*Journal of Chromatography*, 597 (1992) 277–283
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 032

# Artificial immunoglobulin G-binding protein mimetic to staphylococcal protein A

# Its production and application to affinity purification of immunoglobulin G

Yasunori Kihira* and Satiko Aiba

*Nagahama Institute for Biochemical Science, Oriental Yeast Co., Ltd., Kano-cho 50, Nagahama-shi, Shiga-ken 526 (Japan)*

ABSTRACT

Staphylococcal protein A consists of a single polypeptide with five immunoglobulin G (IgG)-binding domains, which are linked as E–D–A–B–C in this order from the amino terminal. The DNA coding domains A–B were polymerized one to six times linearly, taking advantage of the non-palindromic nucleotide sequence of the *Acc*I recognition site and the resultant DNAs were inserted in pTRP vector carrying *trp* promoter. The artificial IgG-binding proteins [$pA(AB)_{1-6}$], which had been expressed in *Escherichia coli* JM109, were purified by methods involving IgG–Sepharose affinity chromatography. Among $pA(AB)_{1-6}$ immobilized on cyanogen bromide–Sepharose, $pA(AB)_4$–Sepharose was the highest in IgG-binding capacity at the same level of mg protein per ml gel, about 30% higher than protein A–Sepharose. At 8 mg protein per ml gel, it bound and eluted about 24 mg of IgG from rabbit serum. Its IgG-binding capacities were the highest with porcine, rabbit, human and guinea pig sera, intermediate with bovine, horse and sheep sera and the lowest with mouse, goat, rat and chicken sera.

## INTRODUCTION

Protein A is localized in *Staphylococcus aureus*, bound to the cell wall. It shows a specific affinity to the Fc portion of immunoglobulin G (IgG), but not to the antigen-binding Fab portion, binding 2 mol of IgG per mol [1]. It consists of a single polypeptide (relative molecular mass $M_r$ = 42 000), in which six domains are linked as E–D–A–B–C–X in this order from the amino terminal. Domain X is for anchoring the protein to the cell wall, whereas the others can bind IgG of various animals. Its molecular shape seems to be markedly extended [2]. It is significantly stable against various reagents because of a relatively high content of α-helix (about 50%). Domains E, D, A, B and C are highly homologous in amino acid sequence (see Fig. 1) and similar in IgG binding.

Recently, expression vectors for fusion proteins containing protein A have been developed. Owing to the IgG-binding domains of protein A, the fusion proteins can be effectively purified with a column of immobilized IgG. It is known that hydroxylamine and cyanogen bromide (CNBr), respectively, bring about specific cleavages of bonds between asparagyl and glycyl residues, and carboxyl terminals of methionyl residues in polypeptides. Nilsson *et al.* [3] synthesized an artificial protein containing various numbers of domain B, in which the original amino acid residues AsnGly and Met were replaced with AsnAla and Leu, respectively. The resultant protein can be separated from the domain B, provided that it does not contain AsnGly or Met residues.

This paper reports the production and purification of artificial proteins capable of binding IgG. The proteins thus produced consist of one to six of domains A–B, which are linearly polymerized,

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Fig. 1. Amino acid sequences of five IgG-binding domains of staphylococcal protein A. The amino acid sequences refer to Uhlén *et al.* [6]. Identical amino acid residues among the five domains are boxed.

called $pA(AB)_n$ ($n$ = 1–6). After being immobilized on Sepharose, $pA(AB)_n$ were compared with one another in IgG-binding capacity. It was found that $pA(AB)_4$ was the highest, about 30% higher than protein A.

## EXPERIMENTAL

### *Materials*

Protein A gene fusion vector plasmid (pRIT2T), multi-functional vector phagemid (pTZ19U), human IgG–Sepharose and CNBr-activated Sepharose 4B were obtained from Pharmacia LKB Biotechnology (Uppsala, Sweden). Molecular mass markers for high-performance liquid chromatography (HPLC) and for sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Oriental Yeast (Tokyo, Japan) and Life Technologies (Gaithersburg, MD, USA), respectively. DNase I was purchased from Takara Shuzo (Kyoto, Japan), Block Ace (commercial blocking solution composed of milk protein) from Yukijirushi Nyugyo (Sapporo, Japan) and goat anti-rabbit IgG conjugated with horseradish peroxidase from Bio-Rad Labs. (Richmond, CA, USA).

### *Bacterial strains*

*Escherichia coli* JM109 [recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1, $\lambda^-$, $\Delta$ (lac-proAB), (F′, traD36, proAB, lacI$^q$, lacZ$\Delta$ M15)] was used for gene expression and other cloning experiments.

### *Synthesis of DNA*

DNAs were synthesized with a Model 8750 DNA synthesizer (Biosearch, San Rafael, CA, USA), followed by purification using an oligonucleotide purification cartridge (OPC) cartridge (Applied Biosystems, Foster City, CA, USA). Those synthesized were the sense and antisense strands as a gene cassette for *trp* promotor (5′-AGCTTCCCTGTTGAC AATTAATCATCGAACTAGTTAACTAGTAC GCAAGTTCACGTAAAAAGGGTAG of 67-mer and 5′-AATTCTACCCTTTTTACGTGAACTTG CGTACTAGTTAACTAGTTCGATGATTAAT TGTCAACAGGGA of 67-mer, respectively), the sense and antisense primers for polymerase chain reaction (PCR) to introduce *Acc*I site into multiple cloning site (5′-GCAATTCATGAAAGTAGACA CTGGCCGTCGTTTTACAA of 38-mer and 5′-GGAATTCTACCCTTTTTACGT of 21-mer, respectively) and the sense and antisense primers for

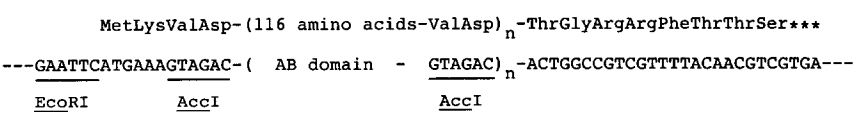


Fig. 2. Amino acid and nucleotide sequences of artificial proteins to be produced. Domains A–B consist of 116 amino acids. The polymerization-fold of domains A–B is indicated by *n* (1–6).

PCR to perform the cloning of domains A–B of protein A, which carry *Acc*I sites at both ends (5′-GGTAGACGCTGATAACAATTTCAACA AA of 28-mer and 5′-GGTCTACTTTTGGTGCTT GAGCATCATTTA of 30-mer, respectively).

*Construction of DNAs*

pTZ19U, being a multi-functional phagemid useful for DNA cloning, DNA sequencing, *in vitro* mutagenesis and *in vitro* mRNA synthesis, was used as a fundamental vector in order to construct an expression vector containing *trp* promoter. The sense and antisense strands synthesized as the gene cassette for *trp* promoter were mixed and inserted between the sites for *Hin*dIII and *Eco*RI of pTZ19U. The resultant DNA is called pTRP. Directly downstream of the *Eco*RI site, the initiation codon (ATG), Lys-codon (AAA) and the *Acc*I-recognition sequence (GTAGAC) were inserted in this order (see Fig. 2). The Lys-codon (AAA) is known to allow a high expression and the *Acc*I-recognition sequence is a cloning site for the ligation of a gene of interest. The DNA thus synthesized is called pTRP–ACC. The above procedures were carried out by PCR using the primers described in the above section.

A DNA which had a nucleotide sequence corresponding to domains A–B was synthesized by PCR, using the plasmid pRIT2T as a template. pRIT2T is an expression vector with $\lambda$ $P_R$ promoter capable of expressing an objective protein as a fusion protein containing IgG-binding domain of protein A, and in *E. coli* (N4830-1), which contains the temperature-sensitive $\lambda$ cI857 repressor, the fusion protein is inducible by shifting the temperature from 30 to 42°C. The resultant DNA coding domains A–B had the non-palindromic restriction site of *Acc*I (GTA GAC) at both ends. It was linearly polymerized one to six times. The GTAGAC at both ends could eliminate head-to-head or tail-to-tail ligation during polymerization [3]. The polymerized DNAs were then ligated to pTRP–ACC. The resultant DNAs are called pTRP–PROT–AB1–6, in which the numbers show the polymerization-fold.

*Transformation of E. coli and its culture*

*E. coli* JM109 cells were transformed with respective pTRP–PROT–AB1–6. The six kinds of cells thus transformed were respectively grown in 10-l Luria–Bertani medium with a Microferm fermenter (New Brunswick Scientific, Edison, NJ, USA). In the culture, the desired protein is spontaneously expressed without an inducer, 3-indoleacrylic acid, because of insufficient amount of tryptophan for the repression of *trp* promoter.

*Extraction and preparation of crude sample*

The grown cells were suspended in three volumes of water. To the resultant suspension was added 6 *M* NaOH to make its final concentration 0.1 *M*. The alkaline solution was supplemented with 0.5 *M* potassium phosphate (K-Pi) buffer (pH 7.5) to a final concentration of 10 m*M*, followed by adjusting the pH to 7.5 with 6 *M* HCl. The solution thus obtained was incubated at 4°C for 30 min in the presence of DNase I (0.1 U/ml) and 10 m*M* $MgCl_2$ followed by centrifugation at 12 000 *g* for 20 min. The resultant clear supernatant was supplemented with ammonium sulphate up to 80% saturation. The precipitate thus collected was dissolved in 10 m*M* K-Pi buffer (pH 7.5). The resultant solution was desalted using a column of Sephadex G-25 previously equilibrated with the same buffer. The desalted solution was used for further purification, called a crude sample.

*Immobilization*

CNBr-activated Sepharose 4B (0.3 g) was suspended in 1 m*M* HCl and allowed to stand at room temperature for 15 min for swelling. The swollen Sepharose was placed in a column (10 cm × 7 mm I.D.), which was washed with 60 ml of 1 m*M* HCl and then with 10 ml of the coupling buffer (0.1 *M*

$NaHCO_3$–0.5 *M* KCl, pH 8.3). An objective protein (1–16 mg) was dissolved in 2 ml of the coupling buffer and then applied to the column. The charged column was sealed at both top and bottom and then shaken horizontally at 4°C for 18 h. After removing the coupling buffer, the resultant column was shaken at 4°C for 2 h with 2 ml of 0.2 *M* glycine (pH 8.0). The column was then washed first with 10 ml of the coupling buffer, second with 10 ml of 0.1 *M* sodium acetate buffer containing 0.5 *M* NaCl (pH 4.0) and finally with 10 ml of the coupling buffer. The efficiency for binding the objective protein to Sepharose was estimated from the absorbance at 280 nm ($A_{280}$) of the washings. The $A_{280}$ of the objective protein, $pA(AB)_{1-6}$, is taken as 0.18 at 1 mg/ml; the conversion factor was calculated from the $A_{280}$ of a 1 mg/ml aqueous solution of $pA(AB)_{1-6}$, whose protein concentrations are determined by amino acid analysis.

*Enzyme-linked immunosorbent assay (ELISA)*

ELISA was carried out essentially by the method of Engvall and Perlmann [4]. In brief, 50 μl of each fraction were applied to a 96-well plate and allowed to stand for 2 h followed by blocking with Block Ace. After washing with phosphate-buffered saline (PBS) containing 0.05% Tween 20, 50 μl of goat anti-rabbit IgG conjugated with horseradish peroxidase (1:3000 dilution with PBS) were applied and allowed to stand for 2 h. After washing with PBS containing 0.05% Tween 20, 200 μl of development solution [4 mg of *o*-phenylene-diamine in 10 ml of 0.1 *M* citrate–NaOH (pH 4.5) containing 5 μl of 30% $H_2O_2$] were added. After 10 min, 50 μl of 3 *M* $H_2SO_4$ were added to stop the reaction. All these procedures were done at room temperature. The $A_{492}$ was measured of the reaction products with an A4 microplate reader (Tosoh, Tokyo, Japan).

*Quantitative analysis of IgG*

IgG-binding capacities of immobilized $pA(AB)_{1-6}$ were determined as follows. An excess volume of sample serum was applied to the respective column of 1-ml bed volume. The overcharge of IgG was confirmed by monitoring its concentration by ELISA. The charged column was washed and then eluted. The content of IgG eluted was determined with the use of $A_{280}$ = 1.5 at 1 mg/ml.

*SDS-PAGE*

SDS-PAGE was carried out at 60 mA for about 1.5 h by the method of Laemmli [5] with an electrophoresis apparatus (Daiichi Pure Chemicals, Tokyo, Japan), using gel slabs (84 mm high, 90 mm wide and 1.0 mm thick) having a concentration gradient from 10 to 20%. The gel slabs were stained with Coomassie Brilliant Blue R-250 by a conventional method.

## RESULTS AND DISCUSSION

*Amino acid sequence of artificial IgG-binding proteins*

The IgG-binding proteins corresponding to the nucleotide sequences of the artificial gene PROT-AB1–6 are called $pA(AB)_{1-6}$, where pA represents protein A, AB for domains A–B of protein A and the numbers for the polymerization-fold. Fig. 2 shows their amino acid sequences. The basic set for the

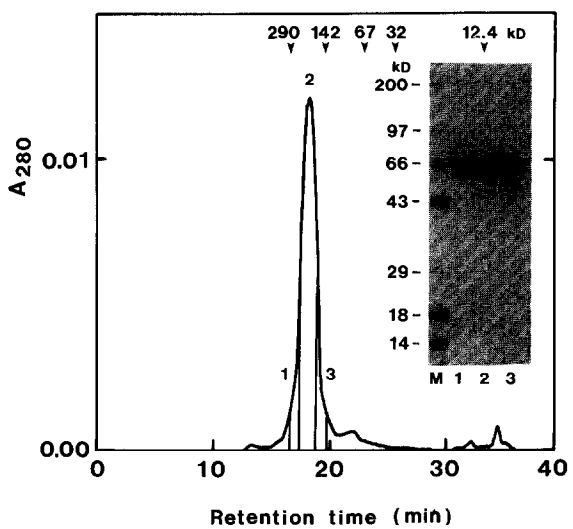


Fig. 3. Further purification of $pA(AB)_5$ by high-speed molecular-sieve chromatography on a TSKgel G3000SW column and purity analysis by SDS-PAGE. The fractions from IgG–Sepharose affinity chromatography were subjected to HPLC on a TSKgel G3000SW column (69 × 0.75 cm I.D.). The purity of the main peak was determined by SDS-PAGE (inset). Molecular mass marker proteins for molecular-sieve HPLC are glutamate dehydrogenase (290 000), lactate dehydrogenase (142 000), enolase (67 000), adenylate kinase (32 000) and cytochrome *c* (12 400), and those for SDS-PAGE are myosine (200 000), phosphorylase *b* (97 400), bovine serum albumin (66 200), ovalbumin (42 700), carbonic anhydrase (29 000), β-lactoglobulin (18 400) and lysozyme (14 300). kD = kilodalton.

polymerization is the polypeptide of domains A–B composed of 116 amino acid residues that is linked with the dipeptide ValAsp at the carboxyl terminal. $pA(AB)_{1-6}$ have 1–6 sets, which are linearly linked to one another, with the tetrapeptide MetLysValAsp and the octapeptide ThrGlyArgArgPheThrThrSer linked at the amino and carboxyl terminals, respectively.

*Purification of $pA(AB)_{1-6}$*

The crude samples (see Experimental) were subjected to ion-exchange chromatography on a DEAE-Sepharose column (12.2 × 2.5 cm I.D.) using a linear gradient (0–1 *M*) of NaCl in 10 m*M* K-Pi buffer (pH 7.5). The fractions containing each objective protein were further purified by affinity chromatography on an IgG–Sepharose column (2 × 2.5 cm I.D.); 10 m*M* K-Pi buffer (pH 7.5) containing 0.15 *M* NaCl and 0.05% Tween 20 was used as the equilibration and washing buffers and 0.5 *M* ammonium acetate buffer (pH 3.4) as the elution buffer. The resulting fractions were further purified by molecular-sieve HPLC on a TSKgel G3000SW column in the 0.1 *M* K-Pi buffer (pH 7.0) containing 0.2 *M* NaCl; Fig. 3 shows a typical result, using $pA(AB)_5$. The main peak of $A_{280}$ in the last chromatographic step was divided into three fractions. In SDS-PAGE followed by dye staining, the first and second fractions exhibited a single band at $M_r$ *ca.* 68 000, whereas the third fraction was accompanied with a contaminant at $M_r$ *ca.* 50 000. The first two fractions were mixed and the mixture was used as a purified sample in further experiments. $pA(AB)_{1-4}$ and $pA(AB)_6$ were also purified in a similar manner. The results obtained were essentially the same as those with $pA(AB)_5$, except for migration rates in molecular-sieve HPLC. The yields of $pA(AB)_{1-6}$ were about 1 g from the cells cultured in media of 10 l.

In molecular-sieve HPLC, $pA(AB)_5$ was eluted with fractions equivalent to $M_r$ *ca.* 210 000, considerably higher than the value in SDS-PAGE. $pA(AB)_{1-4}$ and $pA(AB)_6$ also showed $M_r$ values much higher than those determined in SDS-PAGE and from amino acid composition.

*Purification of IgG from sera of various animals with immobilized $pA(AB)_{1-6}$*

*Capacities of binding IgG from rabbit serum.* An excess volume of rabbit serum was applied on a column of Sepharose, to which 3.8 mg of $pA(AB)_{1-6}$ per ml gel had been immobilized. The overcharge of IgG was confirmed by monitoring its concentration by ELISA. Fig. 4 shows a typical result with $pA(AB)_4$–Sepharose. The charged column was washed and then eluted. In SDS-PAGE followed by dye staining, the resultant eluate showed two bands at $M_r$ *ca.* 55 000 and 25 000. These values are almost identical with those of the heavy and light chains of IgG, respectively. Essentially the same results were obtained with Sepharose immobilizing $pA(AB)_{1-3}$ and $pA(AB)_{5,6}$. The IgG-binding capacities of Sepharose immobilizing $pA(AB)_{1-6}$ and protein A were compared at a constant amount of protein per constant volume of the gel, using rabbit serum (Fig. 5). Of the seven kinds of Sepharose used, $pA(AB)_4$–Sepharose was the highest in IgG-binding capacity: the capacity was 13 mg of IgG, about 30% higher than that of protein A–Sepharose. On the other hand, a commercial protein A–Sepharose was reported to be able to bind about 20 mg of IgG per ml gel. However, protein A–Sepharose which had been prepared by us or obtained commercially could bind about 10 mg of IgG. The reasons for this difference are not known.

As the above experiments were carried out with

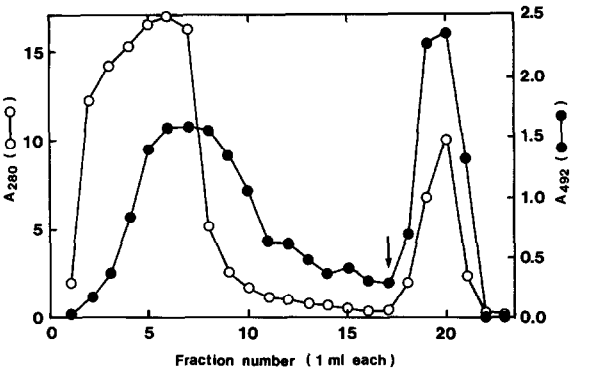


Fig. 4. $pA(AB)_4$–Sepharose column chromatography of rabbit serum for determination of IgG-binding capacity. Rabbit serum (2 ml) was diluted by adding 4 ml of 0.1 *M* K-Pi buffer (pH 7.0) and applied to $pA(AB)_{1-6}$–Sepharose columns (1-ml bed volume) equilibrated with 0.1 *M* K-Pi buffer (pH 7.0). The columns were washed with 10 ml of the above buffer. Elution was then started (at fraction number 17) by developing with 7 ml of 0.3 *M* KCl–HCl (pH 2.3). Eluates were collected in vessels containing 0.1 ml of tenfold concentrated PBS. $A_{492}$, ELISA.

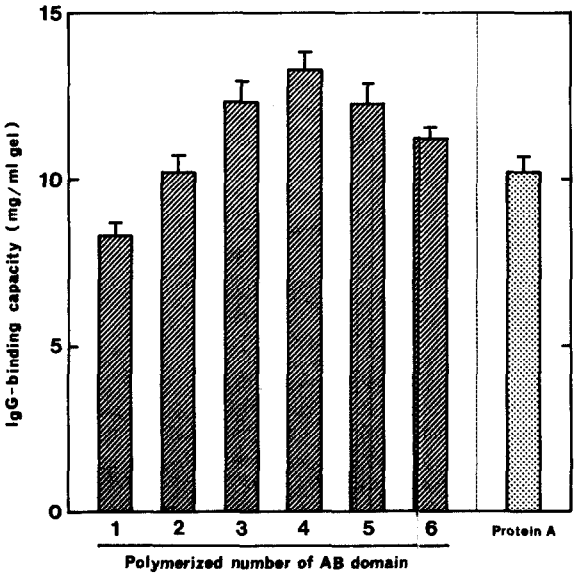


Fig. 5. IgG-binding capacities of immobilized $pA(AB)_n$: effect of polymerization-fold of $pA(AB)_n$ ($n$ = 1–6) on their binding capacities for rabbit IgG. The amount of IgG eluted from each column was determined as described under Experimental, and were compared with one another. Mean values of five experiments are shown and the bars indicate the standard error of the mean.

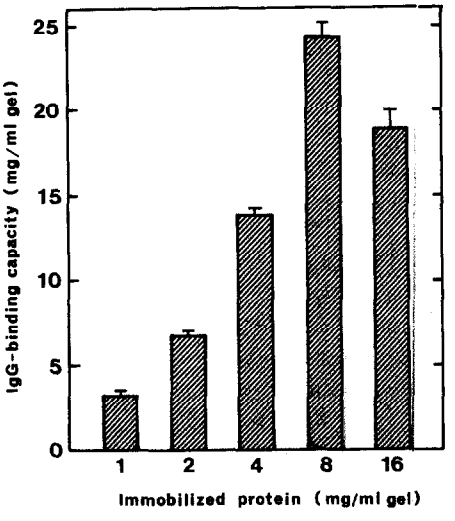


Fig. 6. Effect of amount of immobilized $pA(AB)_4$ on IgG-binding capacity for rabbit IgG. Mean values of three experiments are shown and the bars indicate the standard error of the mean. As the immobilization efficiencies were almost 100%, the amounts of $pA(AB)_4$ added in the immobilization experiments were indicated as immobilized protein.

TABLE I

IgG-BINDING CAPACITIES OF IMMOBILIZED $pA(AB)_4$ FOR IgGs FROM VARIOUS ANIMALS

Sample sera (3 ml) from various animals were diluted threefold by adding 6 ml of PBS and applied to a $pA(AB)_4$–Sepharose column (1-ml bed volume). Other conditions as in Fig. 4.

| Kind of serum | IgG bound (mg/ml) | Kind of serum | IgG bound (mg/ml) |
|---|---|---|---|
| Porcine | 28.0 | Sheep | 6.3 |
| Rabbit | 24.0 | Mouse (ascites) | 3.5 |
| Human | 23.5 | Goat | 3.0 |
| Guinea pig | 21.9 | Rat | 1.3 |
| Bovine | 12.4 | Chicken | 0.2 |
| Horse | 10.3 | | |

constant volumes of Sepharose immobilizing constant amounts of $pA(AB)_{1-6}$ and protein A, the total numbers of IgG-binding domains should be virtually the same among all the ligand–Sepharoses used. It seems mostly likely, therefore, that the IgG-binding capacities depend on the spatial and structural availabilities of the immobilized ligands for the binding of IgG.

*Effect on IgG-binding capacity of amounts of IgG-binding ligands immobilized on Sepharose.* Various amounts of $pA(AB)_4$ were immobilized on Sepharose and compared with one another in IgG-binding capacity using rabbit serum. Fig. 6 shows that the capacity increased with increasing amounts up to 8 mg/ml, whereas it decreased at higher amounts.

*IgG-binding capacities of $pA(AB)_4$–Sepharose from sera of various animals.* Using $pA(AB)_4$–Sepharose at 8 mg protein/ml gel, IgG-binding capacities from sera of various animals were compared. Table I shows that the capacities were the highest with porcine, rabbit, human and guinea pig, intermediate with bovine, horse and sheep and lowest with mouse, goat, rat and chicken sera. This suggests that the Sepharose is useful for affinity purification of sera from all the animals tested except the last two.

ACKNOWLEDGEMENTS

We are grateful to Drs. Y. Matuo, J. Yama-uti and T. Horio for discussions and encouragement.

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