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In vitro Phosphorylation of Soluble Proteins from Soybean (*Glycine max*) Root Nodules: Inhibition of Protein Kinase Activity by Zn^{2+}

HARI B. KRISHNAN and STEVEN G. PUEPPKE*
Department of Plant Pathology, 108 Waters Hall, University of Missouri
Columbia, MO 65211, USA
Tel. 314-882-2418

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Abstract

Soybean [*Glycine max* (L.) Merr.] root nodules contain soluble, Ca^{2+}-dependent and Ca^{2+}-independent protein kinases that phosphorylate several endogenous proteins. Polyacrylamide gel electrophoresis of the *in vitro* phosphorylation products in the presence of SDS resolves 72, 63, 50, 41, and 17 kDa phosphoproteins. Each can be further separated by two dimensional electrophoresis into a cluster of phosphorylated proteins. The 50 and 17 kDa phosphoproteins are synthesized by both nodule and root extracts, but the 72, 63, and 41 kDA phosphoproteins are synthesized only by nodule extracts. The protein kinase activity is influenced by Mg^{2+}, with maximal activity at 10 mM $MgCl_2$. Micromolar concentrations of Zn^{2+} inhibit the phosphorylation of the 72, 63, 50, and 41 kDa phosphoproteins, but maximal inhibition is at concentrations greater than 750 μM. Even at 5 mM, Zn^{2+} has no effect on phosphorylation of the 17 kDa polypeptide. Sodium molybdate and sodium fluoride, two potent inhibitors of protein phosphatases, are not effective in reversing the Zn^{2+}-mediated inhibition of phosphorylation.

Keywords: *Glycine max*, nodulins, protein kinase, phosphoproteins, soybean, zinc

Abbreviations: CaM — calmodulin, DTT — dithiothreitol, EDTA — ethylenediaminetetraacetic acid, EGTA — ethyleneglycol-bis(β amino-ethyl ether)-N,N,N′,N′-tetraacetic acid, PAGE — polyacrylamide gel electrophoresis, SDS — sodium dodecyl sulfate

*To whom correspondence should be sent

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1. Introduction

Root nodules are specialized N_2-fixing structures that form when leguminous plants are invaded by soil bacteria of the genera *Rhizobium* and *Bradyrhizobium*. Nodule biogenesis is a complex, multi-step process involving (a) attachment of bacteria to root hairs, (b) root hair curling, (c) cortical cell proliferation, (d) infection thread formation, and (e) release of bacteroids into the host cytoplasm. The released bacteroids always are surrounded by peribacteroid membranes, which are of plant origin (Verma et al., 1978) and seem to play a pivotal role in determining the effectiveness of nitrogen fixation. By virtue of its location, the peribacteroid membrane regulates the flow of carbon and nitrogen compounds between the bacteria and the host. Indeed, nodules that are deficient in N_2 fixation often lack peribacteroid membranes or contain peribacteroid membranes that deteriorate early in nodule development (Werner et al., 1980).

The presence of rhizobia as discrete endosymbiotic entities, separated from the host cytoplasm by the peribacteroid membrane, implies that communication between the host and the bacteria must be carefully coordinated. Protein kinases, a key class of regulatory enzymes, have been documented to play such a role in signal transduction in animal systems (Nishizuka, 1986). Protein kinase-mediated phosphorylation of several unidentified plant proteins has been documented (Krishnan and Pueppke, 1987, 1988; Polya et al., 1986; Poovaiah et al., 1987; Ranjeva et al., 1984; Veluthambi and Poovaiah, 1984), suggesting the existence of a similar mechanism in plants. Recently, Ca^{2+}-dependent protein kinase activity in peribacteroid membranes from soybean nodules has been detected (Bassarab and Werner, 1987). The nodule cytosol, however, has not yet been examined for the presence of such activity. Furthermore, it is not known whether any nodule-specific proteins, e.g., nodulins, are phosphorylated. We report here the presence of protein kinase activity in the cytosol of soybean nodules. This activity phosphorylates several endogenous proteins *in vitro*. Some of these phosphoproteins are nodule-specific, and their phosphorylation is inhibited by Zn^{2+}.

2. Materials and Methods

Soybean [*Glycine max* (L.) Merr. cv. Williams 82] seeds from the Missouri Seed Improvement Association (Columbia, MO, USA) were germinated for 2 days on moist filter paper then planted in 6-liter plastic pots containing Perlite. The seedlings were treated with commercial inoculum (Urbana Laboratories, Urbana, IL) at the time of planting. Plants were grown in a

greenhouse and fed with a nitrogen-free nutrient solution as described by Triplett et al. (1980). Nodules were separated from the upper taproot of each plant 6 weeks after inoculation and stored at –70°C until use. The residual, nodule-free tissues from the same region of each root were retained as a control.

Preparation of soluble proteins from nodules

Five grams of frozen nodules were ground to a fine powder under liquid nitrogen with a mortar and pestle. This procedure efficiently releases plant proteins, with minimal release of bacteroid proteins (unpublished observations and personal communication from Dr. David Emerich). The powder was extracted with 20 ml of grinding buffer, which consisted of 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, and 0.2 mM EGTA. The slurry was centrifuged at 40,000 *g* for 15 min at 4°C. Three volumes of ice-cold, saturated $(NH_4)_2SO_4$ were added to the clear supernatant solution, which then was placed on ice for 60 min. The precipitated proteins were recovered by centrifugation at 30,000 *g* for 10 min. The pellet was redissolved in 1 ml of grinding buffer and dialyzed against 2 l of dialysis buffer, which consisted of 10 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 0.1 mM DTT, and 0.2 mM EGTA. The dialyzed extract was clarified by centrifugation at 30,000 *g* for 10 min, and the resulting clear supernatant solution was designated the soluble protein fraction from nodules. Similar fractions from roots were prepared as controls. Protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

In vitro protein phosphorylation and gel electrophoresis

Protein phosphorylation was carried out essentially as described by Krishnan and Pueppke (1988). Briefly, the assay was performed in 100 μl of dialysis buffer with the final concentration of the chelating agent (EDTA or EGTA) adjusted to 400 μM. Phosphorylation assay mixtures, which included 300 μg of soluble proteins from nodules, were preincubated at 30°C for 2 min before initiating the reaction with the addition of 5 μCi of $[\gamma^{32}P]$-ATP (300 Ci/mmol, from New England Nuclear). The reactions were terminated by the addition of one volume of SDS-sample buffer [120 mM Tris-HCl, pH 6.8, 4% (w/v) SDS, 10% (v/v) 2-mercaptoethanol, and 20% (v/v) glycerol] followed by boiling for 4 min. In a few cases, reactions were terminated by adding one volume of lysis buffer (O'Farrell, 1975).

SDS-PAGE was carried out according to Laemmli (1970). The proteins were resolved on a 15% separating gel overlaid with a 4% stacking gel. Electrophoresis was at constant current (30 mA per 13.5 cm× 16 cm×1.5 mm slab) until the tracking dye reached the bottom of the gel. The gels were stained, dried and subjected to autoradiography as described by Krishnan and Pueppke (1988).

3. Results

Protein kinases present in the soluble extracts of soybean root nodules phosphorylate several endogenous substrates, the most predominant of which have molecular weights of 72, 63, and 17 kD (Fig. 1, lane A). Phosphorylation of these proteins is stimulated by the addition of 1 mM Ca^{2+} to *in vitro* reaction mixtures (Fig. 1, lane B). Additional polypeptides of 50 and 41 kD are phosphorylated in the presence of Ca^{2+}, but are barely detectable in its absence. Occasionally Ca^{2+} stimulated the phosphorylation of a very high molecular weight protein (Fig. 1, lane B).

The activity of protein kinases in the soluble extracts was influenced by the concentration of Mg^{2+} in the reaction mixtures (Fig. 2). When soluble extracts that had been dialyzed against EDTA were used for *in vitro* phosphorylation reactions, the 72 and 17 kDa polypeptides were readily phosphorylated even in the absence of any exogenously added Mg^{2+}. The phosphorylation of the other proteins was dependent on the presence of Mg^{2+} (Fig. 2), with optimal incorporation of label at 5 to 10 mM Mg^{2+}.

In preliminary studies we examined the effects of several other divalent cations on protein kinase activity from nodules. Only Zn^{2+} has a significant effect on protein kinase activity. At concentrations greater than 500 μM, Zn^{2+} profoundly inhibited phosphorylation of polypeptides with apparent molecular weights of 72, 63, 50, and 41 kDa (Fig. 3). Phosphorylation of a 17 kD polypeptide was insensitive to Zn^{2+}, even at the high concentration of 5 mM.

Two dimensional PAGE of *in vitro* phosphorylation products confirmed that the above 72, 63, and 17 kDa phosphoproteins were heterogeneous (Fig. 4). The 17 kDa phosphoprotein, which appeared as a single band on a one dimensional gel, resolved into several proteins having molecular weights of 19 to 17 kDa. The high molecular weight phosphoproteins yielded similarly complex patterns in two dimensional gels. Addition of 1 mM Zn^{2+} virtually abolished the phosphorylation of the cluster of 72 and 63 kDa phosphoproteins.

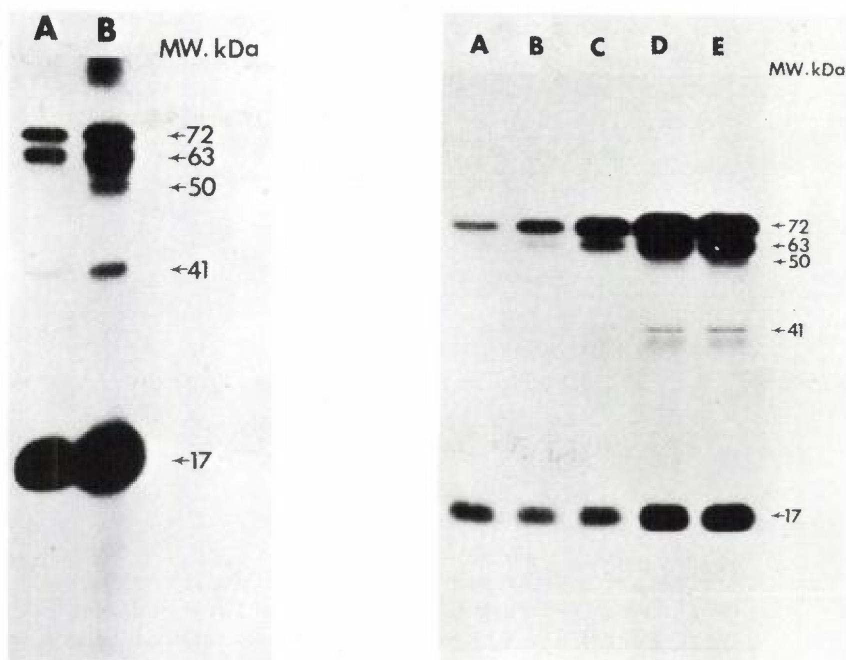

Figure 1. *In vitro* phosphorylation of soluble proteins from soybean root nodules. *In vitro* phosphorylation by endogenous protein kinases was performed in the presence of 400 μM EGTA and 5 mM $MgCl_2$. Lane A: no exogenous Ca^{2+}. Lane B: 1 mM Ca^{2+}. The phosphorylated proteins were resolved on a 15% SDS-PAGE gel and subjected to autoradiography. The exposure time was 16 hr with an intensifying screen at −80°C.

Figure 2. Effect of increasing Mg^{2+} concentrations on *in vitro* phosphorylation of soluble proteins from nodules. Soluble proteins were dialyzed against a buffer that contained 400 μM EDTA, and the reaction was performed at 30°C in 100 μl of dialysis buffer in the presence of 1 mM Ca^{2+} for 2 min. Lane A: No Mg^{2+}. Mg^{2+} was added to a final concentration of 1 mM (lane B), 2.5 mM (lane C), 5 mM (lane D), and 10 mM (lane E).

Some protein phosphatases show Zn^{2+}-dependent stability and catalytic activity (Zubay, 1983), and we thus determined whether Zn^{2+}-dependent protein phosphatases are responsible for the apparent inhibition of protein phosphorylation by Zn^{2+}. This was done by adding either of two known phosphatase inhibitors, sodium fluoride and sodium molybdate, to reaction mixtures before the addition of Zn^{2+}. The presence of either inhibitor at 10 mM did not prevent the Zn^{2+}-mediated inhibition of phosphorylation of 72, 63, and 41 kD polypeptides (Fig. 5).

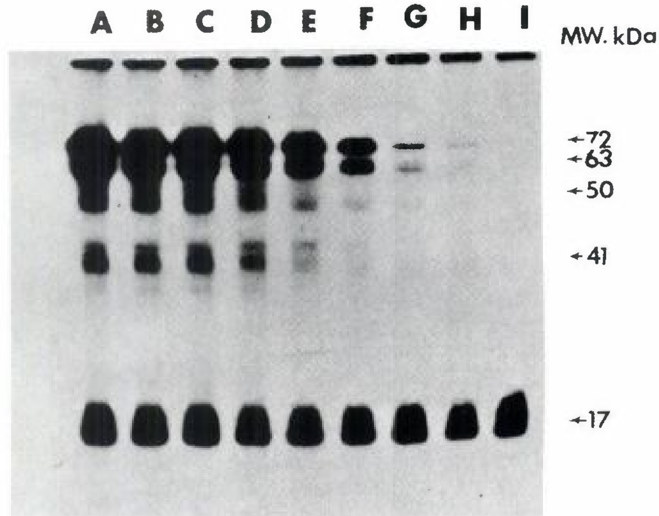

Figure 3. Inhibition of protein kinase activity by increasing concentrations of Zn^{2+}. The reactions were performed in a total volume of 100 μl of dialysis buffer that contained 400 μM EDTA, 1 mM Ca^{2+} and 5 mM $MgCl_2$. Lane A contained no Zn^{2+}. Zn^{2+} was added to the assay mixture to give a final concentration of 1 μM (lane B), 10 μM (lane C), 100 μM (lane D), 250 μM (lane E), 500 μM (lane F), 750 μM (lane G), 1 mM (lane H) and 5 mM (lane I).

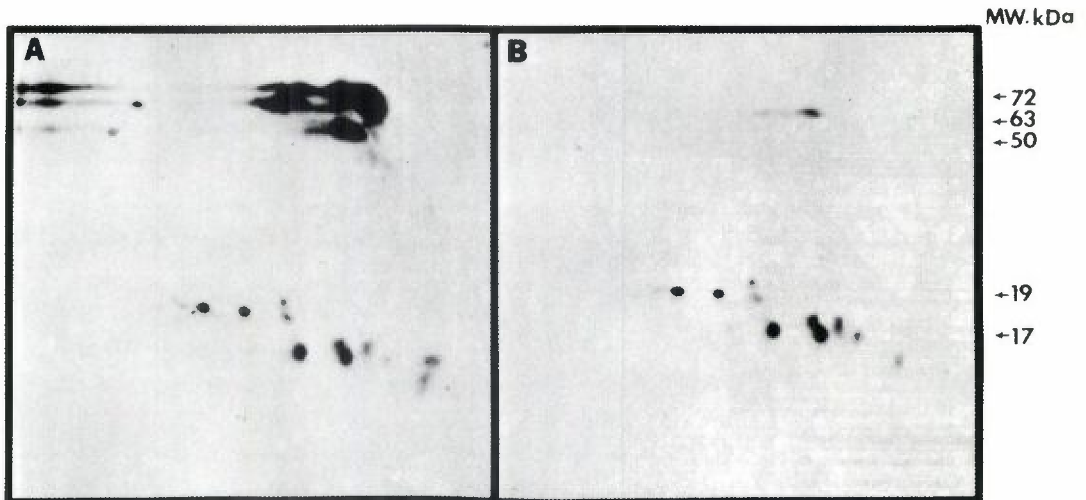

Figure 4. Two-dimensional analysis of *in vitro* protein phosphorylation products obtained in the absence (Panel A) or presence (Panel B) of Zn^{2+}. Equal amounts of proteins (100 μg) were subjected to electrophoresis and autoradiography as described in Materials and Methods. The exposure time was 36 hr with an intensifying screen at $-80°$C.

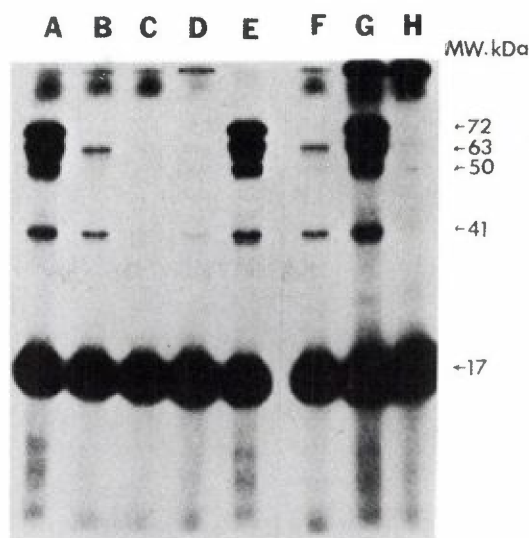

Figure 5. Effect of sodium molybdate and sodium fluoride on Zn^{2+}-mediated inhibition of protein kinase activity. *In vitro* phosphorylation was performed in the presence of 400 μM EDTA and 5 mM $MgCl_2$ as described in Materials and Methods. Lane A: 1 mM Ca^{2+}. Lane B: 1 mM Zn^{2+}. Lane C: 1 mM Ca^{2+} and 1 mM Zn^{2+}. Lane D: 1 mM Ca^{2+} + 1 mM Zn^{2+} and 10 mM $NaMoO_4$. Lane E: 1 mM Ca^{2+} + 10 mM $NaMoO_4$. Lane F: 1 mM Zn^{2+}. Lane G: 1 mM Ca^{2+} + 10 mM NaF. Lane H: 1 mM Ca^{2+} + 1 mM Zn^{2+} and 10 mM NaF.

To determine if any of the phosphoproteins from nodules were nodule-specific, we isolated soluble proteins from nodules and roots of the same age. Figure 6 shows stained soluble proteins from roots (lane A) and nodules (lane B). The most obvious difference between the two is the presence of a predominant 14 kD leghemoglobin band in the nodule extract (lane B*). *In vitro* phosphorylation of root and nodule proteins by endogenous protein kinases is shown in Fig. 6, lanes C and D. The 50 and 17 kDa phosphoproteins were present both in roots and nodules. In contrast, the 72, 63, and 41 kDa phosphoproteins were present only in nodules. Two dimensional SDS-PAGE of *in vitro* phosphorylation products from roots resolves the 17 kDa polypeptide into a cluster of proteins having the same migration pattern as that from the nodules (data not shown).

4. Discussion

Soluble protein extracts from soybean root nodules contain both Ca^{2+}-dependent and Ca^{2+}-independent protein kinases. Such protein kinases have been reported from several sources (Hepler and Wayne, 1985), and some are

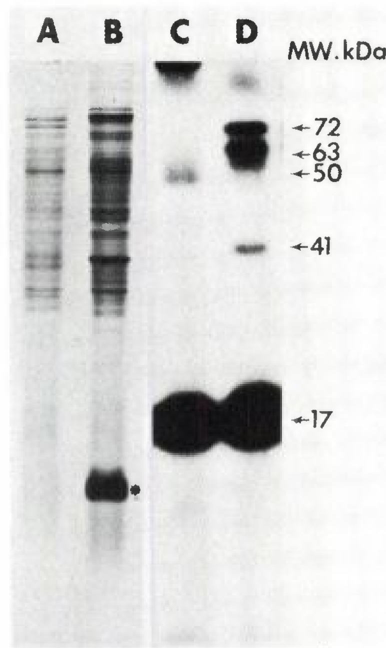

Figure 6. Comparison of *in vitro* protein phosphorylation in root and nodule extracts. Lanes A and B are soluble proteins from roots (50 μg) and nodules (100 μg), respectively, stained with Coomassie Blue. Lanes C and D are phosphoproteins from root and nodule extracts, respectively. The numbers on the right refer to the molecular weights of the phosphoproteins.

stimulated by the calcium binding protein, CaM (Cheung, 1980). In preliminary experiments, we could not detect an effect of exogenous CaM on phosphorylation in nodule extracts. Bassarab and Werner (1987) similarly failed to detect such effects on protein kinases from peribacteroid membranes, suggesting that CaM may not be important in regulating protein phosphorylation in nodules.

Like most other protein kinases from plants (Davies and Polya, 1983; Krishnan and Pueppke, 1988; Polya and Davies, 1983; Polya and Micucci, 1984; Veluthambi and Poovaiah, 1984), protein kinases from soybean nodules require millimolar concentrations of Mg^{2+} for maximal activity. The 72 and 17 kDa polypeptides nevertheless are phosphorylated in the absence of Mg^{2+}. This reaction is not without precedent, because a kinase that phosphorylates a cytokinin binding protein from wheat germ also exhibits about 25% of its maximal activity in the absence of Mg^{2+} (Polya and Davies, 1983).

Some protein kinases from germinating wheat endosperm appear to be similar (Krishnan and Pueppke, 1988). This indicates that the phosphorylation of some nodule proteins may be Mg^{2+}-independent and regulated in a unique way.

The inhibition of protein kinases from soybean root nudules by Zn^{2+} is intriguing. The inhibition appears not to be mediated by Zn^{2+}-activated protein phosphatases, because both sodium molybdate and sodium fluoride, known inhibitors of phosphatases, fail to prevent the inhibition. The inhibitory effect is detected with 250 μM Zn^{2+} in the assay mixture. Because the equilibrium constants of Zn^{2+}-EDTA can be affected by unknown components in the nodule extracts, we have not attempted to calculate the amounts of free Zn^{2+} in our reaction mixtures. Nevertheless, the reactions were carried out in the presence of 400 μM EDTA, so it is likely that Zn^{2+} inhibits protein kinase activity at concentrations much lower than 250 μM. Phosphorylation of the 17 kDa polypeptides is not influenced by Zn^{2+} even at a concentration of 5 mM. This suggests the presence of at least two types of protein kinases in nodules, one influenced by Zn^{2+} and the other insensitive to Zn^{2+}.

Some divalent cations are known to inhibit protein kinase activity (Davies and Polya, 1983; Polya and Davies, 1983; Polya and Micucci, 1984). A purified kinase for a cytokinin binding protein from wheat germ is completely inhibited by 1 mM zinc acetate (Polya and Davies, 1983). Similarly, a casein--phosphorylating protein kinase from wheat germ is almost totally inhibited by the addition of 1 mM Zn^{2+} to the phosphorylation assay mixture (Davis and Polya, 1983). Protein kinase C, an enzyme which is activated by calcium and phospholipid (Elliott and Kokke, 1987), also is regulated by cooperative interaction of Zn^{2+} and Ca^{2+} (Murakami et al., 1987). Purified protein kinase C from rat brain is inhibited by Zn^{2+}, but in the presence of 5 μM Ca^{2+}, Zn^{2+} actually stimulates the protein kinase activity. In contrast, when the Ca^{2+} concentration is greater than 50 μM, Zn^{2+} greatly inhibits the kinase activity (Murakami et al., 1987). We also have observed that the inhibitory effect of Zn^{2+} is enhanced in the presence of 1 mM Ca^{2+}, but we have not examined the effect of phosopholipids on the soluble protein kinase activity from soybean nodules. A protein kinase C-like enzyme, which is dependent on calcium and phospholipids, has been detected in peribacteroid membranes from soybean nodules (Bassarab and Werner, 1987). Protein kinase C can be translocated from cytosol to membranes in response to external signals (Nishizuka, 1986), and thus it would be interesting to know whether the kinase described in our study is the same as that detected in peribacteroid membranes.

In vitro phosphorylation of the 72, 63, and 41 kDa polypeptides in nodules

but not in root extracts implies that these phosphoproteins may be "nodule specific". Verma et al. (1986) have identified at least 18 to 20 polypeptides, in addition to leghemoglobin, which are synthesized nodule-specifically in soybean. However, a comparison of the two-dimensional gel electrophoretic migration of soybean nodulins (Gloudemans et al., 1987) and nodule-specific phosphoproteins fails to reveal any obvious similarities. This suggests that the proteins themselves are not synthesized specifically in nodules, but rather are present both in roots and nodules. Alternatively, these proteins may actually be nodule-specific, but perhaps because of their low abundance, they were not detected in earlier studies.

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