ELSEVIER

Journal of Chromatography A, 731 (1996) 293–298

JOURNAL OF
CHROMATOGRAPHY A

Fast and sensitive staining technique for glucose oxidase in polyacrylamide gel

C. Obinger*, S. Pfeiffer, W. Hofstetter, R. Wutka, R. Ebermann

Institut für Chemie, Universität für Bodenkultur, Gregor Mendelstr. 33, 1180 Vienna, Austria

Received 20 March 1995; revised 27 June 1995; accepted 16 October 1995

Abstract

The described staining techniques for glucose oxidase activity on polyacrylamide gels are based on the fact that glucose oxidase reacts with one-electron scavengers in a reaction dependent on pH and pO_2. Glucose oxidase separated in PAGE shows reduction of ferricyanide, cuprisulfate, ferricytochrome c and nitrotetrazolium blue reduction leading to sharp coloured bands under aerobic conditions. In contrast to the conventional peroxidase/*o*-dianisidine-assay, the staining procedures only need about 30 min and are specific for glucose oxidase using glucose as electron donor. Enzyme activity can be detected down to 0.2 units.

Keywords: Staining technique; Glucose oxidase; Enzymes

1. Introduction

Glucose oxidase (β-D-glucose: oxygen oxidoreductase, EC 1.1.3.4) is a flavoprotein produced by different types of molds mainly. The enzyme is known to mediate the transfer of two electrons from glucose to oxygen. Glucose is oxidized to D-glucono-δ-lacton and oxygen is reduced to hydrogen peroxide [1–9]. The conventional staining technique for visualization of the catalytic activity of glucose oxidase on gel electropherograms needs a second enzyme, namely horseradish peroxidase, which consumes hydrogen peroxide evolved by glucose oxidase mediated glucose oxidation. At the site of H_2O_2 generated *o*-dianisidine is oxidized by peroxidase leading to brown bands on a colourless background. Because the reaction should take place inside the gel only, to yield coloured zones, peroxidase must be enabled to diffuse into the gel. Therefore, the gel has to be swollen in distilled water for at least two hours, before the staining reaction can be carried out successfully.

Looking for activity staining procedures, which are independent on the addition of peroxidase and therefore less time-consuming, we have seen that zones of hydrogen peroxide production in the gels coincided with zones stained with typical one-electron scavengers like ferricyanide, cuprisulfate, ferricytochrome *c* and nitrotetrazolium blue. The only report which appeared so far in the literature indicating the occurrence of a reducing species in the glucose oxidase reaction is the paper by Jos et al. [10]. The authors described the reduction of nitrotetrazolium blue in the presence of phenazine-methosulfate (PMS) during glucose oxidation. The reaction of glucose oxidase was used for the detection of glucose in tissues.

In the present paper we describe several staining

*Corresponding author.

0021-9673/96/$15.00 © 1996 Elsevier Science B.V. All rights reserved
SSDI 0021-9673(95)01181-1

procedures which are more sensitive and time-saving than the usual peroxidase/*o*-dianisidine assay. The described staining techniques are compared and discussed in their dependence on pH, pO_2 as well as in their partial inhibition by superoxide dismutase.

2. Experimental

2.1. Chemicals

Three different preparations of glucose oxidase (EC 1.1.3.4) from *Aspergillus niger* were tested without any significant difference in the results presented below. All were purchased from Sigma (G-6641, G-6891, G-7141). Cu/Zn-superoxide dismutase (EC 1.15.1.1, Sigma S-2515), horse-heart cytochrome c, *o*-dianisidine (3,3′-dimethoxybenzidine), nitro blue tetrazolium (NBT), acyl-CoA-dehydrogenase, flavodoxin, xanthine and xanthine oxidase were also purchased from Sigma. All other chemicals were of analytical grade (Merck, Darmstadt, Germany).

The specific activity of the commercial sources of glucose oxidase (micromolar units per mg glucose oxidase) was assayed by the peroxidase/*o*-dianisidine method [11]. In the present paper we use the following unit definition: one unit will oxidize 1.0 μmol of β-D-glucose to D-gluconic acid and H_2O_2 per minute at 35°C and pH 5.1 (if the reaction is saturated with oxygen, the activity may increase 50–100%).

2.2. Polyacrylamide gel electrophoresis (PAGE)

Gel electrophoresis was performed in a vertical slab gel apparatus (Mini-Protean II Dual Slab cells from BioRad) according to Ebermann & Bodenseher [12]. A separation gel containing 15% acrylamide, pH 8.0, cross-linked 1:125 with N,N′-bis-methyleneacrylamide was used. The electrophoresis buffer system consisted of Tris-glycine, pH 8.9. The separation time was 2 h with a constant voltage of 200 V and a starting current of 120 mA. After electrophoresis the gels were washed in distilled water for 30 min.

2.3. Staining procedures

Scanning and computer-aided quantitative evaluation of the gels was done with a soft laser densitometer (Biomed Instruments). If the staining was performed under anaerobic conditions, nitrogen was bubbled for at least 30 min through the buffer solutions. Finally, incubation of the gel was performed in a desiccator which was evacuated and filled with nitrogen deoxygenated by a pyrogallol solution. Inhibition of the reactions was investigated by addition of 5 mg superoxide dismutase (Sigma, 3500 U/mg).

2.4. Peroxidase/o-dianisidine assay

Localization of glucose oxidase in the gels was performed via its hydrogen peroxide production determined by peroxidase catalyzed *o*-dianisidine oxidation. The reaction should take place inside the gel only to yield coloured zones. The peroxidase (M_r about 50 000) in the buffer solution must be enabled to diffuse into the gel. Therefore, the gel has to be swollen in distilled water for 2 h, before the staining reaction can be carried out successfully. The gels were inserted into 50 ml 0.1 *M* acetate buffer (pH 5.0) or 0.1 *M* phosphate buffer (pH 8.0) which contained 50 mg glucose, 2.5 mg horseradish peroxidase (Sigma 200, U/mg) and 5 mg *o*-dianisidine. Brown zones of enzymic activity on a colourless background were obtained.

2.5. Ferricyanide assay

Incubation solution (50 ml): 50 mg glucose, 80 mg potassium-hexacyano-ferrat(III) and 50 mg ferrichloride dissolved in 0.1 *M* acetate buffer (pH 5.0). After ca. 1 h blue zones of Thurnbulls Blue appear at the location of glucose oxidase.

2.6. Cuprisulfate assay

Incubation solution (50 ml): 50 mg glucose, 40 mg Cu(II)sulfate and 50 mg ammonium reineckate $NH_4[Cr(SCN)_4(NH_3)_4]$ dissolved in 0.1 *M* acetate buffer (pH 5.0). Within about half an hour yellow precipitations appear at the location of glucose oxidase.

2.7. Nitrotetrazolium assay

In order to stain the gels with NBT the reaction was carried out in 0.1 *M* acetate buffer (pH 5.0) or 0.1 *M* phosphate buffer (pH 8.0). The incubation solution contained 50 mg glucose and 20 mg NBT. In both of the variants blue zones of formazan reduction products are appearing at the locations of glucose oxidase in the gel.

2.8. Ferricytochrome c assay

Detection of superoxide anion production with cytochrome c was performed in 0.1 *M* acetate buffer (pH 5.0) containing 10 mg cytochrome c. After 14 h 50 mg of glucose, dissolved in a small volume of buffer, were added. One hour later the gel was inserted into 50 ml of acetate buffer containing 10 mg of *o*-dianisidine and 0.02 *M* H_2O_2. As a result the gel was stained brown with the exception of the glucose oxidase locations which remained colourless. Oxidized cytochrome c acts on *o*-dianisidine in a peroxidase-like reaction whereas the reduced form does not.

3. Results and discussion

Glucose oxidase so far is known as an enzyme which mediates the two-electron transfer from glucose to molecular oxygen. The only report indicating the occurrence of a reducing species in the glucose oxidase reaction is the paper by Jos et al. [10] showing NBT reduction in the presence of phenazine-methosulfate (PMS) during the glucose oxidation. We have reinvestigated this reaction without the addition of PMS and have been able to show activity staining of glucose oxidase after PAGE with both NBT and other one-electron acceptors like ferricyanide, cuprisulfate and ferricytochrome *c* in a wide pH-range. In contrast to aqueous solutions polyacrylamide electrophoresis has the advantage that the mobility of the protein, which is present in a highly purified state, is diminished and that enzyme activities can be detected rapidly and sensitively even in crude extracts without expensive chromatographic steps.

Polyacrylamide electrophoresis of the commercial glucose oxidase preparations used in our investigations was performed routinely to estimate the purity of the commercial enzyme preparations. Gel scanning of Coomassie brilliant blue stained gels showed that about 98–99% of total protein is recovered in a single band (Fig. 1A). In Fig. 1B,C the staining of glucose oxidase results from the peroxidase catalyzed reaction of *o*-dianisidine with H_2O_2 at pH 5 (B) and pH 8 (C). This conventional glucose oxidase staining method takes several hours and enables the detection of activity down to 0.5 units at pH 5. At pH 8 the staining is less intense as compared to staining at pH 5 indicating a diminished hydrogen peroxide production at alkaline pH.

Fig. 1D shows the reduction of $K_3Fe(CN)_6$ by glucose oxidase. During this reaction glucose oxidase mediates the transfer of one electron two times to $Fe(CN)_6^{3-}$, which is reduced to $Fe(CN)_6^{4-}$. The stain results from a reaction of ferrocyanide with Fe(III)chloride yielding Berlins blue. The uncatalyzed reduction of ferricyanide by aldoses is known as Hagedorn–Jensen reaction in sugar analysis. Catalysis by glucose oxidase makes it possible to perform the Hagedorn–Jensen reaction at pH 5 and room temperature instead of pH 12 and 100°C in the uncatalyzed variant. The sensitivity of the method was determined by using serial dilutions of glucose oxidase. Using gels with 0.75 mm thickness glucose oxidase activity can be detected down to concentrations of 0.5 units.

Another typical one-electron transfer reaction is the reduction of Cu(II) salts to Cu(I). Cu(I) ions precipitate with Reineckes salt. In this way the formation of Cu(I) can be visualized in the polyacrylamide gel. Fig. 1E documents the results obtained by glucose oxidase mediated oxidation of glucose in the presence of Cu(II) at pH 5.0 and room temperature leading to yellow precipitations at the site of the enzyme. Practically, the reaction is an enzyme-catalyzed variant of the Fehling reaction often used in sugar analysis. With this method glucose oxidase can be visualized down to about 1 unit.

In order to test the specificity of the staining procedures presented in this paper, we also tested varied flavoproteins, especially the so-called 'dehydrogenases' or 'transhydrogenases' and 'electron transferases' known to produce superoxide radicals

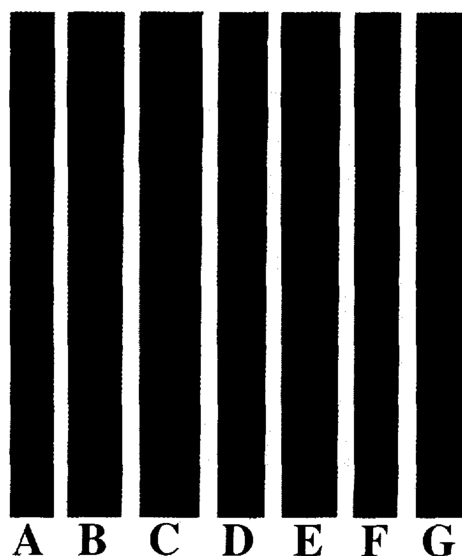

Fig. 1. Electropherogram and activity staining of the glucose oxidase/glucose-system with: (A) Coomassie brilliant blue (10 units); (B) *o*-dianisidine/peroxidase (2 units, pH 5); (C) *o*-dianisidine/peroxidase (2 units, pH 8); (D) ferricyanide (2 units, pH 5); (E) cuprisulfate. Lane (F) shows staining of a mixture of flavoproteins containing acyl-CoA-dehydrogenase, flavodoxin and xanthine oxidase with nitro blue tetrazolium (pH 8); (G) as (F) but with addition of 1 unit glucose oxidase.

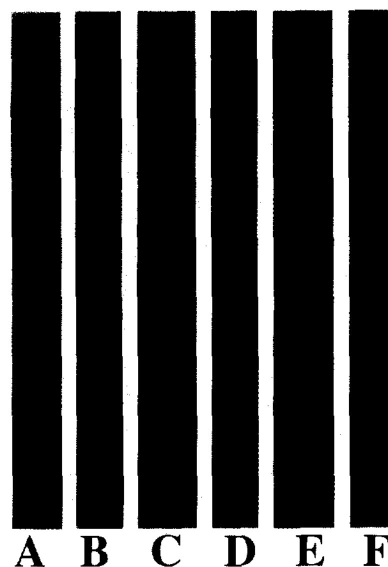

Fig. 2. Electropherogram and activity staining of the glucose oxidase/glucose-system with: (A) Coomassie brilliant blue (10 units); (B) nitro blue tetrazolium (1 unit, pH 5); (C) nitrotetrazolium blue (1 unit, pH 8); (D) nitrotetrazolium blue (1 unit, pH 8, anaerobic incubation); (E) nitro blue tetrazolium (2 units, pH 8, and incubation with 5 mg superoxide dismutase). Lane (F) ferricytochrome c/*o*-dianisidine/H_2O_2 (5 units, pH 5).

[13], with the same staining techniques. Fig. 1F shows an electropherogram of a flavoprotein mixture containing acyl-CoA dehydrogenase, flavodoxin and xanthine oxidase stained with nitrotetrazolium blue (pH 8). In contrast to lane G (Fig. 1), where 1 unit glucose oxidase from *Aspergillus niger* was added to the mixture, no staining is visible as long as glucose was used as electron donor. The same control tests have been done with ferricyanide, cuprisulfate and ferricytochrome c as electron acceptors. Again only on addition of glucose oxidase coloured bands appeared (not shown) and thus confirming the specificity of the staining procedures presented in this paper.

Besides ferricyanide and cuprisulfate, nitrotetrazolium blue (NBT) can be used to detect glucose oxidase on polyacrylamide gels. In contrast to Jos et al. [10] reduction of NBT by glucose oxidase takes place even without PMS. Fig. 2B,C shows that glucose oxidase catalyzed reduction of NBT in a polyacrylamide gel at pH 5.0 and at pH 8.0 leads to blue bands of formazan reaction products within the first half hour even with enzyme activities down to 0.2 units. At alkaline pH reduction of NBT is more effective than at pH 5.0.

Fig. 2F shows the results of the glucose oxidase catalyzed reduction of cytochrome c in a polyacrylamide gel at pH 5. Like other heme proteins cytochrome c looses its peroxidatic function when heme iron is reduced from its three-valent state to the two-valent form. The oxidized form of cytochrome *c* is capable of oxidizing *o*-dianisidine, which results in the formation of a brown background stain. Colourless zones appear at the location of glucose oxidase, which means that the enzyme catalyzes cytochrome c reduction simultaneously with glucose oxidation. In contrast to the three staining techniques presented above, reduction of ferricytochrome c is time-consuming, expensive and less sensitive. At least 5 units glucose oxidase are necessary for a regular detection.

In order to decide whether oxygen is necessary as an electron transfer mediator in the reactions described in the present paper, all staining procedures were also carried out under anaerobic conditions. Staining the gels with nitrogen-saturated solutions leads to inhibition of the reactions indicating that molecular oxygen is necessary as an electron mediator (shown in Fig. 2D for NBT reduction).

In order to look for superoxide radicals as possible intermediates we have incubated the gels with solutions containing superoxide dismutase (SOD). As Fig. 2E shows, reduction of NBT is influenced only to a small extent by SOD (at least under the staining conditions performed). Scanning and quantitative evaluation of the blue zones of formazan reaction products showed that SOD was able to inhibit about 15% of NBT-reduction by glucose oxidase in the gels. The dependence of the staining techniques on oxygen and their partial inhibition by superoxide dismutase has been shown also for ferricyanide, cuprisulfate and cytochrome c as electron acceptors (not shown).

Finally, we have also added hydrogen peroxide scavengers to the soak solutions to decide if the colour developments are based on H_2O_2 rather than superoxide radicals. Neither addition of bovine liver catalase (10^4 units) showed an effect on the colour developments (probably because of its high molecular mass which makes it unable to diffuse into the gel) nor addition of the low molecular α-ketoacid pyruvate (tested in a concentration range from 1 to 100 m*M*).

In contrast to HRP (M_r about 40 000) glucose oxidase staining procedures using ferricyanide, cuprisulfate and NBT need less time. When gels containing concentrations of acrylamide up to 15% are used it takes several hours until HRP soaks homogeneously into the gel matrix. The low molecular mass of the electron acceptors used in our work offers the possibility of completing the stains within half an hour. With glucose as electron donor to glucose oxidase the techniques presented are very specific.

Although a one-electron reduction product of oxygen could not be detected by ESR so far [16,17], the presented data give evidence for its occurrence as an intermediate. It is difficult to decide if there are two ways of electron efflux from glucose oxidase, one of which results in the generation of superoxide anions, whereas the other must be involved in the direct reduction of dioxygen to hydrogen peroxide, or if glucose oxidase like other flavoenzymes transfers the two electrons resulting from glucose oxidation to oxygen in two single steps and hydrogen peroxide production derives from the spontaneous dismutation of O_2^-.

Nevertheless the dependence of reduction of ferricyanide, cuprosulfate, NBT and ferricytochrome c by the glucose/glucose oxidase system on oxygen and pH and the partial inhibition of the stains by addition of superoxide dismutase gives some evidence for the occurrence of superoxide radicals in the reaction. Reduction of cytochrome c and NBT by superoxide radicals and their inhibition by superoxide dismutase are established chemical methods for the detection of these radicals [14,15]. The dependence on oxygen and the increased sensitivity of all staining procedures at alkaline pH (shown in Fig. 2C for NBT reduction, but also valid for ferricyanide, cuprisulfate and ferricytochrome c) are consistent with the increased stability of superoxide radicals at alkaline pH. Inhibition by superoxide dismutase is explainable only with the occurrence of superoxide radicals during the reaction. The inhibition was only partial, probably because the gels have not been swollen in distilled water and therefore the enzyme has not been enabled to diffuse into the gel. Further experiments have to be done, above all in aqueous solutions.

But besides the fact of having presented new fast and sensitive staining techniques of glucose oxidase in polyacrylamide gels our work could also be of strong interest in amperometric glucose biosensor techniques, where oxygen concentrations are often too small for reliable measurements over an extended time period. Probably one of the electron acceptors presented in our paper is a suitable potential redox couple to act as a mediator between oxygen and an electrode or (though not confirmed by our investigations) as an oxygen bypass, as has been reported for the most successful class of mediators based on ferrocene [18–20].

Acknowledgement

This work was supported by the Jubilaeumfond of the Austrian National bank (P4828).

References

[1] T. Nakamura and Y. Ogura, J. Biochem. (Tokyo), 52 (1962) 214.
[2] Q.H. Gibson, B.E.P. Swoboda and V. Massey, J. Biol. Chem., 239 (1964) 3927.
[3] B.E.P. Swoboda and V. Massey, J. Biol. Chem., 240 (1965) 2209.
[4] S. Nakamura and S.Y. Ogura, J. Biochem., 63 (1968) 308.
[5] H.J. Bright and M. Appleby, J. Biol. Chem., 244 (1969) 3625.
[6] B.E.P. Swoboda, Biochim. Biophys. Acta, 175 (1969) 365.
[7] S. Nakamura and Y. Ogura, J. Biochem., 64 (1968) 439.
[8] J.H. Pazur, K. Kleppe and A. Cepure, Arch. Biochem. Biophys., 111 (1965) 351.
[9] K.R. Frederick, J. Tung, R.S. Emerick, F.R. Masiarz, S. Chamberlain, A. Vasavada, S. Rosenberg, S. Chakraborty, L.M. Schopter and V. Massey, J. Biol. Chem., 265 (1990) 3793.
[10] J. Jos, J. Frezal, J. Rey and M. Lamy, Nature, 19 (1967) 516.
[11] Sigma Chemical Co. (1993), Assay procedure, Glucose oxidase No. 6891, P.O. Box 14508, St. Louis, MO 63178, USA.
[12] R. Ebermann and H. Bodenseher, Experienta, 24 (1968) 523.
[13] P.C. Engel, in F. Müller (Editor), Chemistry and Biochemistry of Flavoproteins, Vol. III, CRC Press, Boca Raton, FL, 1992, p. 597.
[14] I. Fridovich, in Handbook of Methods for Oxygen Radical Research, 4th edn., CRC Press, Boca Raton, FL, 1989, p. 121.
[15] C. Auclair and E. Voisin, in Handbook of Methods for Oxygen Radical Research, 4th edn., CRC Press, Boca Raton, FL, 1989, p. 123.
[16] V. Massey, S. Strickland, S.G. Mayhew, L.G. Howell, P.C. Engel, R.G. Matthews, M. Scuman and P.A. Sullivan, Biochem. Biophys. Acta, 36 (1969) 891.
[17] V. Massey, J. Biol. Chem., 269 (1994) 22459.
[18] E.A.H. Hall, Biosensors, Advanced Reference Series, Prentice Hall, Englewood Cliffs, NJ, 1991.
[19] A.E.G. Cass, G. Davis, G.D. Francis, H.A.O. Hill, W.J. Aston, I.J. Higgins, E.V. Platkin, D.L. Scott and A.P.F. Turner, Anal. Chem., 56 (1984) 667.
[20] J.E. Frew and H.A.O. Hill, Anal. Chem., 59 (1987) 933.