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Short Communication

Retaining in-gel zymographic activity of cysteine proteases via a cysteine-supplemented running buffer

Zymography is a powerful technique to separate and identify different enzymatic activities on a standard acrylamide gel. For oxidation prone enzymes such as cysteine proteases however, the oxidizing species generated by electrolysis of the gel running buffer may result in partial or complete inactivation, thus compromising the final readout. This can be only partially remedied by subsequent treatment of the gel with reducing agents. We demonstrate the generation of reactive oxidizing species during electrophoresis and discovered that supplementation of the gel running buffer with a minimum of 5 mM cysteine prevents enzyme inactivation and allows retention of proteolytic activity as measured by zymography on model substrate *N* α-benzoyl-L-arginine *p*-nitroanilide, without at the same time altering the mobilities of the gel proteins.

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Zymography is the general term given to techniques able to visualize enzymes by means of a detectable product of their activity. It can be applied to electrophoretic gels (in gel zymography), biological sections (in situ zymography) and even, more recently, to living organisms (in vivo zymography) [1]. In gel zymography is a well-established experimental tool to separate the proteases responsible for various enzymatic activities in a mixture. It consists of a traditional nonreducing acrylamide gel electrophoresis followed by a proteolytic step on an appropriate substrate, which localizes the separated enzymes on the gel and allows comparison with known proteases or subsequent purification by band extraction. This procedure can be carried out either by embedding gelatin in the actual gel and subsequent protein staining of the gel itself to reveal light spots where the gelatin has been hydrolyzed, or, in overlay zymography, by overlaying on the gel a membrane containing an appropriate substrate, such as L-BApNA (*N* α-benzoyl-L-arginine *p*-nitroanilide) [2]. The yellow reaction product (*p*-nitroaniline) is revealed on the membrane itself in correspondence with the position of the protease bands either directly or by diazotization to a more visible, purple product [3].

Overlay zymography is particularly useful in the analysis of complex proteolytic systems such as the plasminogen activator/plasminogen activator inhibitor (PA/PAI, [4]) as a variety of components (cofactors, cosubstrates, and inhibitors) can be incorporated in the indicator support and tested independently after the electrophoresis. Putative or protease class specific substrates and inhibitors can also be easily screened by this technique.

While this technique is directly applicable to the majority of proteases, we have observed that when cysteine proteases such as papain are subjected to overlay zymography the enzyme activity is often lost (see Figure 2B). We hypothesize that this loss of enzyme activity is associated with the chemical lability of cysteine in the enzyme's active site.

Cysteine protease inhibition is usually caused by modification of cysteine at the active site of the enzyme, which can be either reversible or irreversible. Reversible inhibitors such as 2-PDS (2,2′ dithiodipyridine) [5, 6] covalently bind the cysteine sulfhydryl group forming a disulfide bridge that can be cleaved by a sulfur containing reducing agent such as dithiotreitol or cysteine. Irreversible inhibition, on the other hand, involves the formation of covalent bonds to other atoms such as carbon or oxygen, which cannot be reversed by reducing agents, and leads to permanent inactivation of the enzyme. The irreversible cysteine protease inhibitors E64 or hydrogen peroxide are examples of the latter inhibition mechanisms, leading to a thioether in the first case and oxidation products in the latter [7]. This oxidation of thiol groups proceeds through steps involving the sequential addition of one to three oxygen atoms giving sulfenyl ($-SO^-$), sulfinyl ($-SO_2^-$), and ultimately sulfonyl (SO_3^-) groups, of which only the first step (sulfenic) is generally considered chemically reversible [8].

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Abbreviations: DTT, 1,4 dithiothreitol; **H_2DCFDA**, 2′, 7′ - dichlorofluorescin diacetate

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Colour Online: See the article online to view Fig. 1–3 in colour.

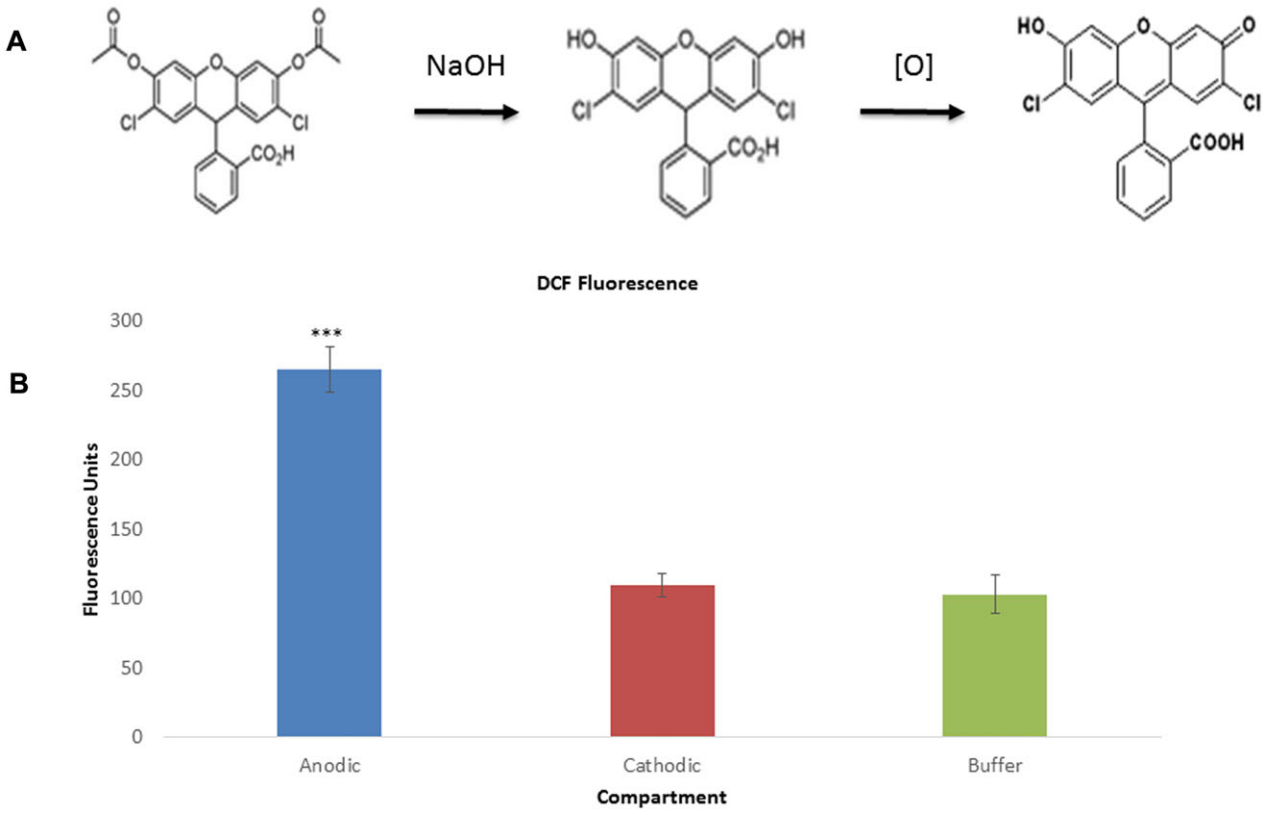

Figure 1. (A) The cellular ROS probe H_2DCFDA and its chemical conversion to the soluble form H_2DCF. (B) Measured fluorescence showing increased ROS in the anodic native gel tank running buffer compartments compared to cathodic and control fresh buffer. Statistical analysis was carried out using Student's *t*-test ($^{***}p < 0.001$). Data are representative of the mean ± SEM for $n = 3$ experiments.

We decided to investigate whether cysteine protease inactivation during electrophoresis was a result of oxidation by means of the radical oxygen species probe 2′, 7′ dichlorofluorescin (H_2DCFDA), a nonfluorescent compound which yields 2′, 7′ dichlorofluorescein following oxidation. The commercial probe contains two additional acetyl groups, which are normally removed by widespread cellular carboxylesterases [9] and cause the accumulation of the hydrophilic species dichlorofluorescin in the cell. We chemically removed these groups by preliminary alkaline hydrolysis of H_2DCFDA in NaOH 0.01 M (Fig. 1A) as previously described [10]. We then ran acidic (reverse polarity) native electrophoresis on an unloaded gel at 200 V for 120 min with a running buffer having the following composition: 0.35 M β-alanine, 0.14 M acetic acid, pH 4.3 (described on The Wolfson Centre for Applied Structural Biology website, http://wolfson.huji.ac.il).

After electrophoresis we extracted 90 μL of buffer from the anodic (top), cathodic (bottom) compartment, or control running buffer not used in the run and added 10 μL of hydrolyzed probe. Following incubation for 1 h in the dark at room temperature, fluorescence was read at 485 nm excitation/528 nm emission wavelengths on a Synergy HT plate reader. Results (Fig. 1B) indicate that the electrolysis of the running buffer results in a significant twofold increase in reactive oxidative species in the anodic side, which could in turn inactivate cysteine proteases since the proteins are initially loaded within this compartment.

Having established that electrophoresis was able to generate oxidative species that are probably liable for the enzymatic inactivation of cysteine protease activity, we sought ways to mitigate oxidation. Oxidative inactivation of cysteine proteases is a well-described phenomenon and in order to reverse this inactivation and allow zymography previous workers have used the reducing agent 1,4 dithiothreitol (DTT) that is added to the membrane at the detection step [2]. However, this would only reactivate the enzyme fraction that had been reversibly inactivated and thus only achieve partial reactivation.

In order to maximize assay sensitivity we therefore decided to investigate whether it could be possible to prevent the oxidation from taking place altogether by supplementing the gel running buffer with cysteine, so as to create a reducing environment that would prevent oxidation. Free cysteine is a good reducing agent ($E_0 = -0.34$ V) [11] and being more easily accessible in solution than the cysteine in the enzyme active site it should ideally react preferably with the oxidizing species. We employed papain, bromelain, and ficin (Sigma) as widely used cysteine proteases and a crude papaya extract

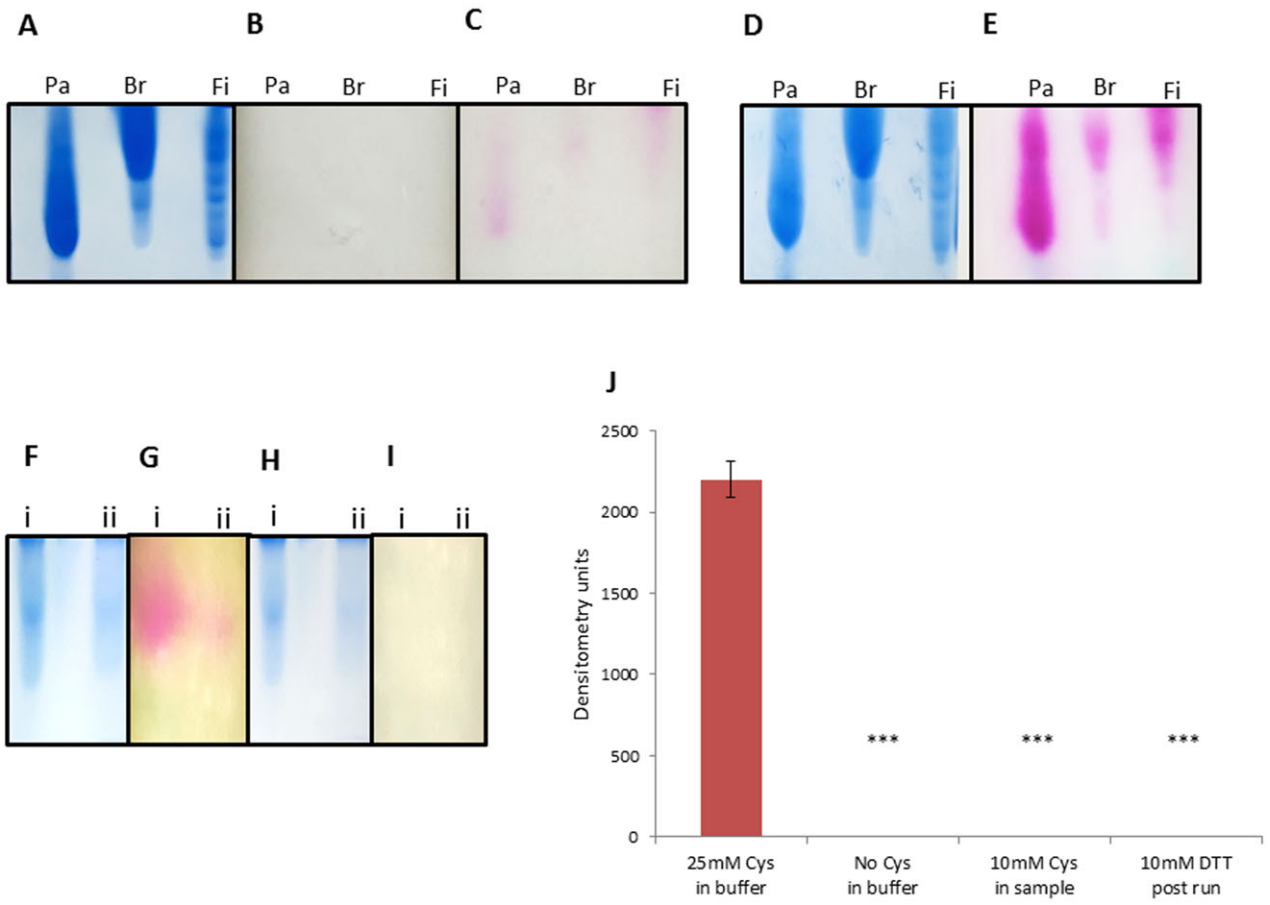

Figure 2. (A–C) Native gel run of papain (Pa), bromelain (Br), and ficin (Fi) in buffer 0.35 M β-alanine, 0.14 M acetic acid, pH 4.3. (A) Coomassie stain, (B) zymography from same gel, and (C) zymography with DTT posttreatment of gel. (D, E) Coomassie stain, and zymography of a native gel run of same enzymes but in buffer 0.35 M β-alanine, 0.14 M acetic acid, pH 4.3 containing 25 mM cysteine. (F–I) Coomassie stain and zymography of concentrated (i, left lanes) and pure (ii, right lanes) papaya extract run in buffer above with cysteine (F, G) or without cysteine (H, I). (J) Densitometric quantification of papain zymography bands under various conditions. Statistical analysis was carried out using Student's *t*-test ($^{***}p < 0.001$). Data are representative of the mean ± SEM for $n = 3$ experiments.

(obtained by pulping and filtering the flesh of ripe fruit) and its evaporated 5× concentrated form as realistic biological samples. The commercial enzymes were dissolved in water at 10 mg/mL, while the papaya extract used as such, and mixed 1:4 with native gel sample buffer (K acetate pH 6.8 50 mM, glycerol 35%, methyl green 0.01%). In parallel we also ran samples with 25 mM cysteine in the loading buffer.

Aliquots (30 μL) of both dilutions were then loaded in lanes on a native PAGE gel and run under reverse polarity (anode to cathode) for 120 min at 200 V in one of two running buffers: 0.35 M β-alanine, 0.14 M acetic acid, pH 4.3 as described above or 0.35 M β-alanine, 0.14 M acetic acid, pH 4.3 with the addition of 25 mM cysteine. One half of the gel was then directly stained with Colloidal Coomassie Blue (Invitrogen) and the other used for zymography.

Zymography was carried out according to the procedure in [2] with some modifications. Briefly, a nitrocellulose membrane was soaked in a 1.2 mg/mL L-BApNA solution in water. The membrane was subsequently left to air dry for 5 min and then laid on top of the acrylamide gels soaked in their respective running buffer (0.35 M β-alanine, 0.14 M acetic acid, pH 4.3 with or without 25 mM cysteine). Two additional experiments were carried out in which membranes were soaked in 0.35 M β-alanine, 0.14 M acetic acid, pH 4.3 buffer with either 10 mM DTT or 25 mM cysteine. All nitrocellulose membranes were then incubated in a closed chamber for 1 h. The membrane was again left to dry for 5 min and the *p*-nitroaniline visualized by diazotization.

Diazotization followed the protocol in [3]: The membrane was soaked in turn for 5 min in a sodium nitrite solution (1 mg/mL in 1 M HCl), then an ammonium sulfamate solution (5 mg/mL in 1 M HCl) and finally a NNED (*N*-(1-naphthyl)-ethylenediamine dihydrochloride) (Sigma) (0.5 mg/mL in 48% v/v ethanol/water), for about 1 min until any diazotized *p*-nitroaniline became clearly visible as purple bands.

Figure 2A shows the control Coomassie staining of a typical protease gel run in 0.35 M β-alanine, 0.14 M acetic acid,

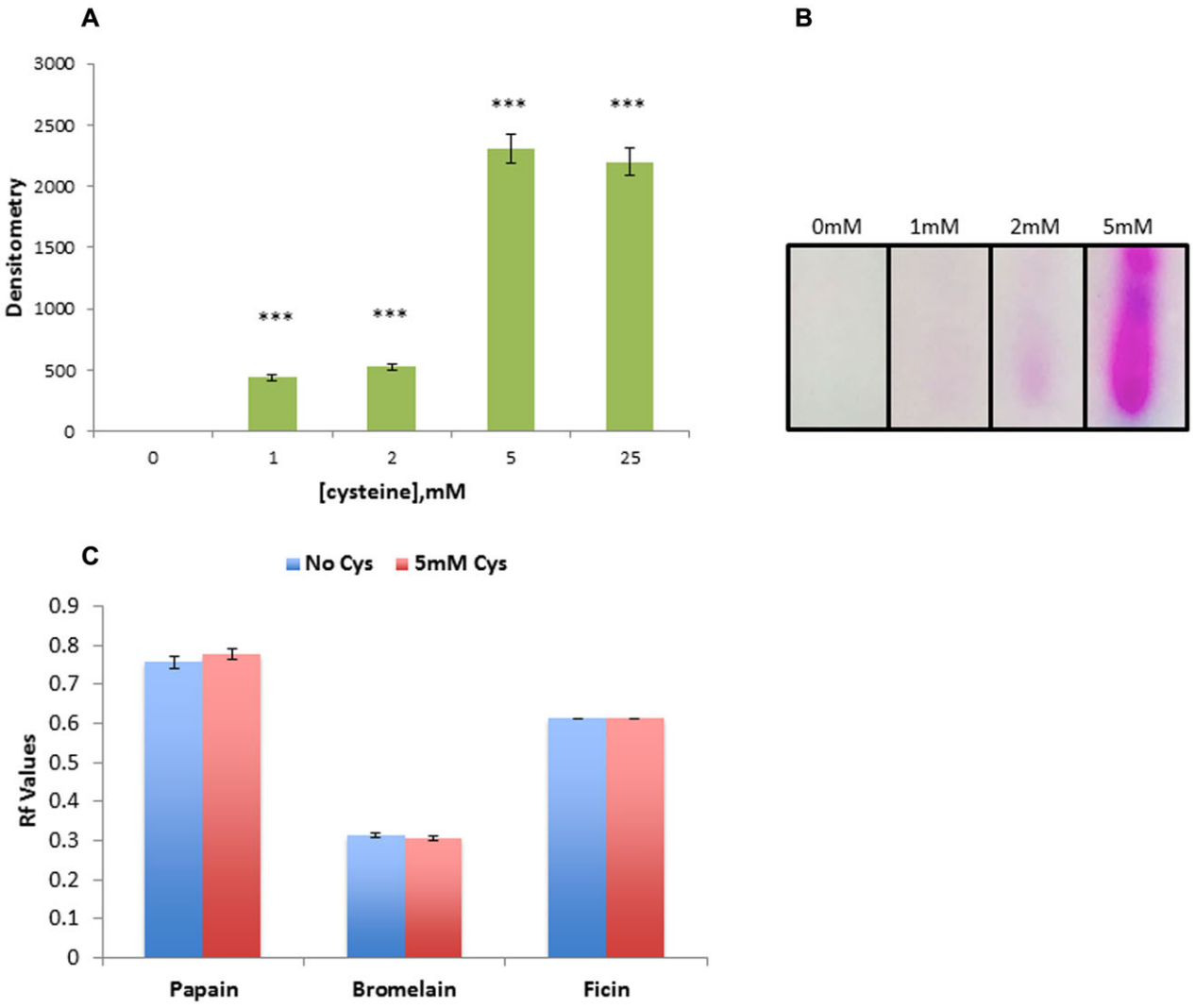

Figure 3. (A) Quantification of papain zymography band intensity with increasing amounts (0–25 mM) of cysteine supplemented to the running buffer. Statistical analysis was carried out using Student's *t*-test ($^{***}p < 0.001$). Data are representative of the mean ± SEM for $n = 3$ experiments. (B) Representative zymographies from same run. (C) Rf values (defined as the ratio between migration distances of protein and front) for papain, bromelain, and ficin measured for the fastest component in each lane native gels run with or without 5 mM cysteine supplementation in the buffer.

pH 4.3. Figure 2B and C shows the zymography bands obtained from the same gel directly (B) or with DTT post treatment (C). Figure 2D and E show similar results (Coomassie staining and zymography) obtained using 0.35 M β-alanine, 0.14 M acetic acid, pH 4.3 buffer supplemented with 25 mM cysteine. This resulted in much stronger bands, thus proving the protective effect of cysteine on papain activity. The fuzziness of the observed bands is most likely due to the grade of the commercial enzymes, which are derived from crude latexes and known to consist of a mixture of proteases resulting in several bands. Figure 2F–I displays an equivalent result carried out on crude papaya extracts both pure and concentrated. The difference in intensities was quantified via densitometry of the zymography bands for the papain analysis with the GelPro 32 software, confirming the significance of cysteine in the running buffer (Fig. 2J). It is evident that posttreatment with either DTT or cysteine only rescued a minor amount of the enzymatic activity suggesting that most of the inactivation undergone during the run is irreversible. Under the same conditions pretreatment of the protein by supplementation of the loading buffer with cysteine had no protective effect. Zymography on both crude extracts was only successful when running the gel in cysteine-supplemented buffer.

We next carried out a dose study to identify the minimum effective cysteine concentration needed to preserve enzyme activity. Figure 3A and B shows the intensity of the papain zymography bands obtained under a range of cysteine concentrations in the running buffer and the bands themselves. We thus identified 5 mM as the optimal concentration required to prevent enzyme inactivation. We finally verified that cysteine supplementation does not change the Rf values of papain, bromelain, or ficin (Fig. 3C).

Taken together these results show that acidic native electrophoresis, the one normally used for the separation of cationic cysteine proteases, results in the generation of significant concentrations of reactive oxidizing species at the anode and that these species in turn cause the partially irreversible oxidative inactivation of cysteine proteases such as papain, ficin, and bromelain during the run. The oxidized active site thiols then impair catalytic activity and make enzymes invisible via zymography. This phenomenon can only be partially remedied via the subsequent treatment of the gel with reducing agents such as cysteine or DTT, as predicted in the literature [8], but it can be significantly prevented, thereby enhancing the sensitivity of the overlay zymographic technique, through an inexpensive and easy modification of the running conditions, i.e. the supplementation of the gel running buffer with a minimum 5 mM cysteine. This alteration of the protocol does not alter protein mobilities and therefore the position of the bands on the gel as measured by their Rf values, but prevents the oxidation of active site cysteine probably by providing an alternative reducing target to the reactive oxidative species generated in the run. This preventive effect leads in turn to a higher retention of protease catalytic activity and to much stronger bands in zymography. This is true both for pure enzymes and for a practical biological sample containing proteases such as a papaya extract. The increased sensitivity of this modified protocol, together with the intrinsic versatility of enzymatic assays, can lead in turn to an extended range of applications of the technique on biologic samples where cysteine proteases are present at concentrations normally below standard zymography detection limits and make possible their visualization without the need for concentration steps.

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The authors have declared no conflicts of interest.

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