Biocatalysis and Agricultural Biotechnology 4 (2015) 327–334

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Yerba mate as a novel inducer for fungal chlorogenate hydrolase production

Ana P. Butiuk [a], Osao Adachi [b], Roque A. Hours [a,*]

[a] Research and Development Center for Industrial Fermentations (CINDEFI; UNLP, CONICET La Plata), School of Science, La Plata National University, 47 y 115 (B1900ASH) La Plata, Argentina

[b] Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, Yamaguchi 753-8515, Japan

ARTICLE INFO

Article history:
Received 9 March 2015
Received in revised form
30 April 2015
Accepted 3 June 2015
Available online 9 June 2015

Keywords:
Chlorogenate hydrolase
Chlorogenic acid
Yerba mate (*Ilex paraguariensis*)
Aspergillus niger
Inducible enzymes

ABSTRACT

The potential of yerba mate (*Ilex paraguariensis*) to induce chlorogenate hydrolase (EC 3.1.1.42, CHase) activity in different fungal strains has been investigated. CHase was highly induced in mycelia of *Aspergillus niger* AKU 3302 grown in Czapek medium containing yerba mate extract as inducer. Different materials derived from yerba mate processing were examined as CHase activity inducers. All yerba mate samples produced CHase activity and a concentrated aqueous yerba mate extract was selected as the most convenient inducer. A response surface methodology was used to assess the simultaneous effect of inducer concentration and induction duration on enzyme production. The experimental design revealed that the optimum CHase production was achieved after long induction times ($\geq$ 25 h) and low inducer concentrations (0.1–0.2% w/v, dry basis).

1. Introduction

Hydroxycinnamic acids are widely distributed in nature, particularly in plant tissues. Some of them, such as ferulic and *p*-coumaric acids, exist as insoluble compounds, generally ester linked to sugar residues and they are thought to play an important role in the structure of the plant cell wall. Hydroxycinnamic acids also exist as soluble ester conjugates of quinic acid (QA); the best known conjugate is commonly referred to chlorogenic acid (CGA, 3-*O*-caffeoyl-quinic acid, 3-CQA). Isomers of CGA include 4-*O*-caffeoyl-quinic (cryptochlorogenic acid, 4-CQA) and 5-*O*-caffeoyl-quinic acid (neochlorogenic acid, 5-CQA). Closely related compounds containing two caffeic acid residues (dicaffeoylquinic acids, diCQA) are called isochlorogenic acids (3,4-diCQA, 3,5-diCQA and 4,5 diCQA). Currently, the term CGA is the trivial name used to describe the mixture of at least these 5 isomers. CGA is present in high concentrations in apples, pears and potato tubers, and mainly in coffee pulp, particularly from green coffee grains, material which is considered to date as the most important CGA natural source (Clifford, 1999, 2000).

Hydrolysis of CGA yields stoichiometric amounts of QA as well as caffeic acid (CA). CA is usually regarded as an antioxidant obtained from plant resources rich in phenolic compounds. It has a high antioxidant capacity, with multiple mechanisms involving free-radical scavenging, metal ion chelation, and inhibitory effects on some specific enzymes involved in free-radical formation (Chen and Ho, 1997; Stanely Mainzen Prince and Senthil Kumaran, 2012).

On the other hand, commercial QA is obtained in limited amounts from cinchona bark, coffee beans, and other plant products. It is considered as a versatile chiral starting material for the synthesis of new pharmaceuticals. However, differently from CA production, a least attention has been directed to date to QA production from plant resources because of more limited utilization of QA than CA. It was only reported that QA is readily converted to shikimic acid via 3-dehydroquinate and 3-dehydroshikimate (Adachi et al., 2006a, 2006b, 2006c). It should be mentioned that, among other synthetic pathways, shikimic acid is important as precursor for the synthesis of Oseltamivir, the potent and selective competitive inhibitor of influenza A and B neuraminidase (Enserink, 2006). Therefore, it would be desirable developing alternative processes for the competitive production of QA for further conversion into valuable fine chemicals, such as shikimic acid.

Chemical acid treatment of plant materials rich in CGA yields a mixture of CA and QA but accompanied with relatively high quantities of by-products, resulting their isolation and purification very laborious and costly. In addition, it produces significant amounts of highly contaminating wastes, difficult to be properly treated before final disposal. Thus, new alternative methodologies for processing the CGA contained in plant materials resulting in a cleaner and more competitive production of CA and QA are needed. In this sense, enzymes allowing the release of these

* Corresponding author. Fax: +54 221 483 3794x112.

http://dx.doi.org/10.1016/j.bcab.2015.06.002
1878-8181/

compounds are of special interest because of their potential industrial applications.

Chlorogenate hydrolase (CHase, EC 3.1.1.42) is the systematic name of an enzyme that catalyzes a site-selected hydrolysis of CGA. This enzyme belongs to the family of hydrolases, specifically those acting on carboxylic ester bonds. Other names in common use include chlorogenase, and chlorogenic acid esterase. It also acts, more slowly, on isochlorogenate. No other substrates are known.

There are limited data in the literature concerning the enzymatic hydrolysis of CGA. Schöbel and Pollmann (1980a, 1980b) isolated and characterized a tetrameric CHase from a pectinolytic enzyme preparation of *Aspergillus niger*. A hydroxycinnamic acid ester hydrolase was purified from *A. japonicus* by Okamura and Watanabe (1982b). Barbe and Dubourdieu (1998) isolated a dimeric cinnamate esterase from an industrial pectinase preparation of *A. niger* able to hydrolyze CGA. Then, Couteau et al. (2001) demonstrated the ability of human colonic bacteria to produce esterase activity against CGA. The purification and characterization of a novel specific CGA hydrolase from *A. niger* BRFM 131 was reported by Asther et al. (2005). These authors demonstrated the efficiency of this enzyme at releasing CA from natural agro-industrial by-products. The identification, isolation, and characterization of the first gene that encodes a CHase were reported by Benoit et al. (2007). Adachi et al. (2008) have developed a practical process for the QA and CA production from vegetable CGA resources. They prepared and characterized a coffee pulp koji as an immobilized microbial catalyst of CHase based on a high CHase induction in fungi by coffee powder or coffee pulp.

Various substrates have been used as culture media components for CHase production. Most of them are complex polysaccharidic materials such as wheat bran, agro-industrial by-products, sugar beet pulp, oat-spelt xylan and coffee derived materials. It is assumed that they contain enough concentrations of esterified compounds inducing CHase production by the microorganism. In these sense, coffee derived materials seem to contain the greatest CGA concentration thus resulting in a high enzyme induction. In addition, different cultivation systems, such as solid substrate fermentations as well as liquid cultures containing insoluble suspended solids, have been used employing the above mentioned substrates.

Ilex paraguariensis St. Hil. (Aquifoliaceae) (yerba mate) is one of the species in South America most employed as a decoction or infusion due to its nutritional and medicinal properties (tonic, choleretic, diuretic, anti-rheumatic, etc.). The main countries which produce yerba mate are: Argentina ($\approx$60% of the worldwide production accounting for 750,000 ton/year in average), Brazil and Paraguay. This species is currently being exported to Europe, USA, Syria and Eastern Asian countries, where it is commercialized as a vegetal drug and extracts. The production process of yerba mate involves the harvest of the older leaves of *I. paraguariensis*, which are dried over fire, milled, stored and packed for their commercialization. The industrialization process may vary among industries although the procedure is basically the same (Isolabella et al., 2010).

Chemical composition of yerba mate, particularly in relation to bioactive compounds, has been reported. Different caffeoyl derivatives were identified and quantified in significant amounts in extracts obtained by decoction of yerba mate samples; among others, CGA including different isomers of isochlorogenic acids (3,4-diCQA, 3,5-diCQA and 4,5 diCQA) (Heck and Demejia, 2007). Due to their closely related chemical structures, all these substances could be considered as potential inducers for CHase production. Concentration of fatty components and dark-colored polyphenolic compounds is apparently lesser in yerba mate than in coffee products. Therefore, it was considered interesting to examine yerba mate as a novel and competitive alternative to coffee products as CHase inducer.

The aim of the present paper was to evaluate the use of different yerba mate preparations as inducers of CHase activity in some fungal strains previously reported as CHase producers. As far as the author's knowledge, this is the first report on the use of yerba mate derived materials as CHase inducers as well as components of culture media.

2. Materials and methods

2.1. Materials

Chlorogenic (CGA), quinic (QA) and caffeic (CA) acids were products of Sigma-Aldrich (St. Louis, USA). Silica gel plates for thin layer chromatography were from Merck (TLC Silica gel 60, Lot: 60 F254, Darmstadt, Germany). All other chemicals employed were commercially available and of analytical grade.

Different yerba mate preparations were obtained from a local yerba mate processing company (La Cachuera S.A., Misiones Province, Argentina) and named as follows: concentrated aqueous yerba mate extract (YME), instant yerba mate powder without maltodextrin (−MD), instant yerba mate powder with maltodextrin (+MD) and yerba mate residue (the powder discarded after yerba mate processing, PD).

Concentrated aqueous extract (YME) and the soluble preparations (−MD and +MD) were prepared as follows. Processed yerba mate leaves (blanched, dried and aged under traditional conditions) were milled and sieved to remove particles of very small granulometry. Water soluble components of yerba mate were extracted in a stainless steel extractor vessel by adding 10 kg of yerba mate leaves to 45 L of hot water (80 °C). The mixture was kept for 10 min with continuous agitation to obtain an extract with a soluble solids concentration around 5% (w/v). Then, the extract was filtrated through a cellulose filter cartridge to remove insoluble particles ($Ø \geq 5$ μm). The resulting clarified filtrate was concentrated in an evaporator at a pressure of 60 mm Hg to obtain a concentrate (so-called YME) with a soluble solids concentration ≅25% (w/v). Then, YME was spray dried (drying air and exhaust air temperatures were 190 °C and 90 °C, respectively). Finally, a dry powder (−MD) with a moisture content of 3% was collected in a cyclone separator. To prepare +MD, enough amount of maltodextrin (D.E. 14–16) as dispersing agent was added with vigorous agitation to YME in order to achieve a soluble solids concentration ≅40% (w/v) and then spray dried as above mentioned. This final preparation (+MD), so-called instant mate tea, is commercially available in Argentina whereas YME and –MD can be obtained from the yerba mate processing company upon special request.

2.2. Fungal strains and culture media

Aspergillus niger AKU 3302, *A. kawachii* IFO 4308, *A. sojae* AKU 3312 and *A. sydowii* IFO 31296 were used in this study. AKU strains used were kindly provided by Prof. J. Ogawa, Kyoto University, Japan. A wild fungal strain isolated from yerba mate in our laboratory was also examined. Fungal spores were stored at −70 °C in a cryoprotectant medium (glycerol, 10 % and skim milk, 5%).

Modified liquid Czapek (MLC) medium contained (g/L): sucrose, 5; $NaNO_3$, 2; K_2HPO_4, 1; $MgSO_4$, 0.5; and yeast extract (Britania, Argentina), 3. The pH of the medium was spontaneously fixed to 7.0. Modified agar Czapek (MAC) medium was prepared by adding 25 g/L of sucrose and 15 g/L of agar to MLC.

2.3. Culture conditions for CHase induction in different fungal strains

Fungal strains above mentioned were evaluated for CHase activity as follows. Strains were propagated in MAC medium at 30 °C for 4–6 days. Cultures were carried out in triplicate, in 1000 mL-Erlenmeyer flasks, containing 200 mL of MLC medium inoculated with 1×10^6 spores/mL of medium and incubated with agitation (120 rpm) at 30 °C. Sucrose content was periodically evaluated in culture filtrates. After sucrose exhaustion (early stationary growth phase ≅24 h of cultivation), 0.50% (w/v, dry basis) YME was added as inducer of CHase activity and cultivation was continued for 24 h. The whole volume of the flasks was recorded and then filtered through a pre-weighed glass-fiber filter (0.45 μm pore size, E04WP04700, MSI, USA). pH of culture filtrate was determined. The mycelium was thoroughly washed with distilled water. Excess of water was carefully removed and wet weight of fresh fungal biomass was measured immediately after all the water has been pulled through. Then, a fraction of wet fungal biomass was separated, weighed, and used for dry weight determination (until constant weight at 80 °C). The remaining wet biomass was resuspended in sodium phosphate buffer (NPB; 2 mM, pH 7.0) (ratio: 1 g wet biomass +2 mL buffer), and milled in a mortar to homogenous gruel. The homogenate thus obtained was centrifuged ($10{,}000 \times g$) and the supernatant, designated as crude enzyme extract, was kept at −20 °C for CHase assay. In the case of *Aspergillus niger* AKU 3302, sampling was also done during the whole cultivation (growth and induction phases) using the above described methodology.

2.4. Effect of different yerba mate preparations as CHase inducers

The effect of different yerba mate preparations (YME, −MD, +MD and PD) as CHase inducers in *A. niger* AKU 3302 was evaluated. For this purpose, this strain was grown as above mentioned and, after sucrose exhaustion (≅24 h of cultivation), 0.25% (w/v, dry basis) of each preparation was added as CHase inducer. Cultivation was continued for 24 h and crude enzyme extract was prepared as above described for CHase assay. Thereafter, the best inducer concentration (in the range from 0.062 to 2.0% w/v, dry basis) and the optimal duration of induction time for CHase production were examined using a similar experimental methodology.

2.5. Measurement of chlorogenate hydrolase activity

CHase activity from crude enzyme extract was determined according to the method of Okamura and Watanabe (1982a) as follows: the reaction was carried out at 30 °C for 10 min, using 1.5 mM CGA solution in NPB (50 mM, pH 6.5) as substrate. The relative difference in absorbance at 350 nm was determined and CGA hydrolysis calculated. A difference in A_{350} of 1 is equivalent to the consumption of 0.14 μmol of CGA based on the molecular extinction coefficient ($\varepsilon_{CGA} = 7200\ mol^{-1}\ L^{-1}\ cm^{-1}$). CA showed no appreciable A_{350}. One unit of CHase activity was defined as the amount of enzyme that hydrolyzes 1 μmol of CGA per minute under the experimental conditions used. All assays were performed in triplicate and standard deviation was less than 5 % of the mean.

2.6. Measurement of chlorogenic acid content in CHase inducers

All yerba mate preparations (YME, −MD, +MD and PD) were dried to constant weight at 103 ± 2 °C and dry weight was calculated (average of triplicates). CGA content was determined by HPLC using a method adapted from Bravo et al. (2006). Samples (50 mg) were dissolved in 5 mL water. In case of PD, samples were extracted with 5 mL hot water (95 °C) during 10 min with shaking. Remaining solids were separated by centrifugation and CGA content was determined in the supernatant. Analysis was conducted using a Shimadzu Prominence gradient liquid chromatograph, equipped with a Shimadzu SIL-20 A HT autosampler and a UV/Vis Shimadzu SPD-M20A photodiode array detector (PDA). Aliquots (20 μL per sample, triplicates) were injected into the HPLC system, and separation was performed on a Phenomenex Prodigy ODS3 C18 reversed phase column (250 × 4.6 mm i.d., 5 μm particle size) protected with an ODS RP18 guard column. Runs were carried out at room temperature at a flow rate between 1–1.2 mL/min. A binary gradient of 1% formic acid in deionized water (solvent A) and acetonitrile (solvent B) was as follows: starting with 10% solvent B, linearly increasing to 17.5% solvent B in 15 min, increasing to 30% solvent B in 2 min and held at 30% solvent B for 8 min, then a linear decrease to 10% solvent B in 1 min. The PDA detector was set at 326 nm. Identification and quantitation of CGA was performed by comparing the retention times, the absorbance at 326 nm (maximum for the CGA) and absorption spectra obtained at 250–400 nm of the peaks of the samples with those of the standard compound and elution patterns with literature (Carini et al., 1998; Fang et al., 2002; Bravo et al., 2006). The CGA concentration was calculated on dry basis of the original yerba mate preparation using a standard curve of commercially available standard ($y = 6 \times 10^8 x - 18511$, $R^2 = 1$).

2.7. Sucrose determination

Sucrose content in fungal culture filtrates was evaluated after acid hydrolysis (0.125% v/v $HCl_{conc.}$), 15 min, in a boiling water bath followed by pH neutralization with $NaHCO_3$ (solid). Glucose liberated from sucrose was determined with a glucose oxidase/peroxidase enzymatic commercial kit (Wiener, Argentina) against a reaction blank without hydrolysis (Bergmeyer and Bernt, 1974).

2.8. Thin layer chromatography

Apparent CHase activity was determined by TLC. Crude enzyme extract was reacted with CGA and incubated under the usual conditions as described above. After standing the reaction mixture in ice-cold water bath, an aliquot of the sample (12 μL) was spotted on aluminum TLC plate and the chromatography performed by using the ascending method with *n*-butanol/acetic acid/water (4:2:1, v/v/v) as the solvent system. Authentic CGA and CA used as standards gave Rf values of 0.72–0.74 and 0.88–0.89, respectively. Disappearance of CGA and appearance of QA and CA in reaction samples were examined on TLC by UV lamp and used to evaluate CHase activity. Since no phenol moiety is present in QA, it does not show absorption in the UV region. Thus, the location of QA was indicated approximately from the trace of authentic QA showing a Rf value of 0.15 according to a previous paper (Adachi et al., 2008).

2.9. Doehlert experimental design

Doehlert experimental design (Doehlert, 1970) was applied to optimize induction procedure of fungal CHase. The number of experiments required in this design (N) is given by $N = n^2 + n + n_0$, where n is the number of variables and n_0 is the number of central points. In our case, the n_0 value was fixed at 3. Thus, with two factors (inducer concentration and induction time duration), the total of points of Doehlert matrix was 9. Replicates at the central level of the variables were performed in order to have an estimation of the experimental variance. For optimization of CHase induction, the central values were fixed to 22 h and 1.05% (w/v, dry basis) of YME. The real values of the independent variables were coded based on a linear functionality between codified (Z) and

actual values (X) according to:

$$X = Z \times \frac{\Delta X}{\Delta Z} + X_0$$

where X_0 is the real value of the central point and ΔX and ΔZ are the difference between the highest and the lowest values of real and coded numbers, respectively.

Multiple regression analysis based on the least square method was performed using Mathcad 2001 software.

For simplicity a full quadratic model containing six coefficients was used to describe the response observed.

$$Y = b_0 + \sum b_i x_i + \sum b_{ii} x_{ii}^2 + \sum b_{ij} x_i x_j$$

where Y is the response variable, b_0 a constant, and b_i, b_{ii}, and b_{ij} are the coefficients for the linear, quadratic and interaction effect, respectively.

3. Results and discussion

3.1. Evaluation of fungal strains producing chlorogenate hydrolase activity

The choice of an appropriate substrate is of great importance for the successful production of any inducible enzyme. In most of the reported cases for fungal esterases, the substrate used was the main component of the culture medium, being present since the beginning of cultivation. Therefore, it not only served as a carbon and energy source, but also providing the necessary inducing compounds for the microorganism. Complex polymeric plant materials containing high amounts of esterified substrates, such as wheat bran, de-starched wheat bran, maize bran, brewers spent grain, sugar beet pulp and others, have been successfully employed (Topakas et al., 2007). These substrates are mainly composed of water-insoluble materials; therefore, it could be assumed that they provide a continuous supply of nutrients (as well as inducers) by slow solution into the medium due to degradation of the original polysaccharide matrix along with cultivation time. In the particular case of CHases, the use of wheat bran and sugar beet pulp as substrates has been reported. Nevertheless, it seems much more reasonable to use plant materials containing significant amounts of CGA or closely related substances as culture media components. Thus, they can provide the necessary inducing compounds to enhance CHase production as it was the reported case for coffee pulp (Adachi et al., 2008). In this sense, yerba mate derived materials also seem to fulfill this condition. But in this case, a different cultivation strategy has to be followed because soluble compounds contained in yerba mate are easily, completely and rapidly dissolved in water. In addition, they display a certain antimicrobial (particularly antifungal) activity due to their high polyphenol content (Filip et al., 2010). Therefore, yerba mate materials have to be added at the stationary growth phase in order to avoid any inhibitory effect on microbial growth. This type of culture comprises two phases, the first or biomass production phase and the second so-called the induction phase. This induction outline was followed in the course of our investigation as below described.

Different fungal strains (*A. niger* AKU 3302, *A. kawachii* IFO 4308, *A. sojae* AKU 3312 and *A. sydowii* IFO 31296 as well as a wild strain locally isolated from yerba mate) were evaluated for CHase production in liquid cultures. All fungi grew well in MLC medium and exhibited a pelleted growth form (Ø≅2–4 mm). In all cultures, sucrose was consumed completely in around 24 h of cultivation (early stationary growth phase). No substantial differences were observed in the culture parameters for the strains tested (biomass

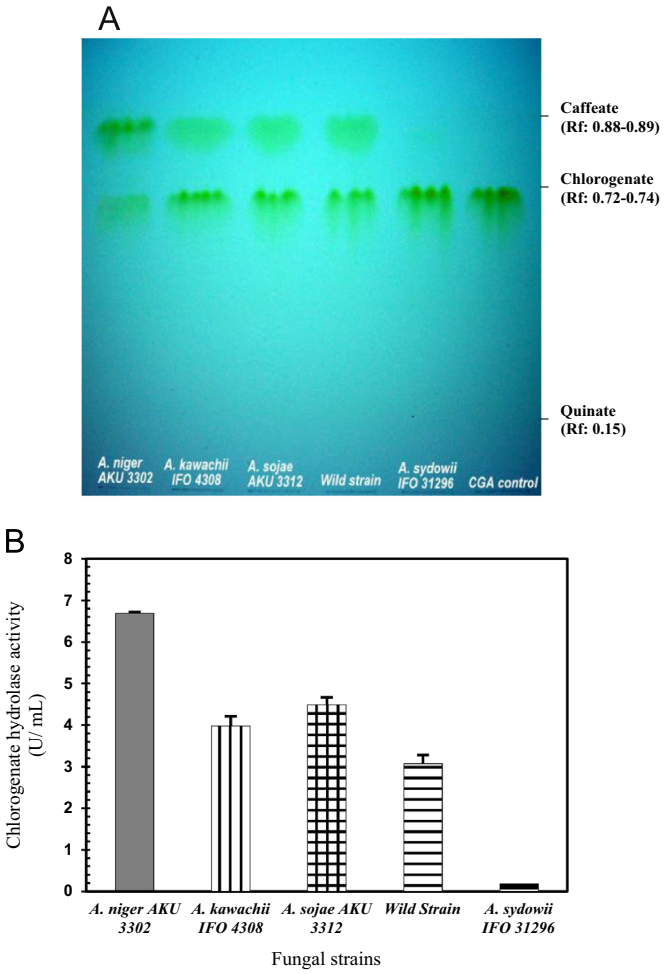

Fig. 1. Screening of CHase producing fungal strains. Enzyme activity was determined by: A) TLC, by checking appearance of caffeic acid and consumption of chlorogenic acid in enzyme reaction mixture, and B) spectrophotometrically, by reading the absorbance change at 350 nm. A: Crude enzyme extract was prepared from mycelia grown in modified liquid Czapek medium containing 0.5% (w/v, dry basis) concentrated aqueous yerba mate extract as enzyme inducer. Crude enzyme extract (15 μL) was incubated at 30 °C for 10 min with 1.5 mM chlorogenic acid in sodium phosphate buffer (50 mM, pH 7.0), in a total volume of 0.265 mL. An aliquot of the reaction mixture (12 μL) was developed with TLC. B: Crude enzyme extract was prepared and incubated with chlorogenic acid under similar conditions as in A and the reaction mixture was stopped with 5 mL of 80% (v/v) methanol. Then, the relative difference of absorbance at 350 nm was determined and CHase activity calculated.

dry weight in a range from 2.0 to 2.2 g/L, pH from 7.8 to 8.2). At this stage, cultures were induced by the addition of YME (0.5%, w/v dry basis). As YMC is a syrupy liquid, it was quantified in mass units. No CHase activity was detected in any case prior to induction both in mycelia and in culture filtrates (data not shown). On the contrary, CHase activity in crude enzyme extracts from fungal mycelia was evident 24 h after induction. It should be mentioned that CHase activity measurement by reading the absorbance of CGA at 350 nm was difficult to do by the presence of highly UV-intensive materials in crude enzyme extract. Therefore, in some cases CHase activity was also examined with TLC by checking appearance of CA and consumption of CGA in order to reconfirm the data. Fig. 1A shows the results obtained by TLC after enzymatic digestion of authentic CGA by mycelial crude extracts from the different strains examined, and Fig. 1B shows the corresponding CHase activities spectrophotometrically determined.

The TLC analysis of CGA hydrolysis indicates that all fungal strains, except for the case of *A. sydowii* IFO 31296, showed detectable mycelial CHase activities. Decreasing intensity of CGA

spots (Rf: 0.72–0.74) and the concomitant increment of CA spots (Rf: 0.88–0.89) are clearly observed (Fig. 1A) and associated to CHase activity. Quantitative determination of CHase activity produced by the different fungi resulted in values ranged from 0.17 U/mL (*A. sydowii* IFO 31296) to 6.49 U/mL (*A. niger* AKU 3302) (Fig. 1B) in well accordance with those visualized in TLC plates.

As similarly seen for fungal CHase induction with coffee derived products (instant coffee powder and coffee pulp) (Adachi et al., 2008), CHase activity was only found after induction, particularly in mycelia whereas very low enzyme activity was detected in culture filtrates. On the contrary, Asther et al. (2005) reported the presence of an extracellular CHase activity in culture filtrates from *A. niger* BRFM 131 grown on sugar beet pulp as carbon and energy source and inducer during 3–12 days. Okamura and Watanabe (1982b) also purified CHase after extraction of *A. japonicus* mycelia developed on wheat bran culture for 8 days. The existence of intra or extracellular fungal CHase seems to be controversial and depends on the strain evaluated, even into the same fungal genus.

In addition, Adachi et al. (2008) screened a set of fungal strains including *A. niger* AKU 3302 and *A. sojae* AKU 3312 for CHase production with coffee derived products. Interestingly, they found a higher CHase activity in *A. sojae* AKU 3312 than in *A. niger* AKU 3302 (determined by TLC analysis), the reverse of our results. We could not find a satisfactory explanation to the difference in behavior of these fungal strains, but it could be associated to the type of inducer used. Nevertheless, it can be concluded that induction mechanisms involved in the utilization of different natural complex materials as inducers are quite complex and the results obtained difficult to extrapolate.

A. niger AKU 3302 exhibited the highest CHase activity among the tested strains induced with YME and thus it was selected for further experiments.

3.2. Effect of different inducers on chlorogenate hydrolase production by A. niger AKU 3302

Different materials derived from yerba mate processing were evaluated and compared as potential inducers of CHase production in *A. niger* AKU 3302. YME, -MD and +MD are instant completely soluble materials whereas PD corresponds to a powder generally discarded from the grinding stage of yerba mate processing. This material contains a high level of water soluble polyphenols including CGA among others (Vieira et al., 2010). Complete solubilization of CGA contained in PD is comparatively rapid, even at 30 °C (data not shown). Thus, due to its null price and interesting chemical composition, it emerges as an interesting candidate as CHase inducer.

Cultures were carried out as above described and after sucrose exhaustion (early stationary growth phase ≅24 h) they were induced by adding 0.25% (w/v, dry basis) of each inducer and incubated during a fixed time (24 h of induction). The amount of inducer used was arbitrary chosen. It should be mentioned that inducer addition was carried out under non-aseptic conditions; nevertheless, no microbial contamination was observed in any case. The antimicrobial activity ascribed to yerba mate could be partially associated to this phenomenon. CHase activity in crude enzyme extracts was examined with TLC by checking appearance of CA and disappearance of CGA as above described. As shown in Fig. 2, all yerba mate materials induced CHase activity. As can be seen, YME and −MD gave very similar results, with an apparent CHase activity slightly higher in the first case. A higher CHase induction was observed with −MD when compared with +MD. The lowest CHase activity was detected in PD induced cultures. Moreover, it is interesting to note that insoluble particles of PD could not be visualized after separation of fungal biomass by glass-

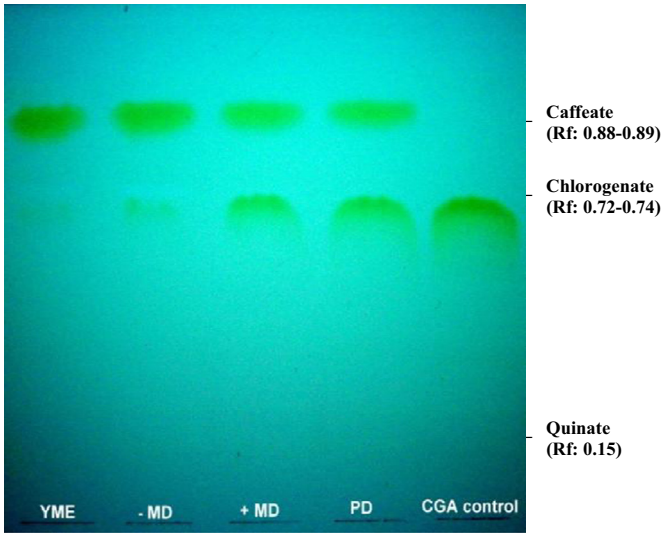

Fig. 2. Different materials derived from yerba mate processing were examined as CHase inducers in cultures of *A. niger* AKU 3302. CHase activity was evaluated with TLC by checking appearance of caffeic acid and consumption of chlorogenic acid in the enzyme reaction mixture. YME: concentrated aqueous yerba mate extract; −MD: Instant yerba mate powder without maltodextrin; +MD: Instant yerba mate powder with maltodextrin; PD: Yerba mate powder (the residue discarded after yerba mate processing).

fiber filtration; thus, it was assumed that *A. niger* AKU 3302 is able to produce cellulolytic (and/or hemicellulolytic) enzymes able to degrade the solid insoluble matrix of PD particles.

In order to clarify the above described results, it was considered important to evaluate the total CGA content in the different materials used as CHase inducers. Fig. 3 shows a representative HPLC chromatogram of YME. In our case, total CGA content was calculated as the sum of the different isomers above mentioned (neochlorogenic acid, chlorogenic acid and crypto-chlorogenic acid). Values shown in Table 1 represent the total CGA content determined by HPLC in each material (on dry basis) as well as the corresponding total CGA concentration in culture media after addition of 0.25% (w/v, dry basis) of each inducer. As expected, no significant differences were found in total CGA content in YME and −MD (≅5.7 g CGA per 100 g). Differences found between total CGA content in −MD and +MD were proportional to the addition of maltodextrin. The lowest total CGA content was found in PD. As far as the authors are aware and believe, this is the first report on the quantification of total CGA content in complex materials used as enzyme inducers for the production of fungal CHase.

Total CGA content in the corresponding inducer was in good accordance with apparent CHase activities detected. Therefore, we concluded that total concentration of the different isomers of CGA in culture media plays a key role on CHase induction in *A. niger* AKU 3302. Due to practical reasons (i.e.: easy preparation, very high stability with time), YME was chosen for the next study.

3.3. Effect of different concentrations of yerba mate extract on chlorogenate hydrolase production by A. niger AKU 3302

The effect of YME concentration on CHase production by *A. niger* AKU 3302 was examined. For this purpose, the culture was induced with different amounts of YME as above described and incubated during a fixed time (24 h of induction). CHase activity obtained from mycelial crude extract is shown in Fig. 4A and B. As can be observed, CHase activity was not detected in crude enzyme extracts from the non-induced culture (CA spot was absent on TLC analysis in good accordance with spectrophotometric determinations). It seems that the expression system for CHase in this fungal

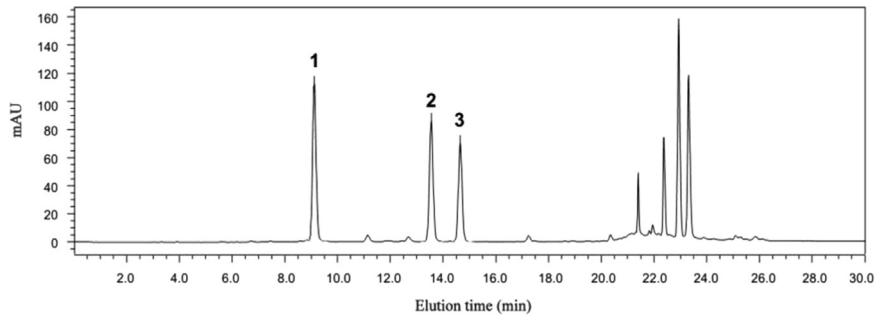

Fig. 3. Representative HPLC chromatogram of yerba mate extract. Signal at 326 nm. (**1**): neo-chlorogenic acid (**2**): chlorogenic acid and (**3**): crypto-chlorogenic acid.

Table 1
Total CGA content in different CHase inducers and the resulting total CGA concentration in culture media after addition of 0.25% (w/v, dry basis) of each inducer.

Inducer	CGA (g per 100 g)	CGA (mg per L of culture medium)
YME	$5.68^{a} \pm 0.02$	$141.86^{a} \pm 0.00$
−MD	$5.72^{a} \pm 0.26$	$143.12^{a} \pm 6.49$
+MD	$3.70^{b} \pm 0.18$	$92.43^{b} \pm 4.57$
PD	$2.22^{c} \pm 0.14$	$55.53^{c} \pm 3.61$

YME: concentrated aqueous yerba mate extract. −MD: Instant yerba mate powder without maltodextrin. +MD: Instant yerba mate powder with maltodextrin. PD: Yerba mate residue (the powder discarded after yerba mate processing).
In each column, different superscripts ($^{a, b, c}$) indicate significant difference ($p < 0.05$).

strain remains silent under non-induced culture conditions even at longer cultivation times (48 h in total). Similar results were obtained by Adachi et al. (2008), who reported that CHase activity was not detected with cell-free extracts prepared from mycelia of *A. sojae* AKU 3312 grown in the absence of inducer (coffee powder or coffee pulp). This fact is a new evidence of the inducible nature of fungal CHases.

Addition of YME resulted in the induction of CHase activity in all concentrations tested. TLC analysis of CGA and its hydrolysis products indicated that when the inducer concentration increased from 0.25 to 2.0% (w/v, dry basis), residual CGA increased with concomitant reduction in CA (Fig. 4A). Spectrophotometric determination of enzyme activity revealed that the highest values of CHase obtained were 11.6 U/mL and 11.3 U/mL, using 0.125% and 0.25% (w/v, dry basis) of YME, respectively (Fig. 4B). Analysis of variance ($p < 0.05$) revealed no significant differences between these figures. Interestingly, CHase activity achieved using 0.125% (w/v, dry basis) of YME is substantially higher when compared with the result obtained using +MD (as shown in Fig. 2). In both cases, CGA concentration in culture media is similar (≅70 mg/L and 92 mg/L, respectively). This apparently contradictory result was tentatively ascribed to the presence of maltodextrin in +MD that could induce the production of fungal amylases with a concomitant negative effect onto CHase induction.

Decreasing CHase activities were found using concentrations of YME higher than 0.25% (w/v, dry basis) in well accordance with TLC results. The lowest value of CHase obtained was 3.8 U/mL, with 2.0% (w/v, dry basis) of YME. These results could be due to an inhibitory effect of YME on CHase production. Similarly, Adachi et al. (2008) reported that addition of coffee powder at concentrations higher than 2% (w/v) to a culture of *A. sojae* AKU 3312 appeared to be growth inhibitory.

Fungal biomass dry weight was determined at the end of biomass production phase (after sucrose exhaustion) and during induction phase using 0.25% (w/v, dry basis) of YME. After 24 h of induction, the biomass produced was 3.1 g/L, which represents a net biomass production of ≅1 g/L during the induction phase. Therefore, it was concluded that organic compounds in YME induce CHase activity but also they can be assimilated as carbon and energy source by *A. niger* AKU 3302.

3.4. Combined effect of concentration of yerba mate extract and duration of induction time on chlorogenate hydrolase production by *A. niger AKU 3302*

Finally, a Doehlert experimental design was applied to assess the combined effect of inducer concentration and induction time duration on CHase production. For this purpose, the culture was induced with different amounts of YME (0.1, 0.57, 1.05, 1.53, 1.57, and 2% w/v, dry basis) as above described and then incubated during variable induction times (11, 22 and 33 h). The matrix used in the Doehlert design and the results of enzyme activity obtained

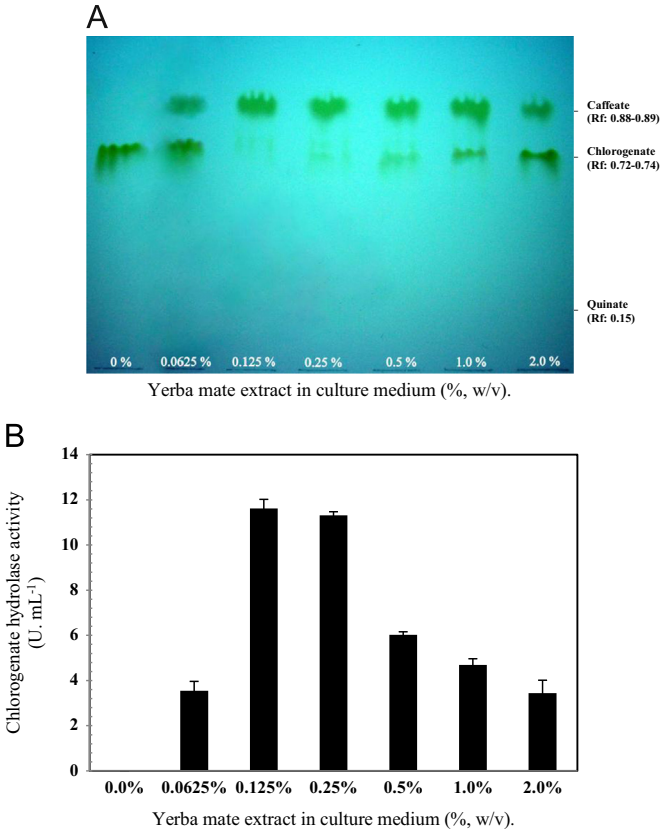

Fig. 4. Effect of different concentrations of yerba mate extract on CHase induction in cultures of *A. niger* AKU 3302. Enzyme crude extracts were incubated with chlorogenic acid under similar reaction conditions as in Fig. 1A. Enzyme activity was determined by: A) TLC, by checking appearance of caffeic acid and consumption of chlorogenic acid in enzyme reaction mixture, and B) spectrophotometrically, by reading the absorbance change at 350 nm.

Table 2
Codified, actual values and enzyme activity attained in the Doehlert design with two variables.

	Codified values		Actual values		CHase activity (U/mL)
	YME (w/v)	Time (h)	YME (w/v)	Time (h)	
1	+1.0	0	2.00	22	3.9
2	+0.5	−0.866	1.53	11	1.3
3	−0.5	−0.866	0.57	11	3.8
4	−1.0	0	0.10	22	10.7
5	−0.5	+0.866	0.57	33	8.3
6	+0.5	+0.866	1.53	33	4.1
7	0	0	1.05	22	5.0
8	0	0	1.05	22	5.0
9	0	0	1.05	22	4.8

YME: yerba mate extract.

Table 3
Significance level and value of the coefficients, calculated by ANOVA, in the full quadratic model obtained according to Doehlert design.

	Coefficient	*P*
Constant	4.94	
Concentration	−3.37	0.0008
Time	2.13	0.0003
Concentration^2	2.41	0.0036
Time^2	−1.54	0.0015
Concentration • time	−1.01	0.0131
R^2=0.99		

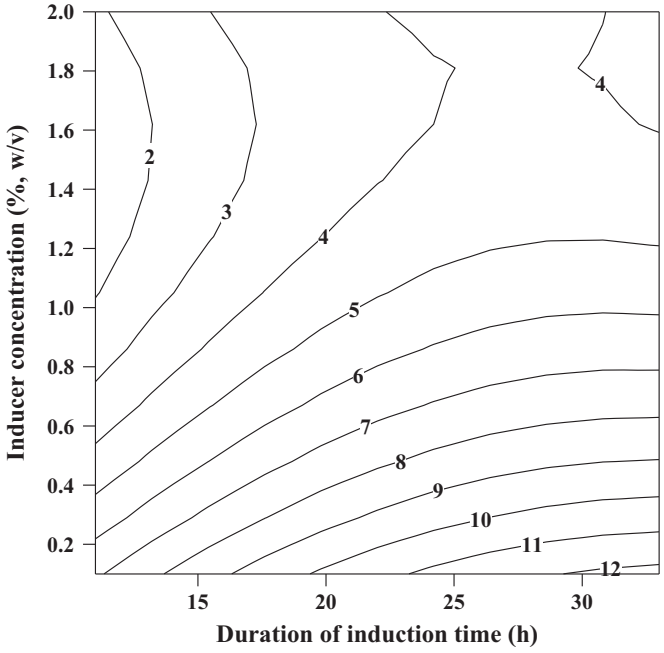

Fig. 5. Contour plot showing the effect of duration of induction time and concentration of inducer (concentrated yerba mate extract) on CHase production by *A. niger* AKU 3302.

in each experiment are shown in Table 2. Data in Table 2 were fitted to a second order polynomial equation. Values of linear, quadratic, and cross coefficients and their level of significance (calculated by ANOVA) are given in Table 3.

The statistics analysis showed that both, YME concentration and duration of induction time, as well as their interactions, had a significant effect on enzyme production, in the range studied. The regression coefficient found (R^2=0.99) indicates that the model was able to explain more than 99% of the observed response.

As can be seen in the contour plot (Fig. 5), the direction of the path of steepest ascent for in CHase activity was towards lower inducer concentrations and longer duration of induction times. The experimental design revealed that the optimal CHase production can be achieved after an induction time ≥ 25 h and an inducer concentration between 0.1–0.2% (w/v, dry basis) of YME. Under these conditions it could be obtained an activity of ~12 U/mL. The model was validated with data obtained from two independent cultures for CHase production. The induction conditions evaluated were 0.15% and 28 h and 0.2% and 32 h for YME (w/v, dry basis) concentration and for induction time, respectively. Values of CHase activity obtained in these cases were 11.5 U/mL and 12.6 U/mL (average of triplicates) indicating that the difference between values of CHase activity obtained with those predicted by the model was less than 10%.

It is interesting to observe that, according to this model, CHase activity tends to increase along with duration of induction time, particularly for low inducer concentrations. For example, 0.2% (w/v, dry basis) of inducer resulted in a CHase activity of 6.0 U/mL after around 10 h of induction, and this value almost duplicated after 30 h of induction. On the contrary, high inducer concentrations such as 1.8% (w/v, dry basis) resulted in low CHase activities which tend to decrease after 30 h of induction. Therefore, it could be assumed that net enzyme synthesis lasts during the whole incubation time and that CHase activity is very stable into fungal mycelia. These results suggest that detailed studies on the mechanism of fungal CHase induction and its intracellular stability are needed in order to clarify the present observations.

4. Conclusions

The inducible nature of fungal CHase was confirmed. CHase activity was detected in mycelia of various fungal strains when induced by different yerba mate materials containing CGA but not in non-induced cultures. Culture filtrates also showed negative CHase activities both in induced and non-induced cultures. *A. niger* AKU 3302 exhibited the highest CHase activity and YME proved to be the most convenient inducer among the different yerba mate materials evaluated. The effectiveness of YME as a powerful inducer of CHase activity was evaluated using a Doehlert design. Optimal induction was obtained at low inducer concentrations and long induction times.

Studies on the preparation, characterization and utilization of a fungal biocatalyst containing CHase activity are in course and results will be published elsewhere.

Acknowledgments

The authors thank Professor J. Ogawa, Kyoto University, and La Cachuera S.A. Inc., Misiones, Argentina, for their generosity in providing fungal strains and yerba mate samples, respectively, all used in this study. This investigation was partially supported by grants from National Institute of Yerba Mate (INYM, Argentina (PRASY N° 049/11)), Argentina National Research Council (CONICET, PIP 0662) and National University of La Plata (UNLP, 11/X650) to R. Hours. Moreover, a part of this work was supported by FY 2011–2013 Grant-in-Aid for Scientific Research (C), JSPS KAKENHI 23580135 to O. Adachi.

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