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The Effect of DTT in Protein Preparations for Proteomic Analysis: Removal of a Highly Abundant Plant Enzyme, Ribulose Bisphosphate Carboxylase/Oxygenase

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Rubisco is a major photosynthetic plant enzyme in the chloroplasts, catalyzing a photosynthetic reaction through carboxylation and oxygenation in the leaves. Despite its biological importance, its high abundance causes difficulties in the proper separation of protein mixtures during 2-dimensional gel electrophoresis (2-DE). Here, we resolved those plant soluble proteins by efficiently removing Rubisco. This resulted in a high quality and resolution of 2-DE gels. Rubisco removal was achieved through aggregation in the presence of a high DTT concentration, which subsequently increased the visualization of less abundant proteins and reduced horizontal streaking. This simple method may provide a means for finding more biologically important protein targets via plant proteomics.

Keywords: 2-DE, abundant proteins, plant proteomics, protein solubilization, Rubisco

Proteomics is a powerful technique for the global analysis of gene expression at the protein level from a whole organism, tissue, or cell (Cortón et al., 2004). Due to recent technical improvements in 2-DE and the increased accuracy of MS, it is possible to characterize various complex protein samples (Cánovas et al., 2004). However, for several reasons, proteomics has its own difficulties in resolving all proteins from the sample of interest on a 2-DE gel (Quadroni and James, 1999; Cho et al., 2006). The main limitation is the loading capacity to IEF (isoelectric focusing). The increased amounts of total proteins often cause a decline in the resolution of gel images (Rothemund et al., 2003). To overcome this, numerous efforts have been made toward devising more efficient reagents and improved equipment: e.g., longer IPG strip gels and suitable holders (from 7 to 24 cm), narrow-range IPG strip gels (Hoving et al., 2002), and the development of an enhanced IEF system (Craven et al., 2002).

Nevertheless, resolution is still challenging because of the obstacle associated with highly abundant proteins (Craven et al., 2002), which are problematic irrespective of their biological functions in performing 2-D electrophoresis. These abundant proteins limit the entrance of low-abundance proteins on IPG strip gels; the large occupancy of the former then occludes the appearance by the latter on gels (Rothemund et al., 2003; Shaw and Riederer, 2003). Furthermore, highly abundant proteins are not suitable for providing reproducible 2-DE gel images because their greater abundance often blocks the migration of other proteins on IPG strip gels during IEF, resulting in horizontal streaking.

In animal cells, albumin and immunoglobulin are two highly abundant proteins that mask potential low-abundance biomarkers. Greenough et al. (2004) have shown that methods such as alcohol precipitation, ultracentrifugation, salting in/out, and affinity chromatography can be efficient for reducing them. In plants, ribulose bisphosphate carboxylase/oxygenase (Rubisco) is the most highly abundant protein, and is strongly expressed during photosynthesis. Kim et al. (2001) have suggested a fractionation method that utilizes polyethylene glycol (PEG) to remove Rubisco, so that more protein detection is possible. Here, we present a simple and effective protein preparation method that involves DTT and 2-DE gels to reduce highly expressed Rubisco.

MATERIALS AND METHODS

Plant Materials and Protein Extraction

Rice (*Oryza sativa* L. 'Dongjin') seedlings were grown for 10 d at 27°C under a 12-h photoperiod provided by fluorescent white lamps (300 μmoL m^{-2} s^{-1}). Rice samples were snap-frozen in liquid nitrogen and ground very finely with a mortar and pestle. This powder was added to two volumes of crude extraction buffer [20 mM HEPES/KOH (pH 7.5), 40 mM KCl, 1 mM EDTA, 1 mM PMSF, 10% glycerol, and various concentrations of DTT] and mixed vigorously. The homogenate was then centrifuged at 20,000 g for 30 min at 4°C and the supernatant was centrifuged five times. Afterward, it was dialyzed for 6 h at 4°C in buffer containing 20 mM HEPES/KOH (pH 7.5), 40 mM KCl, 1 mM EDTA, 1 mM PMSF, 5 mM 2-mercaptoethanol, and 10% glycerol). Following further centrifugation at 20,000 g for 30 min at 4°C, protein samples were frozen in liquid nitrogen and stored at –80°C. The concentrations of all extracts were determined with a BCA protein assay kit (PIERCE, Rockford, IL, USA).

Two-Dimensional Gel Electrophoresis (2-DE)

For 2-DE, 150 μg of each protein sample was precipitated for 20 min at –20°C with four volumes of 100% acetone

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containing 1% DTT. After centrifugation at 20,000 g for 20 min at 4°C, the precipitate was air-dried and solubilized in 20 mL of lysis buffer (9.8 M urea, 4% CHAPS, 40 mM Tris, and 1% DTT). To confirm protein denaturation and solubilization, the sample solution was held at room temperature for 30 min and mixed with 200 mL of rehydration buffer [9.8 M urea, 2% CHAPS, 0.5% IPG buffer, pH 4-7 or pH 3-10 NL (non-linear; GE Healthcare, Milan, Italy), 1% DTT, and a trace of bromophenol blue]. Isoelectric focusing (IEF) was followed with immobilized 18-cm strip gels (pH 4-7 or pH 3-10 NL) at 22°C using IPGphor (GE Healthcare). For the best separation of protein complexes, the IEF procedure was performed according to the gradual steps of rehydration at 50 V for 10 h; pre-separation at 200 V for 1 h, 500 V for 1 h, 1000 V for 1 h; gradient-focusing from 1000 V to 8000 V for 30 min; then steady-state focusing at 8000 V for 8 h. Afterward, the strip gels were equilibrated as described by Phee et al. (2004), applied to 1.5-mm-thick 12% acrylamide gels, and sealed with 0.25% agarose. SDS-PAGE was carried out overnight at 15 mA, with a PROTEAN II xl 2-D Cell (Bio-Rad, Hercules, CA, USA).

Silver-Staining and Image Analysis

Each set of 2-DE gels was silver-stained simultaneously in the same tray. Proteins were fixed for 2 h in a solution of 50% ethanol and 20% acetic acid; this fixation was repeated twice. After three washes in 50% ethanol for 1 h, the gels were sensitized with 0.02% sodium thiosulfate for 2 min and washed with deionized water for 3 min. The sensitized gels were then stained with silver-nitrate solution (0.25% silver nitrate and 0.05% formaldehyde) for 30 min, followed by washing with deionized water for an additional 20 s. Their development was then conducted in a solution of 6% sodium carbonate, 0.012% sodium thiosulfate, and 0.04% formaldehyde until the desired 2-DE gel resolutions were obtained. These developed gels were incubated in a stopping solution (1.46% EDTA) for 30 min. After washing with deionized water for 30 min, the gels were preserved in 35% ethanol. Finally, the stained 2-DE gels were scanned with a Powerlook III flatbed scanner (UMAX).

We used the auto-processing image analysis software ProteomWeaver (Definiens, Munich, Germany) to perform spot detection, matching, normalization, and quantification. For

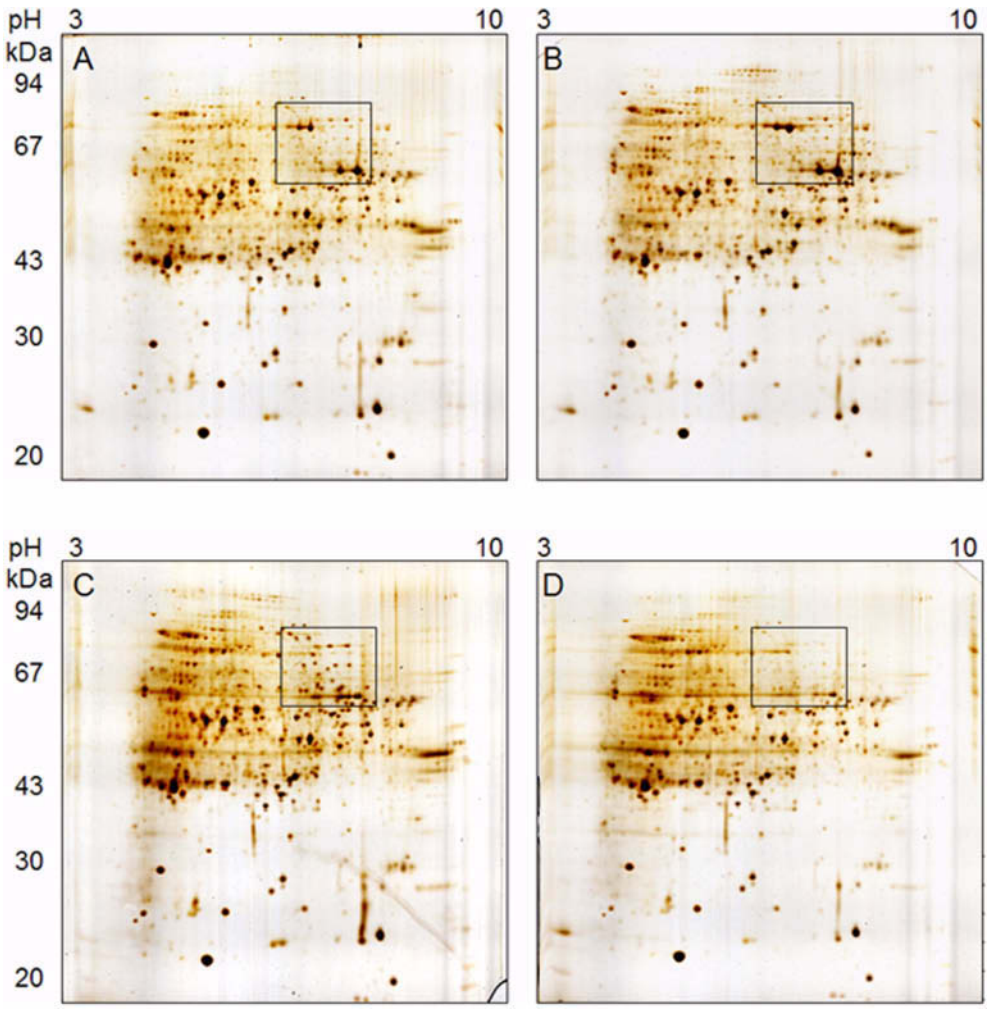

Figure 1. 2-DE gel images of 10-day-old rice seedlings indicating effect of DTT on resolution of pH 3-10 NL gels. Plant proteins were extracted with crude buffer containing 1 mM (A), 10 mM (B), 100 mM (C), or 1000 mM DTT (D). Boxed regions around Rubisco spots are shown with magnified images in Figure 2.

high reliability, we applied the same parameters, based on default settings, to each set of gels.

RESULTS AND DISCUSSION

When compared to microorganisms and animal cells, 2-DE gel electrophoresis of plants is disadvantaged in the preparation of their total proteins (Jacobs et al., 2001). Here, to make protein samples cleaner and more applicable for 2-DE, we performed a crude extraction step prior to the commonly used acetone precipitation step in our sample preparation. This resulted in 2-DE gels of high quality and resolution (data not shown). We also tested various concentrations (1 to 1000 mM) of DTT in the crude extraction buffer. However, we fixed the concentration of DDT at 65 mM in our lysis and rehydration buffers. From our 2-DE, we obtained good separations over a broad pH range of 3 to 10, from the acidic to basic regions, regardless of DTT concentration (Fig. 1). Analyses of protein patterns indicated that overall images were similar, with a minor difference in

Table 1. Number of protein spots obtained in each 2-DE gel region following soluble protein extraction with crude buffer that included 1 mM, 10 mM, 100 mM, or 1000 mM DTT.

DTT concentration	Total number of spots*		Number of changed spots** (increase/decrease)	
	pH 4-7	pH 7-10	pH 4-7	pH 7-10
1 mM	979	125	–	–
10 mM	991	127	25 / 5	7 / 3
100 mM	1020	139	59 / 4	19 / 3
1000 mM	948	115	5 / 39	5 / 17

*Number of reproducible protein spots among three replicate gels.
**Number of protein spots showing > 2-fold difference.

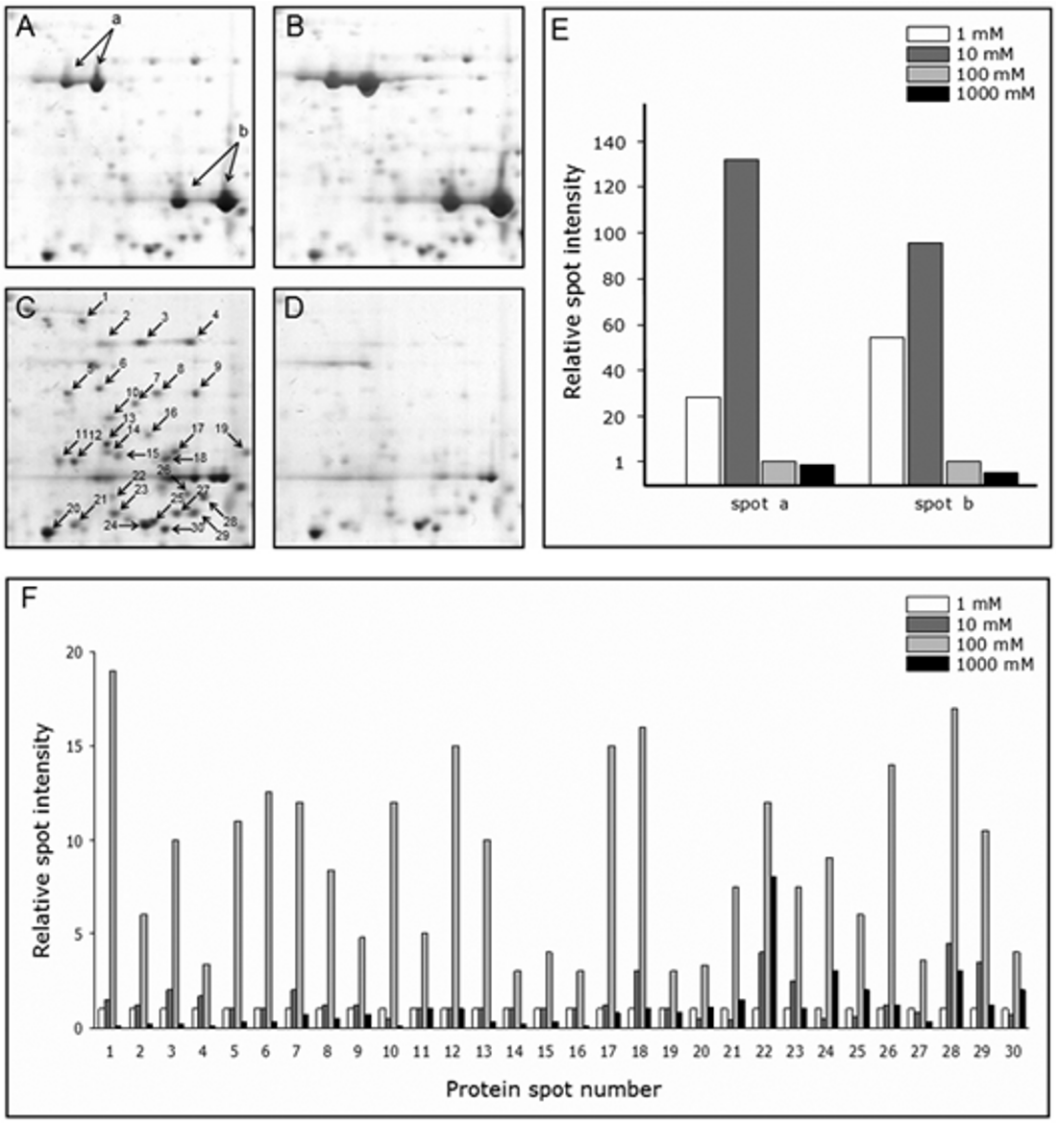

Figure 2. 2-DE gel images around Rubisco spots (spot a and b) at DTT concentrations of 1 mM (A), 10 mM (B), 100 mM (C), or 1000 mM (D) in crude extraction buffer. Protein spots with > 2-fold enhanced intensities via 100 mM DTT are indicated with arrows (C). Intensity of Rubisco spots was quantified with ProteomWeaver software (E). Relative intensity of indicated spots (Nos. 1-30), along with increased DTT concentrations, was quantified with image analysis software (F).

the number of protein spots. Although the total number of those spots was marginally increased, the spot intensity of approximately 80 proteins was remarkably elevated when 100 mM DTT was used in the crude extraction step (Table 1). As shown in Figure 2, the most dramatic change was observed in the abundance of the highly saturated photosynthetic enzyme Rubisco in conjunction with different DTT concentrations in the soluble protein extraction. For example, when DTT concentration reached 100 mM, no saturated protein spot was detected above 50 kDa (Fig. 2C, D). However, increasing the level of DTT up to 1000 mM was no more efficient in removing the Rubisco protein or improving the resolution of our 2-D gels. In fact, DTT concentrations >1000 mM resulted in more opaque protein spots as well as decreased resolution (data not shown).

Using ProteomWeaver, we quantified two Rubisco spots (Fig. 2E). Compared with 10 mM DTT, at which their intensities were highest, those spots were remarkably reduced in

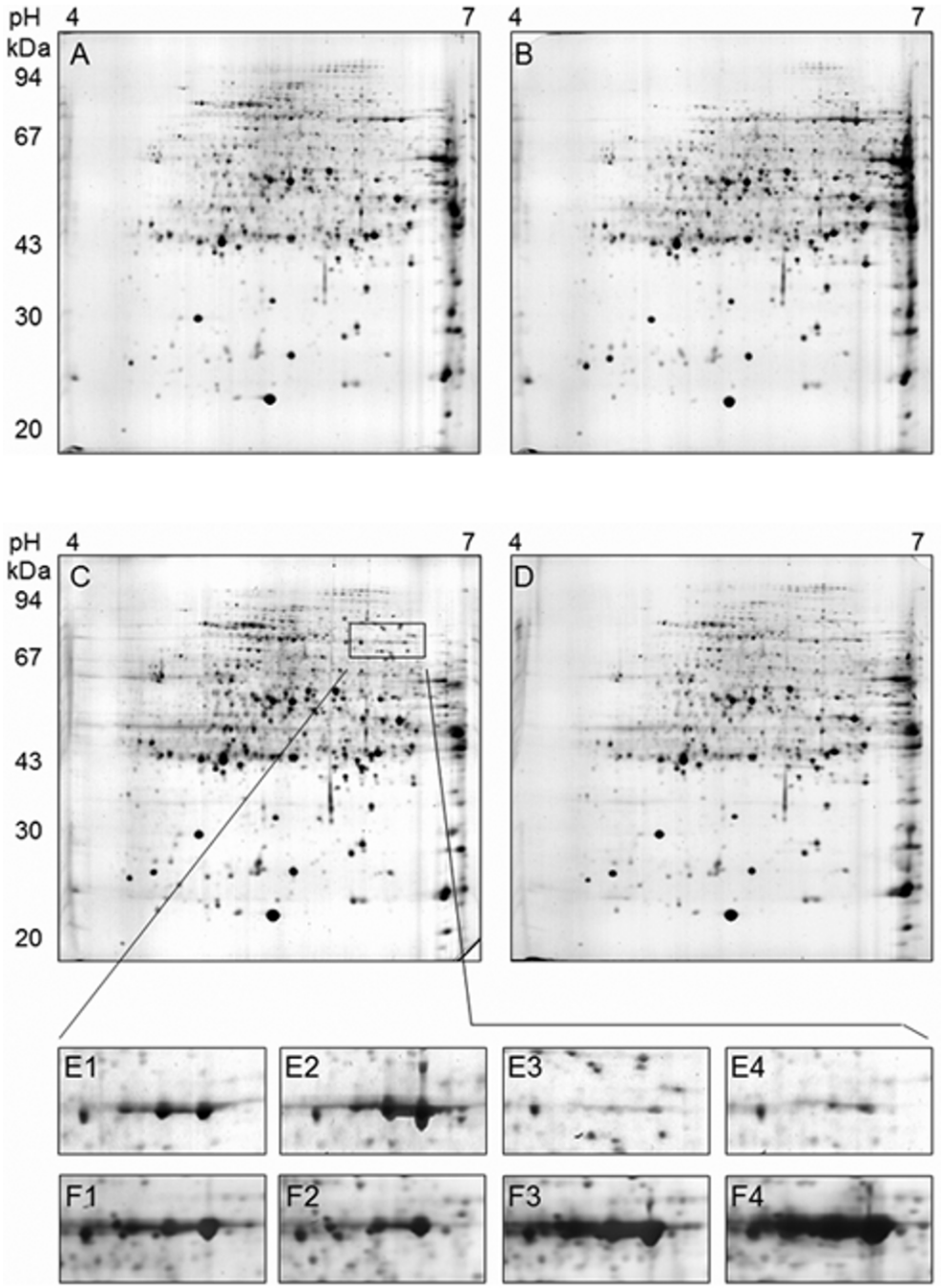

Figure 3. 2-DE gel images of 10-day-old rice seedlings indicating effect of DTT on resolution of pH 4-7 gels. Plant proteins were extracted with crude buffer containing 1 mM (**A**), 10 mM (**B**), 100 mM (**C**), or 1000 mM DTT (**D**). Changes in abundance of Rubisco spots within boxed region are clearly seen in magnified images (**E1-E4**). Rubisco spots extracted from insoluble materials corresponding to upper panels are also shown (**F1-F4**).

intensity when 100 mM DTT was added in the crude extraction step. Therefore, we concluded that this level is optimal for improving 2-DE gel resolution, especially around the Rubisco spots. Most protein spots in that region showed greater intensity along with a remarkable decline in saturated spots. For example, the intensities of 30 protein spots appeared to be increased by more than 2-fold (Fig. 2C, F). Of these, 12 (including Spots 7 and 12) showed a >10-fold enhancement. Moreover, the appearance of low-abundance protein spots, e.g., Nos. 1, 5, and 6, were clearly heightened in response to 100 mM DTT.

To confirm whether this effect of DTT also could be detected in the crowded region, these analyses were extended to the acidic region by using pH 4 to 7 IPG strip gels (Fig. 3). There, the highly abundant Rubisco spots were diminished with 100 mM DTT (Fig. 3E). As expected, the surrounding protein spots became more clearly apparent concurrent with this decreased intensity of Rubisco spots (Fig. 3E). Moreover, 2-DE gel resolution was improved, and had less of the horizontal streaking that had possibly been caused by Rubisco. Thus, we conclude that plant protein mixtures with highly abundant Rubisco can be successfully resolved on a 2-DE gel if one uses an optimized extraction buffer supplemented with 100 mM DTT during the soluble protein extraction procedure.

In general, DTT is added at concentrations of 50 to 100 mM in the lysis buffer or, occasionally, in the rehydration buffer. This is done in order to maintain the reduced state of those denatured protein mixtures during IEF, with the result that 2-DE gel resolution is better, especially in the basic region. In the experiments described here, we used high DTT concentrations in a crude extraction buffer that lacked chaotropes, detergents, or ampholytes. Thus, it became obvious that the significant reduction in Rubisco with 100 mM DTT was due to the additional soluble protein extraction step. However, it is not clear how the abundance of Rubisco was dramatically decreased when the DTT concentration reached 100 mM. We might hypothesize that exposing Rubisco to the abundant free-thiol group made it more susceptible to scrambling compared with other proteins under these buffer conditions. This may have resulted in those scrambled forms with exposed hydrophobic residues perhaps being extruded from the buffer as insoluble aggregates. To confirm that this was a reasonable possibility, we used our lysis buffer to solubilize insoluble proteins from the precipitated pellets. As expected, the decreased Rubisco in the soluble extraction buffer was found in insoluble aggregates (Fig. 3). Therefore, we could prove that this highly abundant photosynthetic enzyme is readily precipitated by excess DTT during the extraction of soluble proteins.

In this study, we used 100 mM DTT in the extraction buffer when preparing the plant proteins. Consequently, the highly abundant Rubisco spots that had previously limited the entrance of low-abundance proteins were successfully reduced, which then led to the production of high-quality and highly resolved 2-DE gels. This optimized method will be valuable in efforts to find more proteins of biological importance and to identify those targets with increased abundance.

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