PURIFICATION AND PROPERTIES OF THIAMINE PYROPHOSPHOKINASE FROM PARSELEY LEAF

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Summary Thiamine pyrophosphokinase was purified about 8,000fold from extracts of parsley leaves. The enzyme, as prepared, was homogenous on polyacrylamide gel electrophoresis. The molecular weight of the enzyme, estimated by gel filtration with Sephadex G-150, was approximately 30,000. In 0.05 M Tris-HCl, the enzymic activity showed a pH optimum over a range of 8 to 9. A least squares analyses of Lineweaver-Burk and Hofstee plots gave $K_m$ values of 0.8 mM and 0.15 $\mu$M for ATP and thiamine, respectively. Thiamine homologues and analogues so far tested, except for cetyl thiamine, were all inactive as substrates. The enzyme was specific for ATP and Mg++, although to a lesser extent a combination with other ribonucleoside triphosphates or divalent cations could replace them. SH reagents, such as PCMB, NEM and iodoacetamide, were potent inhibitors of the enzyme. The inhibition was prevented by the addition of dithiothreitol. Inorganic pyrophosphate exhibited striking inhibition. TMP could not replace thiamine as the substrate, whereas it inhibited the TPP formation from thiamine. These findings are consistent with the views that TMP is not directly converted to TPP but after being dephosphorylated by the action of a monoesterase, thiamine is pyrophosphorylated with ATP by thiamine pyrophosphokinase (EC 2.7.6.2) to form TPP and thus give a clear evidence regarding the mechanism of TPP formation in plant tissues.

Direct pyrophosphorylation of thiamine with ATP has been established in microbial and mammalian systems (1, 2). Thiamine pyrophosphokinase (EC

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Abbreviations: TMP, thiamine monophosphate; TPP, thiamine pyrophosphate; TTP, thiamine triphosphate; OMP, 2-methyl-4-amino-5-hydroxymethylpyrimidine; thiazole, 4-methyl-5,β-hydroxyethylthiazole.
2.7.6.2) which catalyzes pyrophosphotransferation has been obtained as a partially purified preparation from brewers' yeast (1), rat liver (2) and E. coli (3). It is still uncertain whether the enzymic formation of TPP proceeds in two steps with the intermediate formation of TMP, the immediate product in thiamine biosynthesis, or via a direct pyrophosphotransferation to thiamine. In a previous paper (4), the present authors demonstrated that a relatively high activity of thiamine pyrophosphokinase is distributed in the green leaves of a variety of higher plants, and suggested that "TMP kinase," absolutely specific for TMP, is irrelevant to the TPP formation in plants.

The present paper describes properties of a highly purified thiamine pyrophosphokinase from parsley, and offers evidence for direct pyrophosphorylation of thiamine for TPP synthesis in plants.

MATERIALS AND METHODS

Materials. Fresh leaves of parsley were purchased in September at a local market. Thiamine and its mono- and di-phosphates were the products of Sigma Chemical Co. and were purified by recrystallization from acetone and water. OMP and thiazole were kindly supplied by Takeda Chemical Industries; deaminothiamine was obtained from Shionogi & Co., Ltd. Hydroxylapatite was obtained from Clarkson Chemicals Co., Inc. Other analytical grade chemicals were purchased from commercial sources.

Standard assay for thiamine pyrophosphokinase activity. The reaction mixture contained 10 nmoles of thiamine, 4 µmoles of ATP, 20 µmoles of MgCl₂ and an aliquot of the enzyme preparation in 2 ml of 0.05M Tris-HCl buffer (pH 8.0). Incubation was performed at 37°C for 60 min, and the reaction was terminated by adding 2 ml of 1.0 M citrate buffer (pH 6.0), and by heating the resultant mixture for 5 min at 90°C. The TPP content in this deproteineized reaction mixture was determined manometrically as described in the previous paper (4).

Polyacrylamide gel electrophoresis. An aliquot of the enzyme preparation in step 7 was submitted to electrophoresis on a polyacrylamide gel slab (1.5×0.5×17 cm). Electrophoresis was performed at a constant current of 30 mA for 6 hr. After completion of electrophoresis the gel slab was sliced into sections about 5 mm thick. Each was homogenized with 1.0 ml of 0.05 M Tris buffer (pH 7.5) containing 10⁻⁵ M 2-mercaptoethanol and the enzyme was extracted for 15 hr at 4°C.

RESULTS

Purification of the enzyme

All operations were carried out below 4°C unless otherwise noted. Fresh leaves of parsley (800 g) were ground in a Waring blender with 1.5 liters of 0.05 M Tris-HCl buffer (pH 7.5) containing 10⁻⁴ M 2-mercaptoethanol and 10⁻⁸ M
EDTA. The homogenate was squeezed through three layers of gauze [Step 1].

The filtrate (1920 ml) was centrifuged at 5,000×g for 15 min to remove cell debris [Step 2].

Ammonium sulfate to the supernatant (1740 ml) was added to 30 and 45% saturation. The fraction precipitating between 30–45% saturation was collected by centrifugation, dissolved in the buffer, and dialyzed at intervals of 5 hr against two changes of 2.5 liter of the Tris buffer but EDTA [Step 3].

After removal of precipitates formed, the enzyme solution was placed on a DEAE-cellulose column (2.5φ×90 cm) which was previously equilibrated with the Tris buffer. The column was washed with 1 liter of the Tris buffer and eluted by a linear gradient of 0 to 0.5 M KCl in the buffer. Active fractions were combined and brought to 60% saturation with ammonium sulfate and the resultant precipitate was separated by centrifugation [Step 4].

The precipitate was dissolved in the Tris buffer and dialyzed as described above. The dialysate was again chromatographed on a column of DEAE-cellulose (2.5φ×40 cm) equilibrated with the buffer, and then, the protein was eluted by linearly increasing concentrations of KCl to 0.5 M. Active portions were collected and concentrated to a minimal volume by ultrafiltration in a collodion bag [Step 5].

The condensate was fractionated by gel filtration with a Sephadex G–150

Fig. 1. Gel filtration of thiamine pyrophosphokinase with Sephadex G–150. The enzyme preparation at step 5 was passed through the column (2.2φ×95 cm) with Sephadex G–150. The effluent was fractionated in each 5 ml. The enzyme activity in each fraction was assayed according to the same methods as in Table 1. ●—●, activity of thiamine pyrophosphokinase (TPP formed); -----, protein (absorbance at 280 nm).
column (2.2φ×95 cm) [Step 6]. Figure 1 depicts a typical elution pattern by the gel filtration. Thiamine pyrophosphokinase activity was not found in a main protein peak but in a minor one. The combined active fraction was concentrated by ultrafiltration, and an aliquot of the fraction was passed through a hydroxylapatite column (1.1φ×26 cm) which was previously equilibrated with 0.01 M potassium phosphate buffer (pH 7.2) containing 10⁻² M 2-mercaptoethanol. As shown in Fig. 2, most of the enzyme activity was found in the first protein peak, which a majority of contaminant proteins remained on the column [Step 7].

![Fig. 2. Column chromatography of thiamine pyrophosphokinase with hydroxylapatite.](image)

An aliquot of the active fraction separated by Sephadex G–150 gel filtrate was applied on the top of the hydroxylapatite column (1.1φ×26 cm; equilibrated with 0.01 M phosphate buffer of pH 7.2). The effluent was collected by 5 ml portions. The assay conditions were the same as in Fig. 1. ○—○, activity of thiamine pyrophosphokinase (TPP formed); ----, protein (absorbance at 280 nm).

The enzyme preparation after being passed through hydroxylapatite column was subjected to a preparative polyacrylamide gel electrophoresis. The gel slab was cut into small pieces and the respective sections were allowed to extract proteins with the Tris buffer. Figure 3 shows a typical separation pattern of protein on polyacrylamide gel. A single peak having the enzyme activity was formed at the relative mobility \( k_b = 0.3 \) [Step 8]. A purification result is summarized in Table 1. Thiamine pyrophosphokinase was approximately 8,000-fold purified from parsley leaf. The enzyme in the final preparation proved to be homogenous on analytical polyacrylamide gel electrophoresis.

**Molecular weight and isoelectric point**

The molecular weight of thiamine pyrophosphokinase was determined by the
Fig. 3. Separation of thiamine pyrophosphokinase on polyacrylamide gel by electrophoresis. The enzyme preparation at step 7 was finally separated on the preparative polyacrylamide gel (1.5 × 0.5 × 17 cm) by electrophoresis (30 mA, 6 hr in the refrigerator). The gel after electrophoresis was cut into small slices, and then extracted with 1 ml of the buffer. The enzyme activity and protein content in each extract were determined by the respective routine assay methods. ○----○, activity of thiamine pyrophosphokinase (TPP formed); ●----●, protein content determined by the method of LOWRY et al. (17).

Table 1. Purification of thiamine pyrophosphokinase from parsley leaf. The assay conditions were the same as described in "MATERIALS AND METHODS," except for a constant level of ATP (5 mM) and pH 7.5.

| Step | Description                  | Volume (ml) | Protein (mg) | Activity (units) | Specific activityb | Recovery (%) |
|------|------------------------------|-------------|--------------|------------------|-------------------|--------------|
| 1.   | Crude extract                | 1920        | 27200        | 287              | 0.01              | 100          |
| 2.   | Supernatant (5,000 × g, 15 min) | 1740        | 10700        | 243              | 0.02              | 84.5         |
| 3.   | Ammonium sulfate (30–45% satd.) | 63.5        | 5850         | 254              | 0.04              | 88.3         |
| 4.   | 1st DEAE-cellulose           | 50.6        | 1950         | 267              | 0.14              | 92.9         |
| 5.   | 2nd DEAE-cellulose           | 3.9         | 484          | 284              | 0.59              | 98.6         |
| 6.   | Sephadex G–150               | 2.1         | 21.6         | 223              | 10.5              | 77.6         |
| 7.   | Hydroxylapatite              | 3.0         | 7.8          | 195              | 25.0              | 67.9         |
| 8.   | Electrophoresis              | 2.1         | 0.37         | 30.7             | 83.0              | 10.7         |

a One unit of the kinase is explained in terms of the amount of enzyme capable to synthesize 1 nmoles of TPP per 60 min from 5 μM of thiamine under the above conditions.
b Specific activity is expressed as units per mg of protein.

The technique of gel filtration on the Sephadex G–150 column according to the procedure of ANDREWS (5). As shown in Fig. 4, the comparison of the elution volume of thiamine pyrophosphokinase with those of standard proteins allowed an estimation of the molecular weight of about 30,000 for thiamine pyrophospho-
kinase from parsley leaf. Isoelectric focusing in polyacrylamide gel containing 1.0% carrier ampholine (pH 3 to 10) revealed that the enzyme was 4.5 of isoelectric point.

**Time course of kinase reaction**

As shown in Fig. 5, the enzymic reaction proceeded linearly for the first 90 min during incubation. Thus, incubation for 60 min was employed in the standard assay. The TPP formation from thiamine increased with increased con-

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**Fig. 4.** Determination of molecular weight of thiamine pyrophosphokinase by Sephadex G-150 gel filtration. The purified enzyme was chromatographed on the Sephadex G-150 column (2.2×95 cm). The elution volume (Ve) of thiamine pyrophosphokinase was compared with that of trypsin (M.W. 15,000), pepsin (M.W. 35,000), bovine albumin (M.W. 67,000) and hexokinase (M.W. 102,000) as standards.

**Fig. 5.** Time course of TPP formation by catalysis of thiamine pyrophosphokinase. The reaction mixture containing 10 nmoles of thiamine, 4 μmoles of ATP, 20 μmoles of MgCl2 and 250 μg of enzyme protein in 2 ml of pH 8 Tris-HCl buffer, was incubated at 37°C for a desired period. The amount of TPP formed was manometrically determined by the carboxylase action on pyruvate as described in the preceding paper (4).
centrations of the enzyme, but its reaction did not go to completion even after prolonged incubation. This may probably be due to a product inhibition; a presence of large amounts of TPP formed is inhibitory for pyrophosphorylation of thiamine.

**Temperature dependence**

Figure 6 shows the effect of varied temperature on the enzyme activity. The reaction rate reached a maximum at 45°C, and gradually decreased at the higher temperature. Incubation at 37°C for 60 min, the standard assay condition, did scarcely cause inactivation of the enzyme. Arrehenius plot of the relative velocity against the temperature, shown in the inset of Fig. 6, gave an activation energy of 4,500 cal for its reaction.

**pH dependence**

An optimal pH of the enzyme reaction in the presence of ATP and Mg^{++} was observed over the range of 8 to 9, as shown in Fig. 7. The enzyme showed no activity below pH 7. The pH-activity curve was markedly affected by the replacement of ATP or Mg^{++}, or both with other ribonucleoside triphosphates or divalent cations in resemblance with the behavior of thiamine pyrophosphokinase from yeast (J) and rat liver (2). Thus, in the standard assay, the enzyme reaction was allowed to proceed at pH 8.0 with the use of ATP and Mg^{++}. 

![Temperature dependence of thiamine pyrophosphokinase activity and Arrhenius plots.](image)
Substrate specificity

Tables 2 and 3 compare the specificity of the purified enzyme towards thiamine and its analogues as pyrophosphate acceptor and towards ribonucleoside triphosphates as pyrophosphate donor. The enzyme had a limited specificity for thiamine analogues in the presence of ATP and Mg++, as shown in Table 2. After enzymic pyrophosphorylation, thiamine itself and cetyl thiamine among thiamine analogues tested, functioned as the cocarboxylase for pyruvate decarboxylation. Compounds such as dibenzoylthiamine and OMP devoid of a hydroxyethyl group in the thiazole molecule, had no ability of accepting a pyrophosphate moiety from ATP. Other derivatives seemed likely to serve as acceptor for pyrophosphate transfer, but to lack the function as cocarboxylase.

Table 2. Replacable effect of thiamine with its analogues on carboxylase activity.
The experimental conditions were the same as described in the text, except that a variety of thiamine analogues were used instead of thiamine, the principal substrate. The alternative ability was interpreted in terms of the amount of CO₂ evolved for 30 min under the standard assay conditions.

| Substrate (5 μM) | CO₂ evolved during 30 min (μl) | Activity (%) |
|------------------|-------------------------------|--------------|
| Thiamine         | 135.0                         | 100          |
| TMP              | 6.4                           | 4.8          |
| Oxythiamine      | 3.2                           | 2.4          |
| Deaminothiamine  | 9.6                           | 7.2          |
| Cetyl thiamine   | 122.1                         | 90.5         |
| Dibenzyothiamine | 11.2                          | 8.1          |
| OMP<sup>a</sup>  | 0                             | 0            |
| Th<sup>b</sup>   | 6.4                           | 4.8          |

<sup>a</sup> OMP, 2-methyl-4-amino-5-hydroxymethylpyrimidine.

<sup>b</sup> Th, 4-methyl-5,β-hydroxyethylthiazole.
because of the substitution at position 4 in the pyrimidine molecule as indicated
SCHELLENBERGER (6). TMP did scarcely function as the substrate. This fact
reveals that the activity of TMP kinase is not associated with thiamine pyro-
phosphokinase from parsely and that a direct pyrophosphorylation from ATP
to free thiamine takes place in plant leaves. As shown in Table 3, the enzyme
had a broad specificity for a variety of ribonucleoside triphosphates as pyro-
phosphate donor. ATP was the most effective among the triphosphates. GTP,
UTP and CTP followed to it in this order. ITP was also active but to a lesser
extent. ATP could not be replaced by ADP and AMP, nor by mono- and di-
phosphates of other ribonucleosides.

| Nucleotides (2 mM) | TPP formed (nmoles) | Activity (%) |
|-------------------|---------------------|--------------|
| ATP               | 0.74                | 100          |
| ADP               | 0                   | 0            |
| AMP               | 0                   | 0            |
| CTP<sup>a</sup>   | 0.43                | 58           |
| GTP<sup>a</sup>   | 0.52                | 70           |
| ITP<sup>a</sup>   | 0.11                | 14           |
| UTP<sup>a</sup>   | 0.48                | 65           |

<sup>a</sup> Abbreviations: CTP, GTP, ITP and UTP; triphosphates of cytidine, guanosine,
inosine and uridine.

**Michaelis constants**

Figures 8 and 9 show the effects of the concentrations of ATP and thiamine
on the reaction velocity, respectively. The reaction seemed to obey a classical
Michaelis-Menten kinetics in the range of 0.125 to 4 mM ATP. When the con-
centration of ATP exceeded 4 mM, an excess of ATP inhibited the kinase activity,
while thiamine exhibited only a little inhibitory effect even at the concentration
above 5 mM. An apparent $K_m$ value was obtained from the intercept of a
straight line drawn in Lineweaver-Burk (Fig. 8) and Hofstee plots (Fig. 9).
The line was statistically corrected in order to determine a more accurate $K_m$
value. The respective $K_m$ values calculated were 0.8 mM for ATP and 0.15 μM
for thiamine; the latter was much less than 75 μM for the yeast kinase and rela-
tively close to 0.3 μM for the *E. coli* enzyme. These findings imply that in
higher plants a direct pyrophosphorylation of thiamine is preferable to another
pathway.
Fig. 8. Effect of ATP concentrations on thiamine pyrophosphokinase activity. The reaction velocity was expressed as the amount of CO₂ evolved per 30 min under the assay conditions, except for the varied concentrations of ATP. The straight line drawn in the double reciprocal plots had been statistically corrected.

\[
Y = 0.0092X + 0.011 \\
K_m = 0.8 \text{ mM}
\]

Fig. 9. Effect of thiamine concentrations on the kinase activity. The experimental conditions were the same as in Fig. 8, except for a constant concentration of ATP (2 mM) and varied concentrations of thiamine. The straight line in the plots was statistically corrected.

\[
Y = 0.0104X + 0.0016 \\
K_m = 0.15 \mu\text{M}
\]
Requirement of divalent cations

A divalent cation was a requisite for the activation of thiamine pyrophosphokinase. Table 4 shows the effect of various divalent cations on the activity. Mg\(^{++}\) was the most effective among cations tested. Co\(^{++}\) and Mn\(^{++}\) could replace Mg\(^{++}\) in some extent, whereas other cations exhibited little or no stimulating effect. Activation by Mg\(^{++}\) was closely related to the concentration of ATP. The activity increased accompanying with increasing concentrations of Mg\(^{++}\) at a constant concentration of ATP until 10 mM of Mg\(^{++}\) was added. A further addition of Mg\(^{++}\) resulted in a decrease in the activity. The optimum concentration of Mg\(^{++}\) was around 2 mM in the presence of 2 mM ATP. A maximal activation was dependent on the suitable ratio of the concentration of Mg\(^{++}\) to ATP.

Table 4. Dependence of divalent cations on the kinase activity. The enzyme reaction was carried out in the same way as described above. Various divalent cations were utilized in place of Mg\(^{++}\). Before the manometric determination of TPP, MnCl\(_2\) (30 mM) was further added to the routine assay medium.

| Additions (5 mM) | TPP formed (nmole) | Activity (%) |
|-----------------|-------------------|-------------|
| Mg\(^{++}\)     | 1.25              | 100         |
| Co\(^{++}\)     | 0.41              | 32.7        |
| Mn\(^{++}\)     | 0.16              | 12.9        |
| Zn\(^{++}\)     | 0.07              | 6.0         |
| Ni\(^{++}\)     | 0.07              | 6.0         |
| Fe\(^{++}\)     | 0.06              | 4.8         |
| Cd\(^{++}\)     | 0.07              | 5.8         |
| Ca\(^{++}\)     | 0.06              | 5.1         |
| Ba\(^{++}\)     | 0.0               | 0.0         |
| Cu\(^{++}\)     | 0.0               | 0.0         |

Effect of various inhibitors

The effects of a variety of inhibitors are summarized in Table 5. The enzyme activity was completely suppressed by 0.1 mM PCMB and 10 mM of pyrophosphate. Sulphydryl reagents such as NEM and iodoacetamide inhibited fairly the activity, while KCN, NaF and molybdate had little effect. The activity suppressed by PCMB was almost completely restored by adding an excess amounts of dithiothreitol. These facts suggest that the reversible conversion between -SH and -SS- in the protein structure has a significant role on the appearance of the enzyme activity. On the other hand, inorganic monophosphate at a relatively high concentration did not influence on any activity in contrast to the inhibition by pyrophosphate. EDTA, a predominant chelating agent, exhibited a strong inhibition. TMP was not only unable to replace thiamine but also inhibited strikingly the TPP formation from thiamine. Details of this inhibition will be described elsewhere.
Table 5. Effect of various inhibitors on the kinase activity. The experimental conditions were the same as described in the text, except for the addition of various inhibitors.

| Additions          | Final concentration (mM) | TPP formed (nmoles) | Inhibition (%) |
|--------------------|--------------------------|---------------------|---------------|
| None               | —                        | 0.891               | 0             |
| EDTA               | 1.0                      | 0.036               | 66.6          |
| NaF                | 10                       | 0.781               | 12.1          |
| H$_2$MoO$_4$       | 1.0                      | 0.678               | 22.4          |
| KCN                | 10                       | 0.790               | 11.2          |
| Iodoacetamide      | 10                       | 0.656               | 24.6          |
| NEM$^a$            | 10                       | 0.436               | 46.6          |
| Arsenite           | 10                       | 0.808               | 9.4           |
| PCMB$^b$           | 0.1                      | 0.0                 | 100           |
| PCMB$^b$ + DTT$^c$ | 0.1, 10                  | 0.755               | 14.7          |
| Phosphate          | 10                       | 0.900               | 0             |
| Pyrophosphate      | 10                       | 0.0                 | 100           |
| TMP                | 2.0                      | 0.114               | 78.8          |

$^a$ NEM, N-ethylmaleimide.
$^b$ PCMB, $p$-chloromercuribenzoic acid.
$^c$ DTT, dithiothreitol.

**DISCUSSION**

In the previous paper regarding the biosynthesis of thiamine in higher plants (6), the authors ascertained that direct product from condensation of thiazole-P and pyrimidine-PP is not free thiamine but the monophosphate ester as is in microorganisms (8-11), and that the capacity to utilize TMP as the substrate in place of thiamine is mostly removed during the course of purification of thiamine pyrophosphokinase from parsley leaf (4). These facts led to speculate that a TMP kinase is a fortuitous activity and does not account for the enzymic formation of TPP in grown leaves of plants.

On the other hand, a partially purified enzyme preparation from yeasts has been reported by Tokuda (12) to be accompanied by the activity of TMP kinase, using ATP labelled with $^{32}$P. A soluble enzyme from E. coli does not act on free thiamine but specifically on TMP to form TPP (13). It has been found for thiamine triphosphate to occur in germ axes of several higher plants in addition to proliferating cells of yeast by Yusa (14), who states that an active phosphorylative conversion takes place from thiamine into TTP and/or TPP in the earliest period of germination. In microorganisms (15) and animal tissues (16), TTP seems likely to serve as an efficient phosphate donor for the conversion of cellular ADP into ATP.

In the present study, a highly purification of thiamine pyrophosphokinase, scarcely accompanying the TMP kinase activity, has been achieved by means of
chromatographic techniques and electrophoresis. A trace of TPP enzymically produced from TMP is probably regarded as a result of the action of thiamine pyrophosphokinase on free thiamine contaminated in TMP or arising from spontaneous degradation during incubation. The homogeniety and molecular weight of the purified enzyme were determined by gel filtration with Sephadex G-150 and furthermore confirmed by SDS-electrophoresis. It was no longer possible to separate the purified enzyme into more than one component.

The enzyme showed an absolute requirement for ATP or other high energy bond triphosphates as pyrophosphate donor, and for magnesium ion or other divalent cations with a coordination of 6 as a cofactor. Most of enzymes requiring ATP have been known to be activated by a variety of metal ions; the activation is effected by forming a chelate complex among the metal ions, the nucleotide and the substrate. An excess of either ATP or metal ion causes a remarkable inhibition on the reaction of thiamine pyrophosphokinase, based on the hindrance of chelation. EDTA also inhibited the reaction by the competition with ATP. Inactivation mechanism by TMP, pyrophosphate and other inhibitors remained to be further investigated.

The property of thiamine pyrophosphokinase from parsley resembles in many aspects to that of the kinase partially purified from yeast (1) and from rat liver (2). It is consequently safe to conclude on phosphorylation of thiamine in plants that TMP synthesized from the thiazole and the pyrimidine is first dephosphorylated to thiamine by the action of a thiamine phosphate monoesterase and that pyrophosphate moiety of ATP is transferred to the resultant thiamine in the presence of magnesium to form TPP by the kinase action.

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