Factor V Is Activated and Cleaved by Platelet Calpain: Comparison With Thrombin Proteolysis

By Harlan N. Bradford, Anjanayaki Annamalai, Kundan Doshi, and Robert W. Colman

Platelets are known to process human factor V during secretion and/or membrane binding. We studied the functional and structural changes produced in human factor V by purified human platelet calpain (calcium-activated thiol protease) and compared the alterations with those induced by thrombin. A maximum increase in coagulant activity of 2.5-fold was observed when factor V (1 U/mL, 33 nmol/L) was incubated with calpain (0.03 U/mL, 2.7 nmol/L) in comparison with a 8.8-fold increment for α-thrombin (0.7 U/mL, 8 nmol/L) at 25°C. Thrombin additions to reactions initiated by calpain resulted in further activation comparable to that of thrombin alone, whereas the subsequent addition of calpain had no effect on the extent or pattern of the activation of factor V by thrombin. The cleavage pattern of factor V produced by these two enzymes are distinctly different. Although thrombin activation eventually results in four final components designated C₁, (150 kd), D (105 kd), E (71 kd), and F₁F₂ (71 to 74 kd), calpain yields initial components of 200 and 160 kd within one minute.

Factor V is a high-molecular weight procofactor that when activated by thrombin² to factor Va can accelerate 278,000-fold the conversion of prothrombin to thrombin by factor Xa.³ The formation of the prothrombinase complex by the combination of factor Va with factor Xa, calcium, and phospholipid (on the platelet surface) allows for such enormous biologic amplification. Factor Va, moreover, serves as the receptor on platelets⁴ for factor Xa binding, which accelerates factor Xa-catalyzed hydrolysis of prothrombin to thrombin.⁴

Human platelets contain between 4 and 20 μg factor V/10⁸ platelets⁵ localized in the α-granules and released by platelet agonists such as collagen.⁶ The description of hemorrhagic syndromes attributed to the failure of factor Xa to bind to the platelet surface⁷ or a deficiency of platelet factor V⁸ underscores its importance in normal hemostasis. Although factor V binds to platelets, thrombin-activated factor V binds more tightly.⁹,¹⁰ Human factor Va consists of a 105 kd heavy chain (component D) that is bridged through Ca²⁺ to the 71 to 74 kd light chain (component F₁F₂), which binds directly to the unstimulated platelet surface.¹¹ The 150 kd activation peptide (component C₁) described by Suzuki et al¹² does not bind to unactivated platelets.

Calpins are heat-labile (>30°C in vitro), calcium-activated cysteine proteases¹³ occurring primarily in the cytosol of a wide range of mammalian cells.¹⁴,¹⁵ The enzyme is generally isolated in two forms that differ in their calcium requirement to achieve full activation. This enzyme is inhibited in vitro by epoxysuccinate derivatives in the presence of free calcium¹⁶ and in vivo by a naturally occurring inhibitor, calpastatin. Recently calpain has been demonstrated to be inhibited independent of the calcium concentration by human H-kinogen at plasma concentrations.²⁰ Kinogenins have been shown to be identical to α-cysteine proteinase inhibitors by Ohkubo et al and Müller-Estler et al.²¹,²² Moreover, calpain has recently been reported to cleave von Willebrand factor,²³ fibrinogen,²⁴ and cytoskeletal proteins²⁵-²⁸ as well as increase the surface-mediated activity of H-kinogen.²⁹

We now report that purified platelet calpain cleaves and activates human factor V, which results in a different form of activated factor V from that activated by thrombin.

MATERIALS

α-Thrombin was a generous gift of Dr John Fenton. Leupeptin, Trisma (Tris), Temed, Tween 20, ammonium persulfate, 4-chloro-l-naphthol, and rabbit antimon IgG coupled with horseradish peroxidase were obtained from Sigma Chemical Co, St Louis. Dithiothreitol (DTT) was purchased from Boehringer, Mannheim, West Germany; calcium chloride, methanol, silver nitrate, formalin, acetic acid, Coomassie brilliant blue R250, sodium carbonate, disodium ethylenediamine tetraacetate (EDTA), and citric acid were all purchased from Fisher Scientific Co, King of Prussia, PA. Glycine, sodium dodecyl sulfate (SDS), and mol wt standards were purchased from Bio-Rad Laboratories, Richmond, CA. Prestained standards were purchased from Bethesda Research Laboratories (BRL, Bethesda, MD), and nonfat dry milk (NFDM) was obtained from a local food store.

Preparation and characterization of murine monoclonal anti-
man factor V (B10). The murine monoclonal antibody (MoAb) B10 was prepared in our laboratory as described earlier.30 B10, which was typed as an IgG2a, recognizes the activation peptide (Cz) of human factor V and does not affect factor V coagulant activity.31

**Purification of plasma factor V.** Factor V was purified by an immunospecific procedure32 adapted for a murine monoclonal anti-factor V (B10) instead of a human MoAb. The immunosorbent was prepared identically,33 but nonspecifically bound proteins were removed by washing with 0.02 mol/L Tris buffer, pH 7.2, containing 0.37 mol/L NaCl and then washed with the Tris buffer containing 0.15 mol/L NaCl, 1 mmol/L CaCl2, 25% glycerol and 0.5% ethylene glycol. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of the purified factor V showed a major band corresponding to a mol wt of 330 kd, a minor contaminant at 350 kd, and a partial cleavage fragment, component B, at 220 kd. The specific activity ranged between 70 and 100 U/mg and increased 17– to 20-fold upon activation with thrombin at 37°C and eight- to tenfold at 25°C.

**Purification of platelet calpain.** Platelet calcium–activated cysteine protease (calpain) was purified from human platelet cytosol by using polyethylene glycol precipitation, anion-exchange chromatography, hydrophobic chromatography on reactive red agarose, and gel filtration.34 The protein was a single band on nondenaturing 10% PAGE and on 10% SDS-PAGE contained two subunits of 80 kd and a poorly staining 30 kd polypeptide. The assay used to monitor the purification involved the ability of calpain to release percloric acid–soluble peptides from casein as described previously.35 All assays were run in the presence of 5 mmol/L calcium, and blanked against an identical sample substituting 5 mmol/L EDTA for calcium. The specific activity when using α-casein as substrate was 60 U/mg where 1 U/mL was the amount of enzyme that increased the absorbance by 1.0 U/h/mL at 280 nm.

**Factor V coagulant assay.** Factor V coagulant activity was monitored by the one-stage factor V assay of Ware et al36 in which plasma with inactivated factor V37 or congenitally deficient plasma was used. One unit of factor V is defined as the amount in 1 mL pooled normal plasma.

**Activation of factor V by thrombin and platelet calpain.** Aliquots of purified human factor V were incubated with purified platelet calpain at 25°C in all experiments (to circumvent accelerated calpain inactivation at 37°C) in a buffer containing 0.02 mol/L Tris, pH 7.5, containing 0.15 mol/L NaCl and 5 mmol/L CaCl2. Incubations with thrombin, in the same buffer, were performed both at 25°C and 37°C as indicated in the legends and text. For the assay of factor V coagulant activity, at various time intervals aliquots of the incubations were diluted 50-fold into a buffer on ice containing 0.02 mol/L NaCl, 0.1% Tween 20 plus 5% NFDM. An affinity-purified rabbit antiserum IgG conjugated with peroxidase was then incubated with the nitrocellulose paper on a rotating shaker at 23°C for two hours. After washing the nitrocellulose was incubated with 5% NFDM in 0.01 mol/L Tris buffer at pH 7.5, containing 0.1% Tween 20 plus 5% NFDM. An affinity-purified rabbit antiserum IgG conjugated with horseradish peroxidase was then incubated with the nitrocellulose paper on a rotating shaker at 23°C for two hours. After washing with Tris buffer containing 0.1% Tween 20 plus 5% NFDM, immunoblots were developed with a solution consisting of 60 mg of 4-chloro-1-naphthol dissolved in 20 mL of ice-cold methanol; this was followed by dilution to 120 mL with 10 mmol/L Tris and 150 mmol/L NaCl, pH 7.5. To this substrate solution, 60 μL of 30% H2O2 was added. Within minutes a purple stain could be detected, and the nitrocellulose sheet was rinsed in distilled water and then dried on filter paper.

**Calculation of mol wts of polypeptides.** A standard curve for mol wt determination was constructed by plotting the relative mobilities of both the standards and the products of factor V digestion by thrombin38 against the logarithm of the known mol wts. The mol wts of hydrolysis of factor V by calpain was calculated by using relative mobilities on both SDS-PAGE and immunoblots.

**RESULTS**

**Activation of factor V by calpain: comparison with thrombin.** When factor V (1 U/mL, 33 nmol/L) was incubated with purified platelet calpain (0.03 U/mL, 2.7 nmol/L) in the presence of calcium at 25°C (to avoid heat inactivation of the enzyme at a higher temperature), an increase in activity of 2.5-fold was observed by four minutes, with a slight reduction in activity noted by six minutes (Fig 1A). Under identical conditions with thrombin (0.7 U/mL, 8 nmol/L) an increase of 8.0-fold at four minutes and 8.8-fold at six minutes was observed. This partial activation at 25°C has previously been reported for bovine factor V.39 No increase was found in factor V coagulant activity when the protein was incubated in the same buffer without calpain or thrombin for the same time period. When the thrombin reaction was performed at 37°C, a 17-fold increase was observed, which is in agreement with previous data (only the first four minutes of the time course is shown). Higher concentrations of calpain led to a more rapid but not an increased extent of activation; in some cases loss of activity was noted with increasing time.

Analysis of the eight-minute end points of a representative experiment by PAGE (Fig 1B) shows that, compared with the initial factor V preparation (lane 4), thrombin digestion of factor V at 37°C (lane 2) cleaved the 330 kd intact factor...
Activation of factor V by calpain and/or thrombin at 25°C. (A) Factor V (1 U/mL, 33 nmol/L) was incubated with 0.7 U/mL (8 nmol/L) thrombin, with 0.03 U/mL (2.7 nmol/L) platelet calpain (C), or with buffer (A). The factor V activation at 37°C (C) is shown for comparison. At various time intervals the incubation mixture was diluted and assayed for coagulant activity as in Methods. (B) Silver-stained 10% SDS-PAGE of the factor V preparation (lane 4), calpain (lane 3), and eight-minute digests of factor V by either thrombin at 37°C (lane 2) or by calpain at 25°C (lane 1).

Fig 2. Cleavage pattern of factor V by calpain. Factor V (5 U/mL, 165 nmol/L) was incubated at 25°C with thrombin (4.8 U/mL, 38 nmol/L) or calpain (0.15 U/mL, 16 nmol/L). The resulting mol wts were analyzed on 4% to 8% SDS-PAGE and stained with silver. Lane 1 shows the products of thrombin digestion at four minutes for comparison with lanes 2 to 7, which depict the time course of V digestion by calpain at 0.25, 0.5, 1, 2, 4, and 8 minutes, respectively. Mol wts are calculated by comparison with marker proteins (not shown) as in Methods.

V as well as the 220 kd intermediate as previously reported for bovine factor V. In contrast, highly purified calpain (lane 3) hydrolyzed factor V to different polypeptides (lane 1) than did thrombin. These experiments indicate that calpain can activate factor V, but to a lesser extent than does thrombin and with a markedly different pattern of cleavage.

**Time course of hydrolysis of factor V by calpain.** The sequential cleavage of factor V catalyzed by calpain at a 1:10 molar ratio of calpain to factor V is presented in more detail to better visualize the temporal changes in the activation intermediates. The experiment displayed in Fig 2 shows a 4% to 8% gradient SDS-PAGE stained with silver and uses the same molar ratio of thrombin and calpain as in Fig 1A except that the total protein was proportionally increased in the reaction mixtures to minimize the volume loaded for PAGE analysis. This change in the concentration of enzyme and substrate may have affected the reaction rate and therefore may have not directly correlated the changes on this gel with the coagulant activity. For reference, the cleavage pattern of factor V with thrombin for four minutes at 25°C is shown (Fig 2, lane 1) and includes B (220 kd), C, (150 kd), D (105 kd), and F,F2 (74 to 71 kd), similar to that reported by Suzuki et al. In contrast, at 15 seconds of factor V incubation with calpain, components of 200 and 160 kd (Fig 2, lane 2) appear. By one minute the native factor V (330 kd) is completely digested to these two new components as well as a 130 kd polypeptide (lane 4). By four minutes a loss of the 200 kd component and appearance of two new fragments of 140 and 120 kd is evident (Fig 2, lane 5). Further degradation of the 140 kd fragment after eight minutes occurs without any increase in activity. Neither the 105 kd (heavy) or the 74 to 71 kd (light) chain appear. Instead, new components of 100, 87, 65, 58, and 48 kd increase in concentration (lanes 6 and 7).

**Activation of factor V by calpain and thrombin sequentially.** In reactions performed at 25°C, factor V coagulant activity increased 1.3-fold over control after incubation with calpain for two minutes (Fig 3A). After the addition of thrombin at two minutes and after initiation with calpain, a 7.4-fold activation was observed at four minutes and a tenfold activation after six minutes (Fig 3A, 3). This degree of activation was similar to that seen by thrombin alone (Fig 1A). When calpain and factor V were incubated for four minutes, a 1.9-fold activation was observed. With addition of thrombin and incubation continued for an additional two
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minutes, an increase to 7.6-fold was found (Fig 3A, ⑥). These results indicate that, after factor V activation by calpain, thrombin was able to activate factor V and the degree of activation was similar to that of thrombin alone. When the reciprocal experiment was performed, the addition of calpain to factor V exposed to thrombin for either two (Fig 3A, ⑤) or four (Fig 3A, ⑦) minutes gave a similar degree (9.2-fold at six minutes) as exposure to thrombin alone (8.8-fold at six minutes). When leupeptin (100 umol/L), an inhibitor of calpain, was included before calpain addition, no differences were observed from factor V incubations with buffer alone. Thus, after activation by thrombin, calpain has little if any effect on factor V activation.

To further define the molecular changes occurring with calpain action on factor V, the sequential digestion of factor V with calpain followed by thrombin and factor V with thrombin followed by calpain was monitored by SDS gels. The experiment paralleled the changes in coagulant activity seen in Fig 3A. Calpain can cleave the 220 kd (B) and 150 kd (C), fragments of thrombin-digested factor V but does not alter the 105 kd (D) fragment or the 74 to 71 kd (F,F2) fragment (Fig 3B, lanes 2 and 5). Thrombin can cleave the 160, 140, 130, and 120 kd fragments produced by calpain to yield the 105 kd heavy chain and the 74 to 71 kd light chain (Fig 3B, lanes 3 and 4).

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**Fig 3.** Sequential activation and cleavage of factor V by thrombin and calpain. (A) Factor V (1 U/ml, 33 nmol/L) was incubated with thrombin at 25°C for two minutes. Calpain (0.03 U/ml, 2.7 nmol/L) was then added two (②, ③) or four minutes later (④, ⑤). In the inverse experiment, calpain (0.03 U/ml, 2.7 nmol/L) was first added to factor V, and then thrombin (0.7 U/ml, 8 nmol/L) was added after an additional two minutes (⑥, ⑦) or at four minutes (⑧, ⑨). The control reaction consisted of factor V containing 0.5 mmol/L leupeptin before the addition of calpain and assayed for coagulant activity at various times (⑩). (B) SDS-PAGE was performed as in Fig 2 on representative reactions. Mol wt markers are shown (lane 1). Factor V (5 U/ml, 165 nmol/L) was incubated with thrombin (4.8 U/ml, 38 nmol/L) for four minutes, and then calpain (0.15 U/ml, 16 nmol/L) was added to this reaction, which was incubated for two more minutes in the presence of 5 mmol/L CaCl₂ at 25°C (lane 2). Factor V and calpain, in the aforementioned concentrations, were incubated for two minutes, and then thrombin was added and digestion continued for an additional four more minutes (lane 3). Factor V and calpain were reacted for four minutes; this was followed by the addition of thrombin and digestion continued for an additional four minutes (lane 4). In lane 5 thrombin was added for four min and then calpain for four more minutes. Factor V and thrombin, incubated at 25°C for four minutes (lane 6) and calpain alone (lane 7), are presented for reference.

**Fig 4.** Mol wt products of calpain and thrombin: Coomassie blue analysis. Lane 1 is the same digest of factor V by thrombin (eight minutes) that is observed in Fig 1, lane 2, at 37°C. Lanes 2 and 3 are digests of factor V by calpain at 25°C for four minutes and eight minutes, respectively. Each has been electrophoresed on a 10% SDS polyacrylamide gel and stained with Coomassie blue. Mol wt markers appear in lane 4.
Further comparison of factor V digestion products by calpain and thrombin by using Coomassie stain. Calpain reactions with factor V were terminated after four or eight minutes of incubation (Fig 4, lane 2 and 3). Thrombin was incubated at 37°C for eight minutes with factor V to complete the digestion of the 220 kd fragment to its lower-mol wt end products and then analyzed on a 10% SDS-PAGE after staining with Coomassie blue (Fig 4, lane 1). Under these conditions the 150 kd (C₁) product resulting from thrombin and possibly some of the calpain cleavage derivatives resulting from products containing this piece stain poorly because of the high carbohydrate content. Thrombin also yields products of mol wts 105 kd and 74 to 71 kd, whereas calpain gives fragments of 100 and 87 kd (Fig 4, lanes 2 and 3). The smaller fragments produced by calpain below 87 kd (65, 58, and 48 kd) that are observed on silver staining (Fig 2) are below the detection limit for this Coomassie-stained gel. There is no 105 kd (D, heavy chain) or 74 to 71 kd (F₁,F₂, light chain) fragments in the factor V digests resulting from calpain action.

Immunoblotting to identify the domains of factor V cleaved by calpain. To define further the complex pattern of calpain cleavage, we used immunoblotting (Fig 5) with a murine MoAb, B10, that has been shown to recognize C₁, the 150 kd activation peptide from thrombin digests. The antibody recognizes the native factor V (330 kd) and the large digestion product, 220 kd (B, Fig 5, lane 1), present in this preparation of partially cleaved factor V. Both of which contain the 150 kd activation peptide. In the calpain digest the 330 kd moiety is rapidly cleaved by 15 seconds (Fig 5, lane 2), with appearance of a 200 kd piece and another at 160 kd. Further cleavage (Fig 5, lanes 3 to 7) results in the

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**Fig 5.** Immunoblot using MoAb B10 with factor V digested by calpain and thrombin. Factor V (lane 1) was digested with calpain (lanes 2 to 7) at 0.25, 0.5, 1, 2, 4, and 8 minutes, respectively, at 25°C under the same conditions as in Fig 2. The migration of the mol wt standards (BRL) are shown on the left. The calculated mol wts of the fragments of the digest reactions with B10 are shown on the right.

**Fig 6.** Schematic representation of the hydrolysis of factor V by calpain compared with thrombin. Intact factor V is shown by using the nomenclature of Suzuki et al., which designates the thrombin cleavage products as the domains. The scheme for thrombin digestion is also from Suzuki et al. On exposure to calpain, the digestion products of factor V by calpain are arranged according to the data resulting from the 4% to 8% gradient SDS-PAGE (Fig 2) and the 10% SDS-PAGE (Fig 4). Positive reactivity to the MoAb B10 that was determined in Fig 5 is designated as A, which is the epitope contained in the 150-kd C₁ component in the thrombin digests and in the native molecule. The lower-mol wt component reactions with MoAb B10 are presumed to derive from higher-mol wt polypeptides. The alignment is schematic because the N and C terminals of each fragment are not known.
SEQUENTIAL APPEARANCE OF 140 AND 120 KD FRAGMENTS CLEARLY DERIVED FROM THE 160 KD POLYPEPTIDE. NONE OF THE MOL WT SPECIES LESS THAN 120 KD ARE RECOGNIZED BY BlO ON IMMUNOBLOTS, NOR IS THE 130 KD SPECIES RECOGNIZED.

DISCUSSION

Platelet proteases can cleave human factor V as first suggested by Osterud et al. Ittayeh et al demonstrated that plasma factor V was activated twofold by a fresh platelet extract. The suggestion was offered that the cleavage might be due to a calcium-activated protease. This study provides objective evidence that platelet calpain could be the responsible protease. The enzyme used in this study was homogenous, and its action on factor V was inhibited by leupeptin (Fig 1). As in the crude platelet lysate, the maximum activation noted was 2.5-fold.

The cleavage pattern produced by calpain appears to be different from thrombin. Figure 2 summarizes the cleavage by thrombin as defined by Suzuki et al and contrasts it with the pattern produced by platelet calpain that was deduced from the SDS gels and immunoblotting experiments. Calpain first attacks a bond and gives two major intermediates of 200 kd and 160 kd (Fig 2). Even at 15 seconds we observe smaller concentrations of 140, 100, and 65 kd components. The 200 kd component is similar to the thrombin component B as shown by the positive reaction with B10 (Fig 5), which recognizes the thrombin C1 domain (150 kd), and forms part of the 220 kd component. Further cleavage by calpain of the 200 kd polypeptide liberates smaller fragments (160, 140, and 120 kd) that contain an epitope on C1 as shown by blotting with MoAb B10 (Fig 5). The 160 kd fragment seen in Fig 2 may in fact be two different polypeptides. One of these components, presumably not containing the B10 epitope, may be further degraded to fragments of 100 and 65 kd that are unreactive with MoAb B10. The 130 kd and other low-mol wt fragments (87, 58, and 48 kd) do not react with MoAb B10; however, their origin is speculative because they could result from cleavage of the 160 kd component containing the MoAb B10 epitope and be the part of the polypeptide not bearing the epitope. The failure of the 160 kd component to stain well with Coomassie blue (Fig 4) is consistent with the presence of high amounts of carbohydrate because the thrombin-derived C1 (150 kd) also stains poorly. More recently Viskup et al reported the purification of factor V from platelets. Despite the use of inhibitors during platelet collection and washing, the lower–mol wt forms of factor V reported resemble those we observe after exposure to calpain. The inability to obtain intact factor V from platelets could be due to the action of calpain on factor V that is associated with activation of platelets during their isolation from whole blood or during solubilization and subsequent purification. In contrast, in plasma, kininogens would inhibit any calpain escaping from the platelet cytosol.

The failure of calpain to completely cleave the light chain of factor Va (F,F2, 71 to 74 kd) from the 200 kd fragment may account for the incomplete activation observed. Unlike thrombin in which the heavy chain (D, 105 kd) is cleaved off first, calpain removes fragments that may contain this domain late in the digestion and then degrades them further. The failure of calpain to cleave off the polypeptides composing factor Va (D and F,F2) (Figs 2 and 4) is also supported by the ability of thrombin to cleave factor V, which has been previously hydrolyzed by calpain (Fig 3). Both the cleavage and the ability of thrombin to fully activate factor V that is digested by calpain for short times are consistent with this construct. A more detailed analysis of the nature of the cleavage pattern of factor V by calpain awaits the determination of the full amino acid sequence of factor V.

Platelet membrane and cytoskeletal proteins localized in both the plasma and the platelet α-granules, such as fibronogen, von Willebrand factor, and H-kininogen, are also substrates for calpain. Thus, factor V, an α granule protein and a plasma procofactor, also fits well into the group of calpain substrates. The function of activation and cleavage of factor V by platelet calpain is not fully understood. As already stressed, once thrombin is present, calpain cannot activate factor V further; however, calpain may play a role in the early events of hemostasis before the formation of thrombin. Ittayeh et al found that bovine platelet factor V was identical to that in plasma only when purified in the presence of protease inhibitors. When whole platelets were lysed with nonionic detergent or factor V released from platelets after stirring with collagen, these preparations contained cleaved bovine factor V as detected by a change in charge on immunoelectrophoresis. Thus, factor V is cleaved and activated, probably by platelet cytosolic enzymes including calpain, during secretion or injury. Even a low level of activation would facilitate the cleavage of prothrombin by factor Xa and promote the formation of the first few molecules of thrombin. Once thrombin was formed, the feedback loop would rapidly produce factor Va. In this scheme, platelet calpain might serve to allow limited activation of factor V after exposure of platelets to basement membrane collagen in the initial phase of blood coagulation before thrombin formation. Finally, factor V has recently been shown to be a substrate for factor XIIIa. Wang et al have demonstrated that the activation of platelets with thrombin is associated with factor XIII catalysis of the crosslinking of factor V to the cytoskeletal action. The finding that calpain can activate factor XIII suggests an additional indirect interaction between calpain and factor V that is important for expression of this protein on the platelet membrane.

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