ECLÉTICA Química
www.scielo.br/eq
Volume 31, número 3, 2006

Callus cell culture of Pothomorphe umbellata (L.) under stress condition leads to high content of peroxidase enzyme

N. M. Khalil[1,2], M. A. M. Mello[2], S. C. França[3], L. A. A. Oliveira[1], O. M. M. F. Oliveira[2,]*
[1]*Departamento de Análises Clínicas, Faculdade de Ciências Farmacêuticas, UNESP, 14800-900, Araraquara, SP, Brasil*
[2]*Departamento de Bioquímica e Tecnologia Química, Instituto de Química, UNESP, CP 355, 14801-970, Araraquara, SP, Brasil*
[3]*Departamento de Biotecnologia, UNAERP, 14096-380, Ribeirão Preto, SP, Brasil*
[4]*Departamento de Química Geral e Inorgânica, Instituto de Química, UNESP, CP 355, 14801-970, Araraquara, SP, Brasil*
**E-mail address: olga@iq.unesp.br*

Abstract: *Pothomorphe umbellata* (L.) known on Brazil as Caapeba has a number of popular medicinal use, and it has been studied in relation to its pharmacological activity. Peroxidase specific activity (units/mg protein) was evaluated in callus cell culture samples of the *P.umbellata*, grown in two different MS medium (media 1 and media 2), submitted to 16 hours photoperiod or kept in darkness. Cell growth rate curve showed that the best growth indices were observed when media 2 submitted to the photoperiod regime was used, followed by the same media kept in darkness (stress condition). The results obtained also showed that the cell culture grown under stress conditions (darkness) lead to high content of peroxidase enzyme (an increase of 700% was observed). Kinetic constant values of 3.3 mmol.L^{-1} and 2,8 sec^{-1} were obtained for k_M and v_{max},, respectively, using guaiacol as enzyme substrate.

Keywords: Pothomorphe umbellata (L.); cell culture; peroxidase.

Introduction

Pothomorphe umbellata (L.) Miq. of the Piperaceae family, known on Brasil as Caapeba or Pariparoba, has a number of popular medicinal use, due to its soothing effect on choleretics, cholagogues and anti-epileptics activities, diabetes treatment and liver diseases [1]. This vegetal species has been studied in relation to some pharmacological activity, as: anti-edema, anti-PAF (platelet anti aggregation), antimalarial, antiulcerogenic and antioxidant [2-4]. The high antioxidant activity showed by ethanol extracts from *P.umbellata*, higher than the one showed by α-tocopherol, is attributed to 4-nerolidylcatechol (4-NC), a secondary metabolite produced [5]. The possibility of the development of the cell culture from isolated parts of plants makes possible to obtain commercial products of the natural compounds, mainly medicinal substances. The production of these natural substances by suspended cell cultures is more advantageous than the conventional isolation of the same compound from vegetal material of the agriculture products or of the *in nature* source [6]. By using cell cultures or cells reactor, it is possible to induce secondary metabolism to produce the molecule of the interest in large quantity.

Peroxidase (EC: 1.11.1.7) enzymes that catalyse oxidation reactions, involving hydrogen peroxide, are broadly distributed in various tissues and cellular compartments in plants, and plays an important role in plant physiological responses including anti-pathogen defense, modification of cell-wall properties and auxin catabolism [7]. Peroxidases are important

enzymes in living organisms, and have attracted considerable interest for decades due to its activity towards a wide array of chromogenic substrates, good stability, the simplicity of its kinetic analyses and biological implication. The availability and abundance of horseradish root peroxidase (HRP) has made this peroxidase the subject of numerous biochemical studies. Although expensive, purified HRP is the principal source for use as main component in clinical diagnosis, immune assay, bio-technologic research (biosensors), and others [8-12]. Therefore, it is important to study alternatives sources of this type of enzyme with similar physical-chemical properties, mainly specificity (k_M) on most used model substrate comparing with HRP.

The purposes of this work are: i) cell culture development from *P.umbellata*, ii) determination of the peroxidase activity during the period of cell culture growth in different media and light time conditions, and iii) extraction and determination of the kinetic constants of the *P.umbellata* peroxidase, using guaiacol as substrate model.

Experimental details

Plant material

P. umbellata leaves were collected at Biotechnology Laboratory – UNAERP- Ribeirão Preto – São Paulo-SP-Brasil. The voucher specimens are deposited in the Herbarium of the University of Campinas, São Paulo - Brasil.

Callus initiation

Aseptic leaf explants (1.0 cm^2) from 3-year-old micro propagated plantlets of *P. umbellata* [14] were inoculated on MS medium [15], supplemented with KH_2PO_4 (510 mg L^{-1}), sucrose (30 g/L), 2,4- dichlorophenoxy acetic acid (2,4-D; 5.0 mg L^{-1}) and kinetin (0.5 mg L^{-1}). *In vitro* cultures were prepared in 250 mL Erlenmeyer glass flasks containing medium gelled with 0.2% Phytagel (2 g L^{-1}; Sigma, St. Louis, MO, USA) at a pH of 5.7. Flasks were covered with aluminum foil sealed with plastic film. All cultures were kept at 27 ± 2°C, 55-60% of relative humidity, under the following media conditions: 16 hours light regime (40 μmol m^2 s; 85 W cool, white GE fluorescent lamps) or darkness [16]. Aseptic leaf explants (1.5 cm^2) from micro propagated plantlets of *P.umbellata* were inoculated on MS medium, supplemented with: i) 0.5 mg L^{-1} of the ANA (2-naphtaleno acetic acid), and 0.5 mg L^{-1} of the 6-BAP (6-benzylaminopurine), with photoperiod (16 hours light regime) and named media 01; and ii) 0.5 mg L^{-1} of the ANA and 3.0 mg L^{-1} of the 6-BAP, with photoperiod, or darkness, and named media 02. The growth of the callus was accompanied by the increase of its mass, which was measured in periods of 3 days during the total of 30 days of incubation.

Callus cell growth rate

At time 3, 6, 9 12, 15, 18, 21, 24, 27 and 30 days of the inoculation, light regime and darkness, flasks were take out to assay growth rate and POD activity. The growth rate curve was determined by measuring the increase of the cell mass quantity on each flask at time of incubation. (n=3).

Extraction of POD enzyme

Crude extracts were obtained from 13.1g of callus samples. In each sample the mass of the callus was triturated in mortar, with a volume of 40 mL of the 50 mM potassium phosphate buffer, pH 7.0, centrifuged at 3,000 g per 15 minutes at 4°C. Next, it was filtrated in 8 level of gauze tissue. Ammonium sulphate crystals were added to the filtrated solution, until a 70% saturation was achieved, in order to obtain the protein precipitate. After this, it was centrifuged at 9,000 g per 20 minutes at 4°C. The liquid phase was named “POD crude extract”. The solid phase, that showed no enzymatic activity, was discarded.

Elution on Sephadex G-25 column

After the extraction with ammonium sulphate the enzyme crude extract was desalted by elution in a column of Sephadex G-25 (1.6 x 30 cm, Sigma Chemical Co, St. Louis, MO), with phosphate buffer 50 mM at pH, and 2 mL fractions were at collected at a rate of 1.5 mL

min^{-1}. The fractions were assayed to verify the presence of proteins and phenols by measuring the absorbance at 280 nm in a HP Diode Array 8452A UV-VIS Spectrophotometer, coupled to a workstation, using cells of 1.0 cm of optical path. Peroxidase activity was determined on the protein fractions, using guaiacol as substrate. All fractions with peroxidase activity were stored under refrigeration, and named "POD extract".

Protein determination

Protein concentration was determined according to the method of biuret, using bovine albumin (from Sigma Chemical Co., St. Louis, MO) as protein standard [17].

Enzyme P. umbellata POD assay

All POD activity assays were measured by oxidation of guaiacol (Sigma Chemical Co., St. Louis, MO) as substrate. Tipical reaction mixtures contained 100µL of enzyme extract, 1.9 mL of 50 mmol/L phosphate buffer, pH 7.0, 1.0 mL of guaiacol 25 mmol/L, temperature at 25ºC and 20 µL of H_2O_2 0.34 mmol/L. The reaction was started by addition of 20µL of H_2O_2 , and the product formation was followed spectrophotometrically at 470 nm. The initial reaction rate (v_o) was determined by the angular coefficient of the plot of the absorbance at 470 nm versus time (in second), extrapolated to time zero. All reactions were made in triplicate (n=3).

Determination of Unit and Specific Activity of POD

One unit of peroxidase is defined as the quantity of the enzyme that oxidizes 1µM of guaiacol per second at 25ºC of pH 7.0: U.A. = $\Delta A/sec \times 10^6/\varepsilon \times Vt/Va$; where $\varepsilon = 26600\ M^{-1}.cm^{-1}$ at 470 nm , guaiacol molar extintion coefficient; Va = sample volume (mL); Vt = total reaction volume (mL) and ΔA= variation of absorbance at 470 nm.

Determination of k_M and v_{max}.

The effect of substrate concentration was determined by the measurement of the initial velocities (v_0), of *POD* activity, in the presence of various substrate concentrations. The k_M values and the maximum rate (v_{max}) were determined using the Lineweaver-Burk double reciprocal plot.

Statistical analyses

Average values of triplicates were calculated. The data obtained from the studies were analyzed using linear or quadratic regression.

Results and discussion

Callus cell growth rate and peroxidase activity determination

The data of development of *P. umbellata* cell culture samples after elution in Sephadex G-25 column are shown in Figure 1.When the development of the culture was studied, the growth indices of callus from *P.umbellata* in those different growth conditions, it can be seen that the results obtained in media 2 in darkness was similar (small decrease) to media 2 with photoperiod (figure 2).

The data show that the best results for production of peroxidase enzyme are obtained in media 2, kept in darkness. Then, the samples in darkness conditions were preferred to verify the kinetics parameters of its peroxidase enzyme (figure 3).

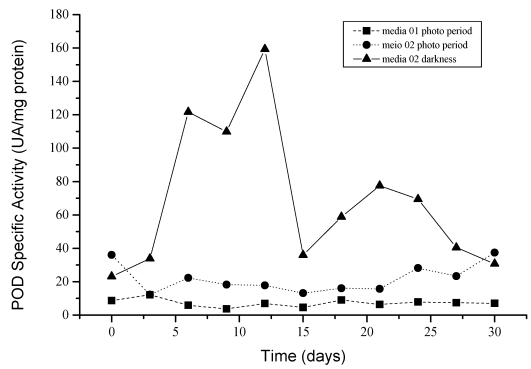

Figure 1. POD specific activity, during one cycle (30 days) of the development of *P.umbellata* cell culture under several culture conditions, after elution by Sephadex G25 column: —■— media n.º 01 with photo , —●— media n.º 02 with photo time, —Δ— media n.º 02 in darkness, n=3.

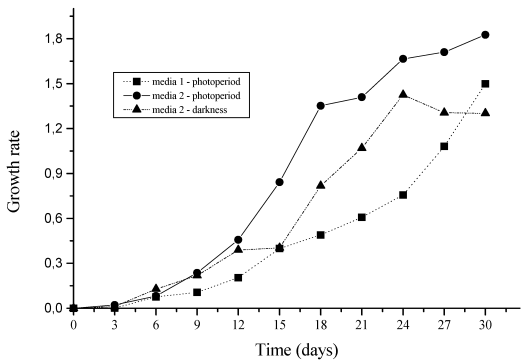

Figure 2. Correlation between growth indices of callus from P. umbellata in different culture and media conditions, during one cycle of 30 days:—■— media 01 (0.5 ANA e 0.5 BAP), photoperiod; —•— media 02 (0.5 ANA e 3.0 BAP),photoperiod; —Δ— media 02 (0.5 ANA e 3.0 BAP) darkness, n=3.

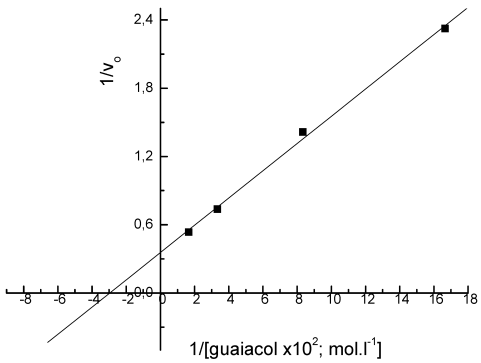

Figure 3. Duple-reciprocal plot of the POD enzyme from *P. umbellata*, using different concentrations of guaiacol, H_2O_2 (0.45 mM) in PBS buffer, 0.1 mM, pH 7.0 at 25º C (y= 0.3561+0.1197x and r^2=0.998), n=3.

Kinetics parameters

*C*rude extracts from *P.umbellata* showing peroxidase activity have small values of k_m, indicating very good specificity of the enzyme for guaiacol substrate, showed by the values of k_m and v_{max}, 3.3 mmol.L^{-1} and 2.8 sec^{-1}, respectively, determined from figure 3.

The *P. umbellata* POD k_m value was similar to turnip peroxidase (3.7 mmol.L^{-1}; ref. [9]), and lower than those found for tomato peroxidase (5-10 mmol.L^{-1}; ref. [18]) and for Korean radish roots peroxidase (6.7-13.8 mol.L^{-1}; ref. [19]). As it is known k_M represents the enzyme substrate affinity, lower values meaning higher specific activity.

Peroxidase enzyme has been considered one biomolecular marker of development of the cell and tissue cultures from plants. Krsnik-Rasol [20] showed that the activity of the peroxidase from selected embroiders has been significant increased before embryogenesis process in callus of the *Cucurbitapepo* (L), suggesting that the rise of the enzymatic activity was due to those tissue in which somatic embryogenesis is initiated.

In vitro, peroxidase mechanistic studies have been assayed with phenolic compounds. Guaiacol is used as hydrogen donator (considered as model substrate; refs.[21], [22]) on most of the assays, including those involving myeloperoxidase enzyme (from polymorphonuclear leucocytes) that is known by its specificity for chlorides ion [13]. Guaiacol is used as tool for identifying the classical cycle of the HRP. Then, it is a possibility to be a future source of the peroxidase for several applications (clinical diagnosis or industry) like HRP (peroxidase from horseradish root).

Conclusions

Cell growth rate curve showed that the best growth indices were observed when media 2 submitted to the photoperiod regime was used, followed by the same media kept in darkness (stress condition). The data also show that the best results for production of peroxidase enzyme are obtained in media 2, kept in stress conditions. Kinetic constant values of 3.3 mmol.L^{-1} and 2.8 sec^{-1} were obtained for k_M and v_{max}, respectively, using guaiacol as enzyme substrate.

Acknowledgments

The authors are grateful to the FAPESP and PROPP/UNESP, Brasil, for financial support, and to CAPES and FAPESP, for a scholarship to M. A. M. Mello and N. M. Khalil,

respectively, as well to Biotechnology Laboratory of UNAERP, Brasil, for helping the development of the cells culture of the *P.umbellata.*

Recebido em: 03/05/2006
Aceito em: 23/08/2006

N. M. Khalil, M. A. M. Mello, S. C. França, L. A. A. Oliveira, O. M. M. F. Oliveira. Cultura de células (calus) de *Pothomorphe umbellate* (L.) sob condições de estresse leva a alta produção da enzima peroxidase.

Resumo: *Pothomorphe umbellata* (L.), conhecida no Brasil como caapeba, possui diversos usos na medicina popular, sendo estudada especialmente em relação a sua atividade farmacológica. A atividade especifica da peroxidase (unidade/ mg proteina) foi determinada em amostras de *callus* (cultura de células) de *P. umbellata*, crescidos em dois tipos de meio MS (meio 1 e meio 2), sob condição de fotoperiodo de 16 horas ou mantidas no escuro (condições de estresse). Pela curva de velocidade de crescimento da cultura de células amostras do meio 2, sob fotoperiodo, apresentaram o melhor índice de crescimento, seguido de amostras do mesmo meio mantidas em condições de estresse. Os resultados também mostraram que a cultura de células crescidas sob condições de estresse levou a um aumento expressivo da quantidade da enzima peroxidase (aumento de 700%). Os valores das constantes cinéticas determinadas foram 3,3mmol.L^{-1} e 2,8 sec^{-1}, respectivamente, para k_M e v_{max}, usando guaiacol como substrato.

Palavras-chave: *Pothomorphe umbellata (L.); cultura celular; peroxidase.*

References

[1] I. Felzenszwalb, J.O.Valsa, A.C.Araujo, R. Alcantara-Gomes, Braz. J. Med. Biol. Res. 20 (1987) 403.
[2] M. de Ferreira-da-Cruz, Y.L.Adami, E. da Espinola-Mendes, M.R. Figueiredo, C.T. Daniel-Ribeiro, Exp. Parasitol. 94 (2000) 243.
[3] S.B.M.Barros, D.S.Teixeira, A.E. Aznar, J.A.J. Moreira, I.E. Ishii, P.C.D. Freitas, Ciência e Cultura 48 (1996) 114.
[4] C.Z. Amorim, C.A. Flores, B.E. Gomes, A.D. Marques, S.B. Cordeiro, J. Ethnopharmacol. 24 (1988) 101.
[5] C. Desmarchelier, S. Barros, M. Repetto, L.R. Latorre, M. Kato, J. Coussio, G. Ciccia, Planta Med. 63 (1997) 561.
[6] B. Deus, M.H. Zenk, Biotechnol.Bioengin. 24 (1982) 1965.
[7] P.J. O'Brien, Chem. Biol. Inter. 129 (2000) 113.
[8] I.L. Brunetti, O.M.M. Faria-Oliveira, Rev. Ciênc. Farm. 16 (1995) 55.
[9] M.A. Duarte-Vazquez, B.E. Garcia-Almendárez, C. Regalado, J.R. Whitaker, J. Agric. Food Chem. 49 (2001) 4450.
[10] J.H. Dawson, Science 240 (1988) 433.
[11] B. Chance, Arch. Biochem. Biophys. 41 (1952) 416.
[12] D. Dolphin, A. Forman, D.C. Borg, J. Fajer, R.H. Felton, Proc Natl Acad Sci U S A 68 (1971) 614.
[13] C. Capeillere-Blandin, Biochem. J. 336 (1998) 395.
[14] M.S. Moraes, F. Oliveira, B. Mancini, M.K. Akissue, G. Akissue, Rev. Ciênc. Farm. 9 (1987) 77.
[15] T. Murashige, F. Skoog, Biochem. 8 (1962) 4159.
[16] Y. Yamada, F. Sato, Plant Cell Physiol. 19 (1978) 691.
[17] R.F. Itzhaki, D.M. Gill, Anal Biochem. 9 (1964) 401.
[18] E. Heidrich, G. Lorenz, P. Schreier, Food Chem. 10 (1983) 285.
[19] M.Y. Lee, S.S. Kim, Phytochem. 35 (1994) 287.
[20] M. Krsnik-Rasol M., Int. J. Devel. Biol. 35 (1991) 259.
[21] K.K. Makinen, J. Tenovuo, Anal.Biochem. 126 (1982) 100.
[22] B. Balen, M. Krsnik-Rasol, V. Simeon-Rudolf, J. Plant. Physiol. 160 (2003) 1401.