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ORIGINAL ARTICLE

Protonography, a new technique for the analysis of carbonic anhydrase activity

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Abstract

All proteolytic enzymes, which are able to renature and reacquire the proteolytic activity on a copolymerized substrate, can be analyzed by zymography upon removal of sodium dodecyl sulfate (SDS). Protonography, the new technique described in this study, unlike zymography, allows the detection of a different protein, not a protease, i.e. of the carbonic anhydrase (CA, EC 4.2.1.1) activity on a SDS polyacrylamide gel electrophoresis gel. CAs are zinc-containing enzymes that catalyze the reversible conversion of carbon dioxide to bicarbonate and protons. Hydrogen ions produced during the catalyzed reaction are responsible for the change of color that appears on the gel around the CA band. For this reason, we named the new technique ''protonography''. The following four salient features characterize this new technique: (a) on the basis of molecular weight markers, recombinant or native CAs with different molecular weights can be detected and quantified rapidly on a single gel; (b) the hydratase activity can be reversibly inhibited by SDS during electrophoresis and recovered by incubating the gel in aqueous Triton X-100; (c) it is possible to separate active oligomeric forms of CAs on the gel enabling their activities to be determined independently of one another. This feature is not possible when using solution assays; and (d) it can be a useful tool to establish if a putative or a newly identified CA in a genome is expressed and enzymatically active. This article outlines the general principles employed in protonography, providing an easy procedure to implement it in laboratories working with CAs. It also presents an overview of its development and current research applications through specific examples.

Keywords

Carbonic anhydrase, hydratase activity, metalloenzymes, protonography

History

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Introduction

In 1978, a new technique was developed to study plasminogen activators, proteins present in the blood and involved in the coagulation cascade; this technique was named ''in gel zymography''[1]. It used a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel to separate plasmin (produced by the proteolysis of plasminogen) from other molecules. Plasmin detection was based on the degradation of fibrin (which is one of the substrates of this protease) contained in an agarose gel, named indicator gel[1,2]. Enzyme diffusion into the indicator gel occurred during incubation of the gel sandwich and resulted in degradation of the protein substrate. The indicator gel was then stained to reveal zones of proteolysis, which appeared as clear bands on a dark background. Subsequently, the method was optimized by incorporating the protein substrate directly into the SDS-PAGE gel[2,3]. Thus, as aforementioned, zymography is defined as a simple electrophoretic method and functional assay for measuring proteolytic activity and is widely applied for investigating proteases belonging to all classes (serine-/threonine-, cysteine, aspartic and metalloproteases)[2,3]. The standard method is based on SDS gels impregnated with a protein substrate, in particular gelatin or casein. All the proteolytic enzymes, which are able to renature and reacquire the proteolytic activity on a copolymerized substrate, upon removal of SDS (acting as a denaturant), can be analyzed by this technique. As described in many literature reports, zymography offers several advantages with respect to other methods because it is feasible with inexpensive materials, and it is also a very sensitive technique, with detection limits of about 10 pg of protease on gelatin zymogram, when compared with other methods, such as ELISA[2–4].

In this article, we introduce a new technique, similar to zymography and derived from it, and named in this study by our group, ''protonography''. Unlike zymography, this new technique allows the detection of the activity of a different class of proteins, the carbonic anhydrase (CA, EC 4.2.1.1) on a SDS-PAGE gel. In the past, Kotwica et al. used a PAGE zymography without SDS to detect the presence of CA activity in the upper vas deferens of the cotton leaf worm, *Spodoptera littoralis*[5]. However, this zymography-like technique applied to CAs did not allow determining of the molecular weight of the enzymes. Moreover, Drescher reported the use of a fluorescent CA inhibitor, 5-dimethylaminonaphthalene-1-sulfonamide, to identify the CA on polyacrylamide gel by an imaging system apparatus[6,7].

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CAs catalyze a simple reaction important in all life kingdoms: the carbon dioxide hydration to bicarbonate and protons: $CO_2 + H_2O \Leftrightarrow HCO_3^- + H^+$[8–18]. The name ''protonography'' was chosen because CA activity produces hydrogen ions (protons) during the catalyzed reaction, which are then responsible of the change of color that appears on the gel, in correspondence with a CA, CO_2 hydrase activity band. The band of the protein with CA activity becomes yellow (see later in the text). The advantage of this technique is that on the basis of molecular weight markers, recombinant or native CAs with different molecular weights can be detected and quantified rapidly on a single gel.

Five different, genetically distinct CA families are known to date, the α-, β-, γ-, δ- and ζ-CAs[10,11,13,19–26]. Whereas α-, β- and δ-CAs use Zn(II) ions at the active site; the γ-CAs are probably Fe(II) enzymes[11,19,27–30] (but they are active also with bound Zn(II) or Co(II) ions)[30], whereas the ζ-class uses Cd(II) or Zn(II) to perform the physiologic reaction[10–14,19–26,31–34]. The molecular weight of these CA classes is in the range of 20–30 kDa on an SDS-PAGE gel[8–11,13,14,22–27,35–49].

In this study, we present the protonography as a new technique for the rapid detection of CA activity, exemplifying it on one α-CA belonging to the mammalian organism *Bos taurus* (bovine α-CA; bCA), and one α-CA from the pathogenic bacterium, *Vibrio cholerae* (VchCA).

Materials and methods

Mammalian bCA

bCA was supplied by Sigma-Aldrich (St. Louis, MO) as crude extract from bovine erythrocytes (lyophilized powder).

Construct preparation, protein expression and purification of VchCA

The GeneArt Company, specialized in gene synthesis, designed the synthetic *V. cholerae* gene encoding for the α-CA, lacking the signal peptide (i.e. the first 20 amino acid residues of the peptide sequence) and containing a *NdeI* and *XhoI* site at the 5′ and 3′ end of the *VchCA* gene, respectively. The resulting plasmid was amplified into *Escherichia coli* DH5 α cells. The *V. cholerae* DNA fragments were separated on 1% agarose gel. The recovered *V. cholerae* gene and the linearized expression vector (pET15-b) were ligated by T4 DNA ligase to form the expression vector pET15-b/Vch. In order to confirm the integrity of the *V. cholerae* gene and that no errors occurred at the ligation sites, the pET15-b/Vch vector was sequenced. Competent *E. coli* BL21 (DE3) cells were transformed with pET15-b/Vch vector, induced with 1 mM IPTG and grown at 37 °C for 5 h. After additional growth for 5 h, cells were harvested and disrupted by sonication at 4 °C. Following centrifugation, the cell extract was placed in conical flasks, and an amount of ammonium sulfate gradually added to obtain saturations of 45%. After resting for 14 h at 4 °C, the sample was centrifuged at 12 000 × *g* at 4 °C for 30 min. The precipitate was resuspended in 20 mM buffer phosphate, pH 8.0, and loaded onto a His-select HF Nickel affinity gel. The protein was eluted with 250 mM imidazole. At this stage of purification, the enzyme was at least 95% pure, and the obtained recovery was of 30 mg of the recombinant bacterial CA.

CA assay

An Applied Photophysics stopped-flow instrument was used for assaying the CA catalyzed CO_2 hydration activity[50]. Phenol red (at 0.2 mM) was used as indicator, working at the absorbance maximum of 557 nm with 10 mM HEPES (pH 7.5) as buffer and 0.1 M $NaClO_4$ (for maintaining constant ionic strength), at 20 °C, following the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s (the uncatalyzed reaction needs around 60–100 s in the assay conditions, whereas the catalyzed ones are of around 6–10 s). The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Enzyme concentrations in the assay system were 9.1 nM for bCA and 8.6 nM for VchCA.

Protonography

SDS-PAGE was performed as described by Laemmli[51]. Wells of 12% SDS gel were loaded with bCA or VchCA mixed with Laemmli loading buffer without 2-mercaptoethanol and without boiling the samples, in order to avoid protein denaturation. The gel was run at 180 V until the dye front ran off the gel. Following the electrophoresis, the gel was removed from glass plates and soaked in 2.5% Triton X-100 for 1 h on a shaker and then twice in 100 mM Tris, pH 8.2 containing 10% isopropanol for 10 min. Subsequently, the gel was incubated in 0.1% bromothymol blue in 100 mM Tris, pH 8.2 for 30 min and then immersed in CO_2-saturated ddH_2O to visualize the hydratase activity of the enzyme. The assay was performed at room temperature, and the CO_2-satured solution was prepared by bubbling CO_2 into 200 mL distilled water for approximately 3 h. The local decrease in pH due to the presence of CA activity was evidenced by the formation of yellow bands due to the change of the indicator color from blue (alkaline pH) to yellow (acidic pH).

Preparation of cell lysates from *E. coli*

We used *E. coli* BL21 (DE3)pLysS cells from Agilent Technologies. Cells were grown for 16 h at 37 °C, then harvested and disrupted by sonication at 4 °C. Following centrifugation, the extract was assayed using the Bradford assay. A total of 30 μg of protein per well was loaded on a 12% SDS-PAGE without 2-mercaptoethanol and without boiling the sample. Gels were made in duplicate. One was stained with Coomassie, and the other was subject to the protonography technique as described in this section.

Results and discussion

The recombinant VchCA prepared as described in ''Materials and methods'' was isolated and purified to homogeneity at room temperature from *E. coli* (DE3) cell extracts. CA activity was recovered in the soluble fraction of the cell extract obtained after sonication and centrifugation. By use of the affinity column (His-select HF nickel affinity gel), VchCA was purified to about 95% of purity.

A stopped-flow CO_2 hydrase assay has been used to measure the catalytic activity of VchCA and bCA in identical conditions. Table 1 shows the catalytic activity of these enzymes for the CO_2 hydration reaction. It may be observed that VchCA has kinetic parameters quite similar to those of the bCA.

Table 1. Kinetic parameters for CO_2 hydration reaction catalyzed by bCA and VchCA at 20 °C and pH 7.5. K_i for acetazolamide was 10 nM for bCA and 6.8 nM for VchCA.

Enzyme	k_{cat} (s^{-1})	K_m (M)	k_{cat}/K_m ($M^{-1}\,s^{-1}$)
bCA[a]	1.62×10^6	12.5×10^{-3}	1.3×10^8
VchCA[b]	8.23×10^5	11.7×10^{-3}	7.0×10^7

[a]Commercial enzyme supplied by Sigma, stopped flow CO_2 hydrase assay method, this work.
[b]Recombinant enzyme.

To investigate the hydratase activity of VchCA and bCA on the SDS-PAGE gel, samples of VchCA or bCA were prepared diluting a stock solution and loaded on the gel in the range of 100–1000 ng/well. As described in the experimental section, the protonography technique is based on the monitoring of pH variation in the gel (protonogram) due to the catalyzed conversion of CO_2 to bicarbonate and protons. The protonogram was stained with bromothymol blue, which is an indicator of pH variation. This dye appears blue in its deprotonated form, while its color changes to yellow in the protonated form. Thus, the production of hydrogen ions during the CO_2 hydration reaction, due to the bCA or VchCA hydratase activity lowers the pH of the solution until the color transition point of the dye is reached (pH 6.8). Figures 1 and 2 show a protonogram obtained with different concentration of VchCA and bCA ranging from 10 to 1400 ng. The yellow band corresponded to the CA position on the gel. The time required for the color change is inversely related to the quantity of CA, which is responsible for dropping the pH from 8.2 to the transition point of the dye in a control buffer. The success of this technique is that the hydratase activity can be reversibly inhibited by SDS during electrophoresis and recovered by incubating the gel in aqueous Triton X-100.

Figure 3 shows a comparison between the protonography and SDS-PAGE stained with Coomassie blue. Both gels were run under denaturing but non-reducing conditions. After the electrophoretic separation of proteins, the gel on the left-hand of Figure 3 was stained with Coomassie blue, whereas the gel on the right-hand of the same figure was resolved by exchange of SDS with a nonionic detergent such as Triton X-100 and then stained with bromothymol blue. The CA activity was detected by immersing the gel in CO_2-saturated ddH_2O. The enzyme activity was detected as yellow bands against the blue background. Moreover, Figure 3 shows that protonography allowed us to identify different oligomeric states of the VchCA when the gel is overloaded (4000 ng of protein per well). This protein, in fact, is a monomer of 25 kDa but the protonogram showed three visible bands corresponding to the dimeric (50 kDa) and tetrameric (100 kDa) forms. At high concentration of protein loaded per well, in the case of bCA, it is possible to see that the protein is always monomeric, with a molecular weight of 30 kDa, whereas for VchCA, it was possible to separate the different oligomeric forms of the enzymes, enabling their activities to be determined independently from one another, which is not possible in the solution assay. Thus, a specific advantage of this system is that the active, oligomeric forms of the enzyme, which can be distinguished on the basis of molecular weight, can be detected by this new technique. As shown in Figure 2, VchCA already showed a band at the position of the dimeric state (50 kDa) in the well containing 1400 ng of enzyme.

Another advantage of this technique is the possibility to reveal CAs in biological systems, such as tissues or bacterial extracts from which only the genome information is so far available. In this case, protonography might be the method of choice for CAs screening, identification and characterization. For example, we prepared a cellular extract of *E. coli*. The genome of *E. coli* encodes for two CA classes: β and γ-CAs, as described in the literature[52,53]. In this study, we want to demonstrate that protonography allowed us to detect hydratase activity in the cellular extract of *E. coli*. Figure 4 shows a SDS-PAGE gel stained with Coomassie blue and a protonogram obtained after loading the bacterial extract on the gels. As expected, when analyzed by protonography, the whole cellular extract of *E. coli* revealed

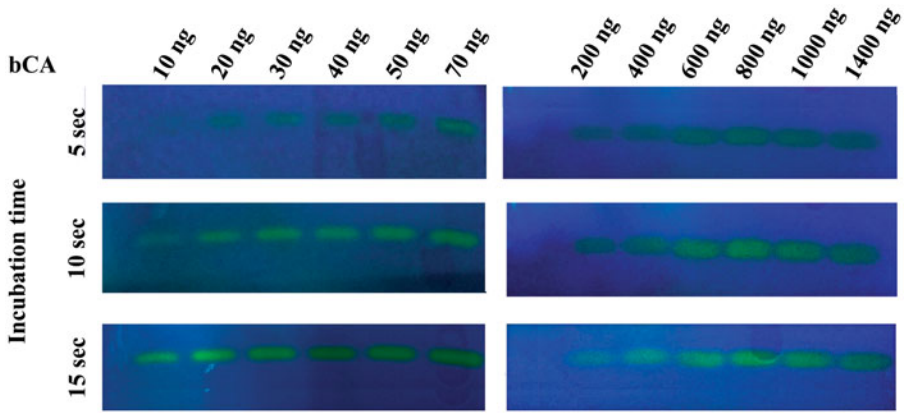

Figure 1. Protonogram obtained at different concentrations of bCA. The yellow band corresponds to the bCA position on the gel responsible for the drop of pH from 8.2 to the transition point of the dye in the control buffer. Three different incubation times are used (5, 10 and 15 s).

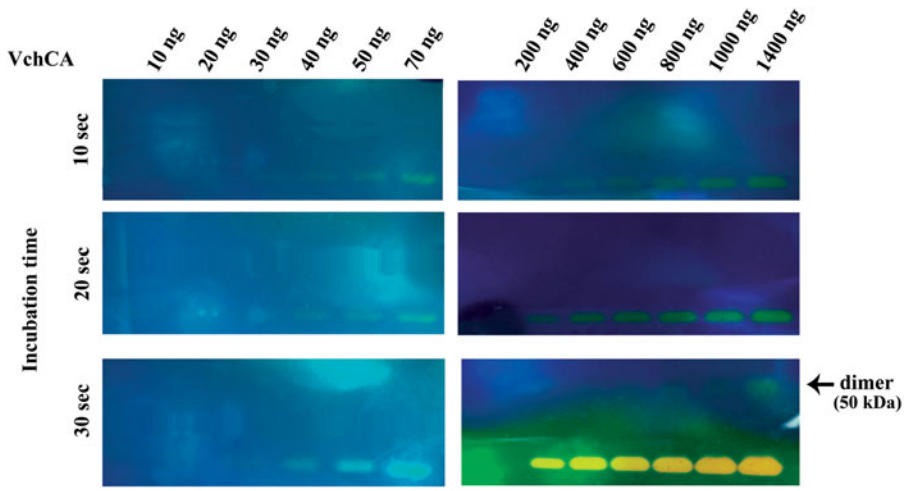

Figure 2. Protonogram obtained at different concentrations of VchCA. The yellow band corresponds to the VchCA position on the gel. Incubation times were 10, 20 and 30 s, higher than those indicated for bCA and shown in Figure 1.

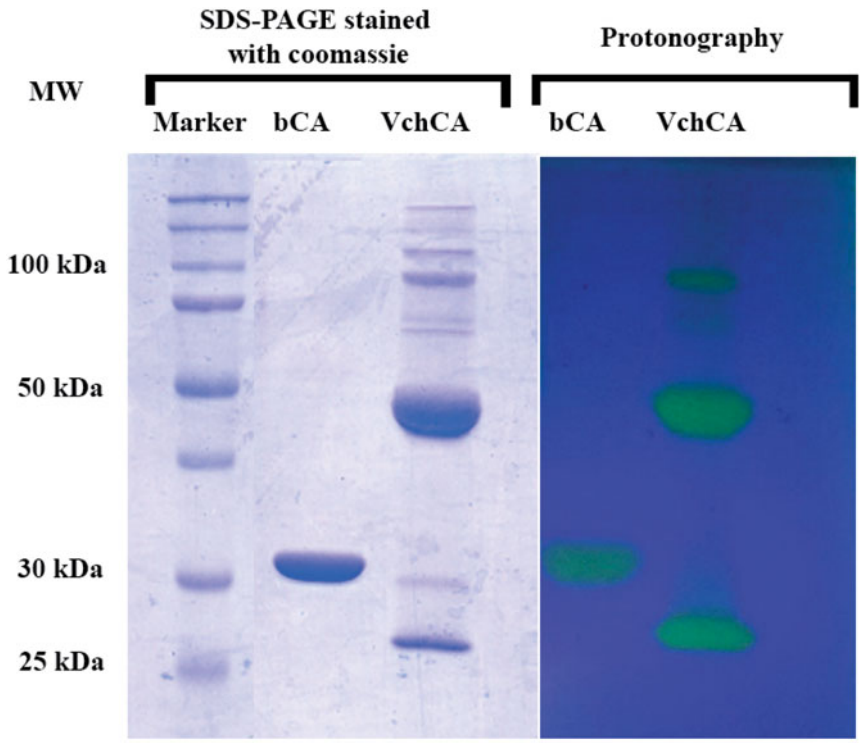

Figure 3. Comparison between the protonography and SDS-PAGE stained with Coomassie blue. Both gels were run under denaturing but non-reducing conditions. Gels were loaded with 4000 ng/well of bCA and VchCA, respectively. The incubation time in this case was 5 s.

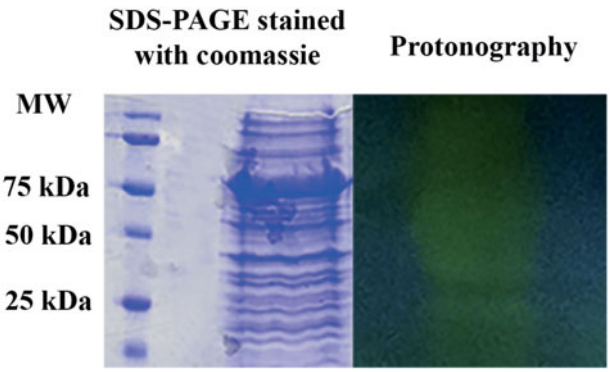

Figure 4. SDS-PAGE protonography using the whole cellular extract prepared from *Escherichia coli* cells. Both gels were run under denaturing but non-reducing conditions. Protonography shows two main yellow bands at a molecular weight ranging from 25 to 50 kDa (see text). Protonography thus evidenced an *E. coli* CA activity as described in the literature.

two main yellow bands at a molecular weight ranging from 25 to 50 kDa, corresponding to a monomeric (25 kDa) or dimeric (50 kDa) β-CA. It was difficult to evidence the band at 75 kDa corresponding to a trimeric γ-CA or, probably, it migrated under these experimental conditions as a monomer with a molecular weight of about 25 kDa. This is an interesting result in the study of organisms with known genome sequences for which we can identify putative CA by bioinformatics analysis, but we cannot ascertained if the ORFs are indeed expressed and active. Protonography can be a useful tool to help with this issue.

Furthermore, protonography coupled with mass spectrometry for protein identification will make possible the broader mapping of active CAs present in protein extract from various sources, allowing the detection of distinct CA isoforms.

Conclusions

Protonography is a new, cheap, reproducible and easy to perform technique for assaying CO_2 hydratase activity on a SDS-PAGE gel. In this study, we presented the basic features of this new technique by using three examples of CAs belonging to a mammal (bCA), a bacterium (*V. cholerae* α-CA) and a bacterium for which the two CAs present in its genome (one β- and one γ-CA), were not investigated in great detail, i.e. *E. coli*. For all these three diverse examples, we were able to demonstrate the CO_2 hydratase activity by using protonography, allowing us to speculate that this technique may be extended also to enzymes belonging to other CA classes than the α one. Indeed, combining protonography with mass spectrometric techniques could be particularly advantageous for the biochemical identification and analysis of putative, new CAs. Furthermore, this technique might reveal the expression of active CAs that has important roles in tumor progression and metastasis, such as CA II, CA IX and XII. In fact, there are many cases of tumors overexpressing such isoforms[54,55]. However, as outlined above, protonography may easily reveal whether CA-like proteins not yet characterized but present in the genome of many organisms, do possess enzymatic activity without the need to clone, overexpress and purify the protein, as exemplified by the *E. coli* experiments reported in this article.

Declaration of interest

The authors declare no conflict of interest.

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