Regulation of Benzyladenine-Induced in Vitro Shoot Organogenesis and Endogenous Proline in Melon (*Cucumis melo* L.) by Exogenous Proline, Ornithine, and Proline Analogues

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Melon (*Cucumis melo* L.) is an excellent system to study the role of proline in regulating benzyladenine-induced shoot organogenesis, which is also an important system in our laboratory for investigating flavor and antioxidant metabolite synthesis in differentiated tissues. We have hypothesized that proline stimulated in vitro shoot organogenesis through the activation of the proposed proline-linked pentose phosphate pathway, which may regulate endogenous cytokinin and auxin biosynthesis. To further investigate this hypothesis, it is essential to first confirm whether benzyladenine-induced shoot organogenesis can be stimulated by enhancing proline synthesis through the use of proline analogues and ornithine (proline precursor). In this study, benzyladenine-induced shoot organogenesis was substantially stimulated by exogenous proline and/or ornithine in combination with proline analogues such as azetidine-2-carboxylate (A2C) and hydroxyproline (HP). Among these treatments, proline in combination with A2C or HP showed the highest stimulation. The stimulation of organogenesis correlated closely with increased levels of endogenous proline content, thereby strengthening the hypothesis that proline-linked activation of purine and aromatic metabolism via the stimulation of pentose phosphate pathway may be important for in vitro organogenesis in melon.

Keywords: *Azetidine-2-carboxylate; benzyladenine; Cucumis melo; hydroxyproline; ornithine; pentose phosphate pathway; proline; purine synthesis; shoot organogenesis; thioproline*

INTRODUCTION

Melon (*Cucumis melo* L.) is being targeted by our laboratory for the expression of novel proteins important to the food and pharmaceutical industries (Shetty et al., 1997). Additionally, we are also exploring the role of proline in the regulation of benzyladenine-induced in vitro organogenesis in melon (Shetty et al., 1992), which is relevant for understanding flavor and antioxidant metabolite synthesis in differentiated tissues. It has been established previously that the addition of exogenous proline had a stimulatory effect on benzyladenine-induced shoot organogenesis (Shetty et al., 1992). This work was based on previous studies showing proline-mediated enhancement of in vitro differentiation in diverse tissue culture systems (Armstrong and Green, 1985; Nuti-Ronchi et al., 1984; Shetty and Asano, 199l).

In the research reported here, we have further investigated the effects of exogenous proline, ornithine (a proline precursor), and proline analogues on benzyladenine-induced shoot organogenesis in melon and examined the correlation between organogenesis and endogenous levels of proline. The model for investigating the effect of proline on in vitro differentiation is based on the proposed role of the proline-linked pentose phosphate pathway in stimulating the synthesis of intermediary metabolites involved in purine and aromatic metabolism (Figure 1). This model is based on the proposed role of the proline-linked pentose phosphate pathway in driving ribose-5-phosphate toward purine synthesis in cancer cells (Phang et al., 1982; Phang, 1985) and possibly in plant tissues (Kohl et al., 1988). In addition to making $NADPH_2$ available for all anabolic reactions, the interconversion of ribose-5-phosphate to phosphoribosyl pyrophosphate (PRPP) and to erythrose-4-phosphate may be critical for the synthesis of natural cytokinins and auxins (via the shikimate pathway), respectively. These metabolic intermediaries have to be derived from the pentose phosphate pathway (Schnarrenberger et al., 1995). The stimulation of the proline-linked redox cycle may also provide an alternative oxidative phosphorylation step using proline as a reductant instead of NADH (Phang, 1985; Rayapati and Stewart, 199l).

If the proline-linked pentose phosphate pathway in regulating cytokinins and auxins is to be investigated, then enhancement of shoot organogenesis coupled to concurrent enhancement of endogenous proline levels in response to exogenous proline and proline precursors in combination with proline analogues should be confirmed. Results reported here confirm this positive correlation between shoot organogenesis and endogenous proline levels.

MATERIALS AND METHODS

Plant Material. Mature seeds of melon (*C. melo* L. var. Hales Best, NK Lawn and Garden Co., MN) were germinated for 48−72 h under 80 μmol m^{-2} s^{-1} at 25 °C on MS medium with 1 mg/L benzyladenine (BA) and 3% (w/v) sucrose (pH 5.8)

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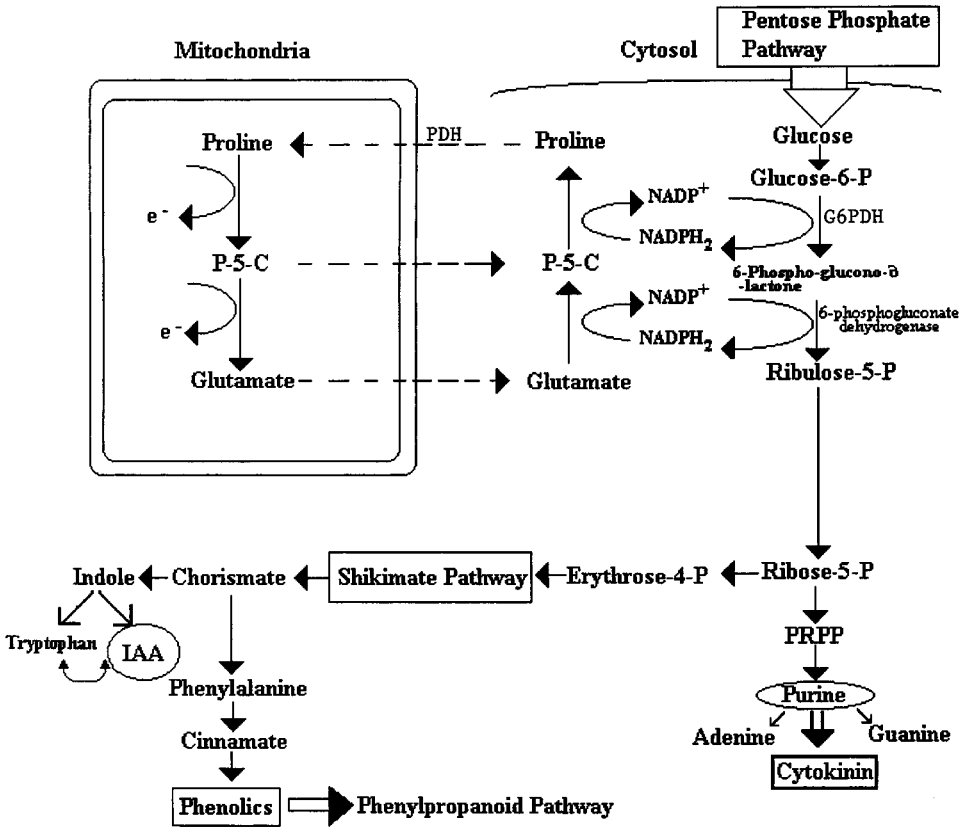

Figure 1. Proposed proline-linked pentose phosphate pathway and role in regulation of benzyladenine-induced organogenesis in melon. (Abbreviations: P5C, pyrroline-5-carboxylate; G6PDH, glucose-6-phosphate dehydrogenase; PDH, proline dehydrogenase; PRPP, phosphoribosyl pyrophosphate; IAA, indole acetic acid.)

after removal of seed coat and disinfection. Disinfection was done by treating the seeds for 45 s in 70% (v/v) ethanol, followed by 25 min in 2% (v/v) sodium hypochlorite and then rinsing by three serial washes in sterile distilled water to remove traces of the disinfectant. Following germination and cotyledon expansion for 48−72 h, sliced cotyledon explants were used to initiate shoot organogenesis (Shetty et al., 1992).

Cotyledon Explants and Initiation of Shoot Organogenesis (Shetty et al., 1992). Expanded cotyledons were cut into 2 mm × 2 mm pieces after the waxy cuticle layer was removed (Dirks and Van Buggenum, 1989). Twelve explants were obtained from each cotyledon (i.e., six explants from each cotyledon half). Individual explants were randomly placed on various shoot organogenesis solid media described below. Every treatment in this study had 36 explants for each of the individual replicates (i.e., 9 explants/plate, 4 plates per individual treatment/replicate). Samples were grown for 30−33 days at 25 °C and 40 μmol m^{-2} s^{-1} (18 h light/6 h dark cycle). Experiments were repeated at least eight times, and results averaged.

Shoot Organogenesis Medium. All in vitro organogenesis media were based on MS medium with Nitsch and Nitsch vitamins (Nitsch and Nitsch, 1955; Shetty et al., 1995) (pH 5.8) containing 1 mg/L benzyladenine (BA) and 3% sucrose. Phytagel (Gellan gum; Sigma Chemical, St. Louis, MO) at 3 g/L was used as the solidifying agent. All treatment concentrations were added to the above medium, and the mixture was sterilized by standard autoclaving: Concentrations of various supplements (Sigma) were as follows: proline (5 mM), azetidine-2-carboxylate (A2C) (0.05 mM), thioproline (TP) (0.5 mM), hydroxyproline (HP) (0.5 mM), and ornithine (Orn) (5 mM). Treatment combinations are shown in Figure 2.

Proline Determination (Bates et al., 1973). Between 50 and 99 mg of plant material (containing both callus and developing tissue) was blotted lightly on tissue, weighed, and placed in 2 mL of 3% (w/v) sulfosalicylic acid solution and homogenized. A total of 1.5 mL of the macerated sample was centrifuged (10 min, 13000*g* in a microcentrifuge). One milliliter of supernatant was extracted and placed into a reaction tube to which 1.0 mL of glacial acetic acid and 1.0 mL of 2.5% (w/v) acid ninhydrin were added. Samples were heated in a 100 °C water bath for 1 h and then cooled for 15 min in an ice bath. A total of 2.0 mL of toluene was added and then vortexed for 20 s. The upper chromophore phase was measured at 520 nm using toluene as a blank. Standards were treated in the same manner as the samples and prepared simultaneously. Three samples per treatment were assayed per individual experiment. Proline is reported as micromoles per gram of fresh weight tissue (Bates et al., 1973). Experiments were repeated eight times with similar trends, and the averages of eight experiments are reported here. Proline content was measured on day 30 following benzyladenine and various supplement treatments.

Organogenesis. Photographs of the individual treatment plates were taken using a stereomicroscope (Olympus SZ40, Tokyo, Japan) to show the extent of callus and/or multiple shoot formation after 30 days of growth under the conditions above, and a magnification of 35× was used (Figure 3).

RESULTS AND DISCUSSION

Endogenous levels of proline were increased the most in cotyledons grown on MS-benzyladenine medium containing a combination of proline plus A2C. Endogenous levels of proline in these tissues were almost 4 times that of the untreated control (Figure 2). Similar high levels of proline were obtained in tissues grown on MS-benzyladenine medium containing proline plus hydroxyproline (Figure 2). Treatment with ornithine resulted in significantly elevated levels of endogenous proline (above that of control) only when given in combination with hydroxyproline but not with A2C and thioproline (Figure 2). Treatment with thioproline alone

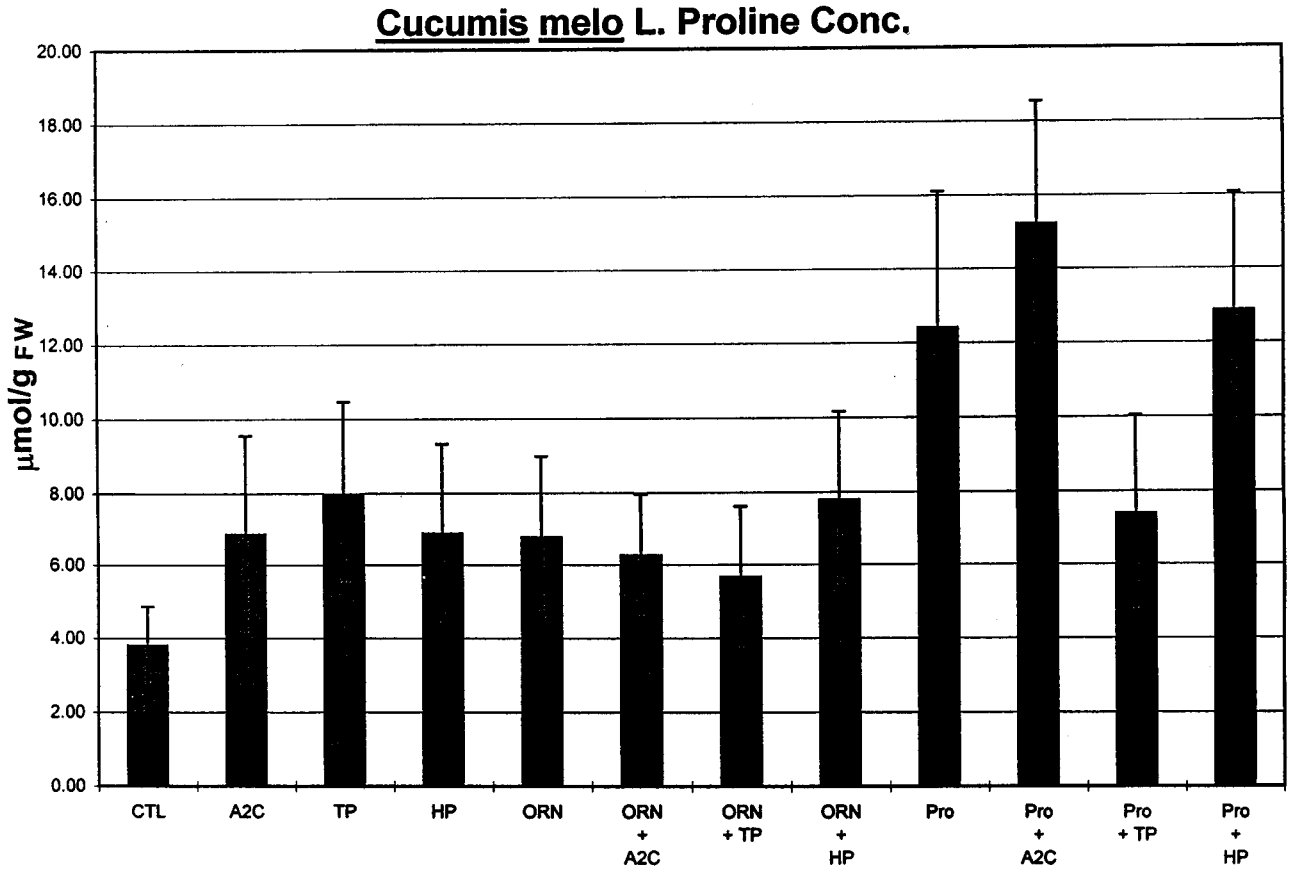

Figure 2. Proline content of multiple-shoot-forming cut cotyledons of melon following 30 days of treatment with benzyladenine and supplements.

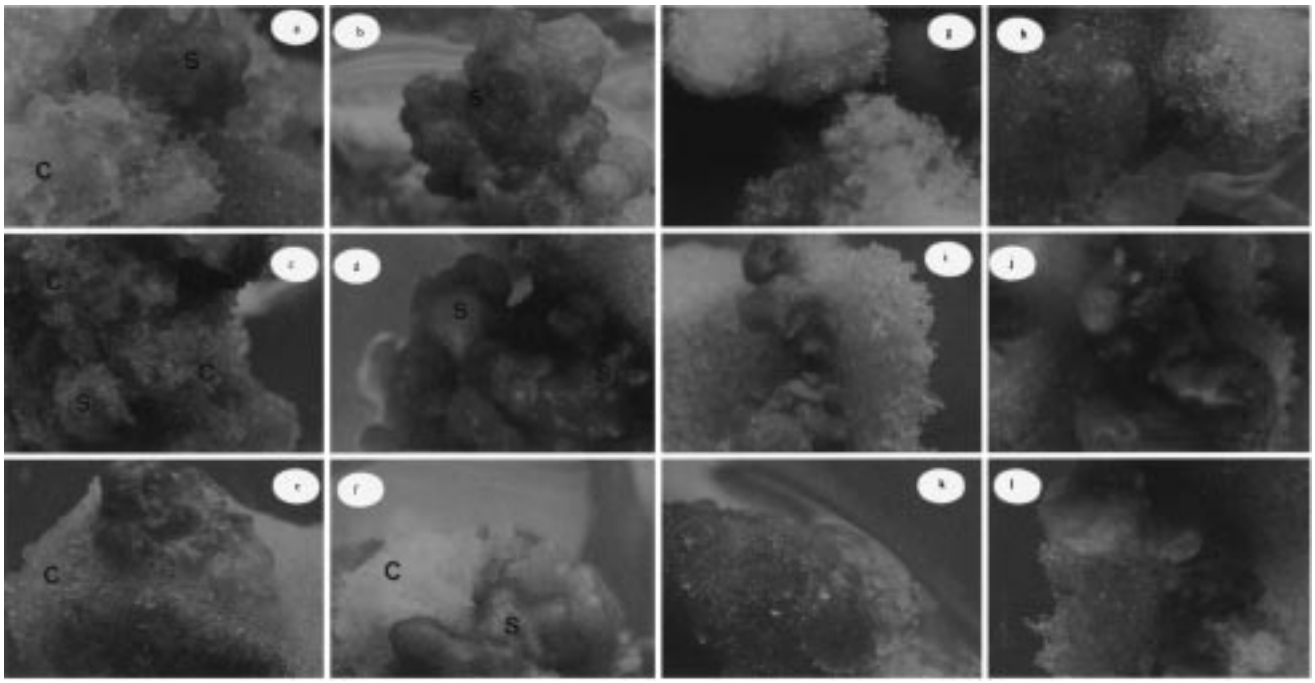

Figure 3. Multiple shoots induced on cut cotyledons after 30 days of benzyladenine treatment with the following treatments: (a) control, (b) proline, (c) A2C, (d) proline + A2C, (e) hydroxyproline, (f) proline + hydroxyproline, (g) thioproline, (h) proline + thioproline, (i) ornithine, (j) ornithine + A2C, (k) ornithine + thioproline, (l) ornithine + hydroxyproline (magnification of 35×). (The figure is reproduced here at 49% of the original size.) (S, shoot; C, callus.)

also resulted in significantly elevated levels of endogenous proline relative to the control (Figure 2). Treatments with the proline analogues A2C or hydroxyproline alone did not result in significant stimulation of endogenous proline.

Poor multiple shoot formation with excess callus tissue was observed when the cotyledon explants were grown on MS medium containing standard inorganic sources of nitrogen with 1 mg/L benzyladenine (control) (Figure 3). Enhancement of benzyladenine-induced in

vitro shoot organogenesis directly from cut cotyledons was seen with treatments of proline, proline plus A2C, and proline plus hydroxyproline (Figure 3), and marginal stimulation was observed with ornithine, ornithine plus A2C, and ornithine plus hydroxyproline. In these cases the enhanced shoot organogenesis correlated directly with increased levels of endogenous proline (Figure 2). A2C alone stimulated multiple shoots with sparsely scattered callus formation (Figure 3). However, A2C-induced stimulation of multiple shoots was not associated with a significant stimulation of corresponding endogenous proline.

The effects of proline and A2C are most interesting. A2C is suggested to be a competitive inhibitor of proline dehydrogenase (Elthon and Stewart, 1984), an inner mitochondrial bound enzyme (Rayapati and Stewart, 1991). A2C is also known to inhibit differentiation of Leydig cells of rat fetal testis (Jost et al., 1988), and their effect can be reversed by treatments with exogenous proline. It is possible that in melon in the presence of A2C, proline synthesis was required to outcompete A2C, which may result in the immediate oxidation of proline to pyroline-5-carboxylate and glutamate and therefore result in low proline accumulation (Figure 2). These proline oxidation products could then be recycled for proline synthesis, thereby continuously stimulating the pentose phosphate pathway and generating $NADPH_2$ and ribose-5-phosphate for purine synthesis (Phang, 1985). In the presence of proline plus A2C, this redox cycle may operate more efficiently, because A2C most likely deregulated proline synthesis, and at the same time there were also high levels of exogenous proline for initiating PDH-based oxidation and maintenance of proline-linked redox cycle that should continuously stimulate the pentose phosphate pathway. In the presence of proline alone, PDH-mediated oxidation may continuously maintain the proline-linked redox cycle required for differentiation without additional synthesis as observed with proline plus A2C.

Hydroxyproline is normally modified from proline during post-translational modification of certain plant cell wall proteins (Varner and Lin, 1989). Exogenous hydroxyproline may compete with proline for incorporation into protein and cause aberrant proteins at the translation level. In response to exogenous hydroxyproline (0.5 mM), stimulation of multiple shoots was not associated with the corresponding endogenous proline level (Figures 2 and 3). It was obvious that free hydroxyproline was inhibitory for shoot organogenesis. Addition of exogenous proline with hydroxyproline reversed the inhibition, and multiple shoot formation was highly stimulated with correspondingly high levels of proline. Likewise, ornithine, a precursor of proline in plants (Rena and Splittostoesser, 1975; Delauney et al., 1993), in combination with hydroxyproline stimulated multiple shoot formation with corresponding stimulation of endogenous proline. In both of the latter cases, proline synthesis in the presence of hydroxyproline continued to occur, and this may have continuously stimulated the proline-linked pentose phosphate pathway and the subsequent critical pathways related to cytokinin biosynthesis (purine synthesis). This stimulation therefore enhanced multiple shoot formation.

Thioproline supplementation resulted in inhibition of multiple shoot formation, even though endogenous proline levels were significantly higher compared to the control (Figures 2 and 3). Thioproline in combination with proline or ornithine did not stimulate multiple shoot formation (Shetty et al., 1992; Figure 3) or endogenous proline levels (Figure 2). Thioproline is known to be a substrate for proline dehydrogenase in *Escherichia coli*, where it is oxidized to *N*-formylcysteine (Deutch, 1992). Thioproline is a simple competitive inhibitor of proline dehydrogenase (therefore increased proline synthesis), but under certain conditions it is oxidized (Deutch, 1992). The presence of proline can inhibit thioproline oxidation (Deutch, 1992) when proline itself is oxidized. The inhibition of benzyladenine-induced multiple shoot formation in melon by thioproline in the presence of proline and the proline precursor (ornithine) may reflect this dual property (competitive inhibition vs oxidation of thioproline). This may offset the proline-linked redox cycle and the subsequent stimulation of pentose phosphate pathway that is required for purine biosynthesis.

In conclusion, the stimulation of multiple shoot formation in melon is clearly associated with enhanced proline synthesis, which is likely the result of the conversion of precursors such as ornithine or glutamate to proline, which is further enhanced in the presence of proline analogues such as A2C and hydroxyproline (Figure 2). It has been suggested that during nodule formation and nitrogen fixation in soybean, proline synthesis may utilize $NADPH_2$ from the pentose phosphate pathway. The NADP is then recycled to stimulate the pentose phosphate pathway, regenerating $NADPH_2$ and phosphorylated intermediates such as ribose-5-phosphate for purine synthesis (Kohl et al., 1988; Figure l). $NADPH_2$ and ribose-5-phosphate biosynthesis through the proline-linked pentose phosphate pathway is closely tied to stimulation of purine synthesis in many animal models (Phang, 1985). Our study suggests that cytokinin (benzyladenine)-induced shoot organogenesis may be stimulated by proline synthesis, and this may be further enhanced through proline oxidation, with glutamate again being recycled for proline synthesis. The $NADPH_2$-requiring steps during proline synthesis may be coupled to the pentose phosphate pathway as in soybean nodules and animal models (Kohl et al., 1988; Phang, 1985; Figure 1). The redox cycle generated by the above proline-linked pentose phosphate pathway (Figure 1) could then stimulate purine metabolism, which may then further enhance natural endogenous cytokinins and result in stimulating multiple shoot formation (organogenesis). Regulation of ribose-5-phosphate levels may be critical in determining how much is diverted to PRPP or erythrose-4-phosphate for purine and aromatic biosynthesis, respectively. This regulation of purine and aromatic biosynthesis may regulate the balance of natural cytokinins and auxin required for multiple shoot formation. Another interesting hypothesis emerging from this study is that the stimulation of the proline-linked redox cycle may also provide an alternative oxidative phosphorylation step using proline as a reductant instead of NADH (Phang, 1985; Rayapati and Stewart, 199l) during in vitro differentiation. On the basis of this study it is now feasible to study the enzymes and corresponding genes (e.g., proline dehydrogenase) associated with the proline-linked pentose phosphate pathway and how these may regulate cytokinin and auxin biosynthesis. This will shed more light on the model proposed for the role of proline in regulating plant in vitro differentiation.

Differentiated tissues are preferred targets compared to the callus system for production of phytochemicals in some in vitro systems (Hilton et al., 1995; Fulzele et al., 1995), and therefore in such systems differentiation could be improved by regulating proline metabolism.

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