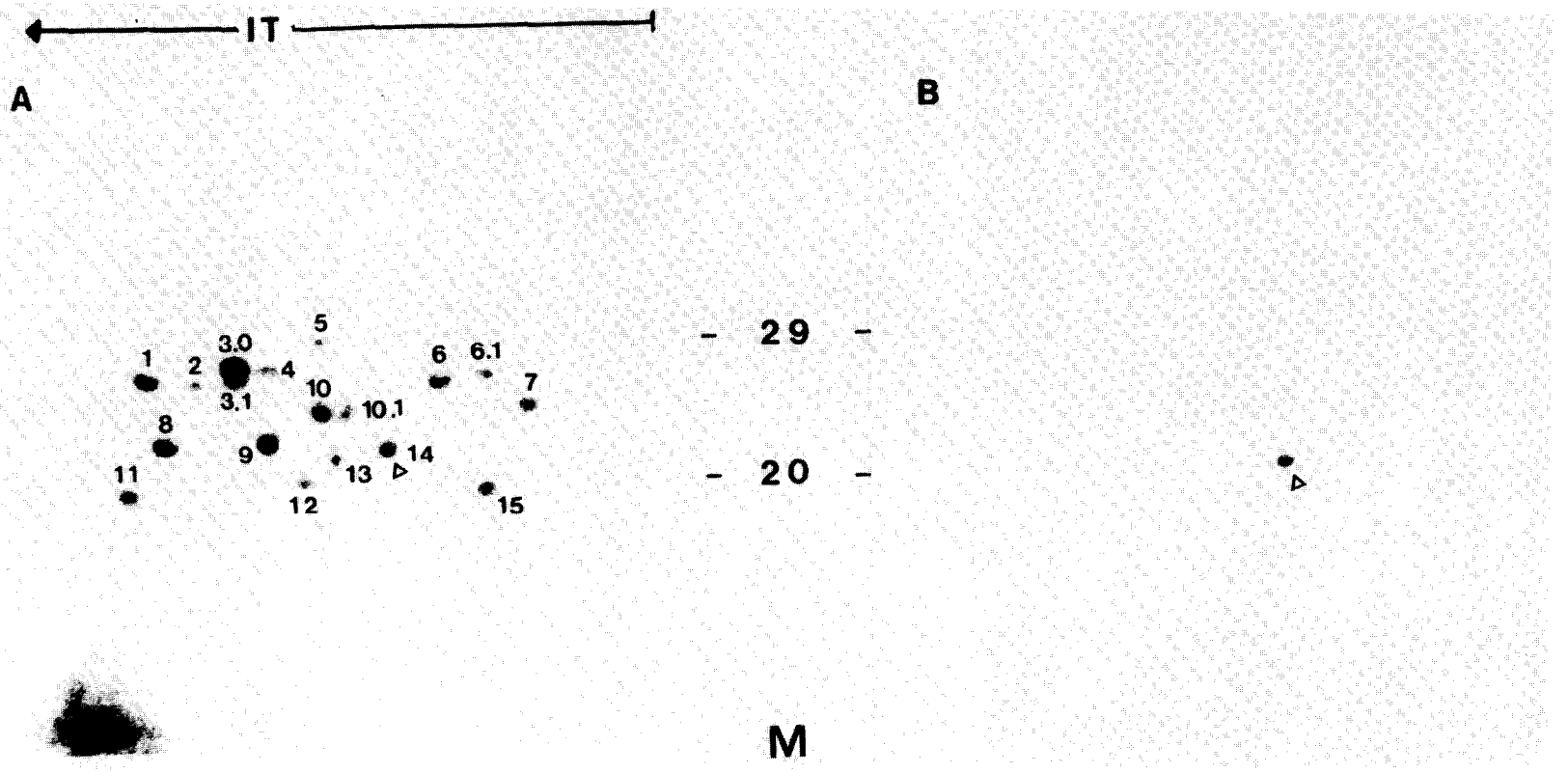

Fig. 3. Two-dimensional protein blot of prosomes probed with STA. Prosomes, purified by gel filtration (Fig. 2) were analysed by two-dimensional gel electrophoresis [14]; then proteins were transferred to nitrocellulose [17]. Blotted proteins were probed with biotinylated STA in lectin buffer. Bound STA was detected by streptavidin-horseradish peroxidase assayed for peroxydase activity. (A) Prosomal proteins stained with Coomassie blue; (B) two-dimensional protein blot probed with STA; (▽) prosomal protein reacting with STA.

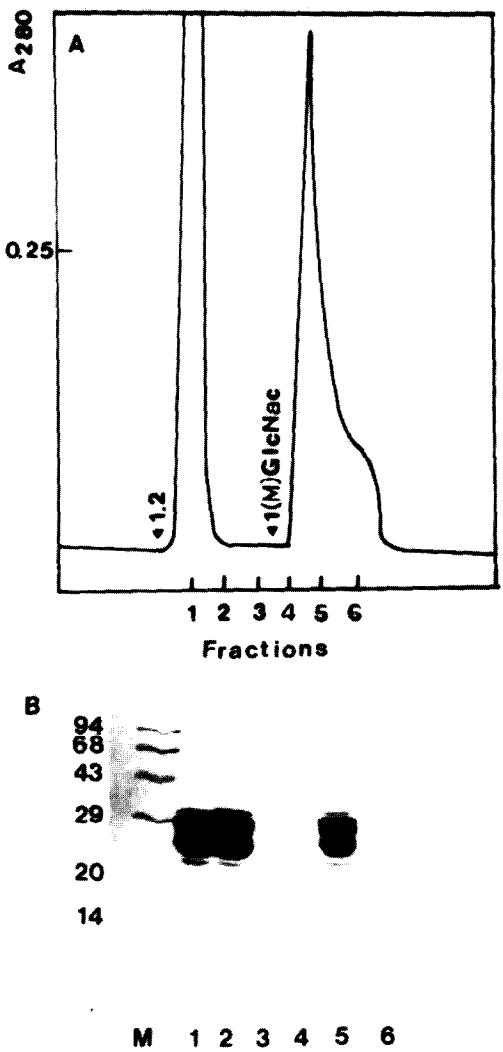

Fig. 4. Affinity chromatography of prosomes on STA agarose. (A) Prosomes (1.5 A_{280} units) purified by gel filtration on a Superose 6 column were applied to divinyl sulphone-activated agarose with immobilized STA. The column was washed with lectin buffer. Bound particles were eluted with 1 *M* N-acetylglucosamine in lectin buffer. 1.2 = Valve position: sample load. (B) Protein composition of bound and unbound prosomes from STA agarose. The proteins were analysed by SDS-PAGE in 12.5% polyacrylamide gel and stained with Coomassie brilliant blue. M = Molecular weight marker proteins in kilodaltons.

particles were eluted as a homogeneous fraction with a retention of 13.5 ml, which corresponds well with a molecular weight of 630 000 dalton and a sedimentation constant of 19S, estimated by ultracentrifugation.

Calf's liver prosomes, purified according to the FPLC procedure, revealed a typical protein pattern when subjected to one- and two-dimensional SDS-PAGE (Figs. 2B and 3A). Differences between the protein patterns of calf's liver prosomes purified via different sucrose gradient centrifugations and FPLC-purified prosomes were not observed (data not shown). In addition, we tested FPLC-purified prosomes for the presence of RNA. As shown in Fig. 2C, they contain RNA in the region of 4S. RNA with the same sedimentation value can be isolated from prosomes purified by sucrose gradient centrifugation [1,2]. This indicates that both methods lead to identical particles. However, the purification by FPLC is faster than the isolation of prosomes by various steps of sucrose gradient centrifugation. With the technique described above we obtained at least 79.8 mg of prosomes from 2030 g calf's liver in 3 weeks.

We propose an alternative method for the purification of prosomes, which is based on the glycosyl residues of prosomal proteins. We recently described the detection of glycosylated proteins of prosomes isolated from erythropoietic mouse cells by the following lectins [11]: Con A, specific for mannosyl- and glycosyl-containing residues; LPA, specific for neuraminic acid; and STA, specific for N-acetylglucosamine.

Interestingly, only one prosomal protein was detected by STA. This corresponds well with the results obtained from calf's liver prosomes, presented in Fig. 3 (protein No. 14). A column with immobilized STA was used for affinity chromatography (Fig. 4). It was found that prosomes were able to bind to immobilized STA via glycosyl residues of one individual prosomal protein. Prosomes were eluted from the matrix with lectin buffer containing 1 *M* N-acetylglucosamine as a competitor. This method could be useful for the isolation of prosomal subcomplexes containing protein No. 14.

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