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Characterization and application of fungal chlorogenate hydrolase to enzymatic breaking down of chlorogenate from yerba mate

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ABSTRACT

The aim of this work was to study the physicochemical and kinetic properties of chlorogenate hydrolase (CHase) (EC 3.1.1.42) of *Aspergillus niger* AKU 3302 by induction in the presence of yerba mate extract. The data obtained could be applicable directly to mass processing for a plant scale production of quinic acid (QA) and caffeic acid (CA) with *koji* CHase. It is fundamentally significant to accumulate catalytic properties of CHase as much as possible with crude CHase, when *koji* CHase is directed to practical industrial processing of breakdown of chlorogenate (CGA). CHase exhibited a high thermal stability without appreciable loss after standing for 6 h at 50 °C and was stable in wide pH ranges from 2.0 to 9.0. The optimum pH and temperature of CHase activity were found at 6.5–7.0 and 45 °C, respectively. The V_{max} and K_m values for CGA hydrolysis were 1.996 unit mg^{-1} and 0.72 mM, respectively. The activation energy of CHase was estimated to be 26 kJ mol^{-1}. Thus, CHase induced with CGA revealed successful for the production of CA and QA, fine chemicals of high interest and commercial values, from natural plant resources rich in CGA such as yerba mate. Several advantageous merits obtained with a crude CHase for the practical formation of QA and CA from yerba mate or yerba mate extract are discussed.

1. Introduction

Phenolics of plant origin are the most widespread dietary antioxidants, and they may play a role preventing of cardiovascular diseases and cancers (Rice-Evans et al., 1996). With its high bioavailability, chlorogenic acid (CGA) is an important and yet overlooked bioactive dietary component. Moreover, the hydrolytic products, quinic acid (QA) and caffeic acid (CA) are important as fine chemicals of high interest and commercial values. CA is produced from plant resources rich in phenolic compounds and has a high antioxidant ability, with multiple mechanisms involving free-radical scavenging, metal ion chelation, and inhibitory effects to some specific enzymes involved in free-radical formation (Chen and Ho, 1997). On the other hand, commercially available QA is limited from cinchona bark, coffee beans, and other plant resources. It is considered as a versatile chiral starting material for the synthesis of new pharmaceuticals (Chen and Ho, 1997; Adachi et al., 2006a, 2006b, 2006c, 2008; Enserink, 2006; Stanely Mainzen Prince and Senthil Kumaran, 2012). CGA is produced by plants in response to environmental stress and it is naturally present in high concentrations in apples, pears and potato tubers, and abundantly in coffee pulp, particularly in green coffee grains (Clifford, 1999, 2000). Yerba mate (*Ilex paraguariensis* A. St. Hil.) is an arboreal species that naturally grows in forests in the sub-tropical climatic regions of Argentina, Brazil and Paraguay. It is widely known and used by the inhabitants of these countries. Branches of less than 10 mm diameter and mainly the leaves of the tree are the basis for the preparation of hot or cold infusions, traditionally known for being tonic stimulants and digestive drinks. The commercial products prepared from yerba mate are also named "yerba mate" or "yerba" and are currently being exported to Europe, US, Syria and Japan. A relatively high CGA content (around 8–10 g/100 g dry weigh) is reported in yerba mate leaves and stems with very low levels of interfering substances for CGA extraction such as fatty materials (Isolabella et al., 2010; Marques and Farah, 2009; Pagliosa et al., 2010).

In our own investigation, we found that the highest CGA content was observed in green leaves and stems of just harvested yerba mate and such high CGA content was superior to those reported in coffee products (Butiuk et al., 2016). It is also worthy to add that relatively

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high CGA content was found with root, stems, saw dust from trunk of yerba mate trees. Thus, the whole parts of yerba mate tree can be used for CGA extraction. In the previous studies, we examined the production of fungal CHase capable of hydrolyzing CGA into QA and CA. We demonstrated that CHase was highly induced in mycelia of *Aspergillus niger* AKU 3302 when grown with YME as inducer (Butiuk et al., 2015). As a consequence, the production of CHase releasing QA and CA from CGA in natural plant resource offers special interest due to their potential industrial applications.

Mold *Aspergillus* is one of the filamentous fungi most widely used in fermentation industries in Japan. The molds *A. oryzae, A. sojae, A. awamori, A. niger* and *A. saitoi* are of great practical importance in fermentation industries, enzyme technologies and food industries (Ichishima, 2012). Significance of crude enzyme preparations to industrial process, where there are crude agricultural products for manufacturing value-added materials, can be seen with fungal mold (*koji*) as the enzyme sources. As a typical example, when *A. oryzae* is grown on steamed rice grains, rice starch is hydrolyzed by the fungal amylases to glucose or maltose. When *A. sojae* is grown on steamed soy beans or soy bean powder, the soy proteins inside are hydrolyzed to a mixture of amino acids. It can be employed for large scale production of enzymes and thus the fungal mycelia can be directly used as the respective hydrolases required instead of purified or partially purified enzyme (Ichishima, 2012). In this sense, it is important to survey thoroughly the enzymatic properties of crude CHase to perform the biotechnological manufacturing with scaled up *koji* mold catalyst.

Favorable knowledge about the stability of an enzyme is an important aspect when considering its application to biotechnological processes, since it can provide information on the structure of the enzyme and facilitate an economical production design. Deactivation mechanisms can be complex, since enzymes have highly defined structures, and the slightest deviation from their native form can affect their specific activity. Moreover, CHase is known as a kind of serine-esterase and stable against inhibitors such as metal chelators. As shown by Okamura and Watanabe (1982a), CHase is only inhibited markedly by an organic phosphorus compounds such as diisopropylfluorophosphate (DFP). Better knowledge of enzyme stability under the operating conditions could help to optimize the profitability of enzymatic processes (Cavalcante Braga et al., 2013). The activity and thermal stability of enzymes are influenced by diverse environmental factors (temperature, pH, reaction medium, shaking) which can strongly affect the specific three-dimensional structure or spatial conformation of the protein (Cavalcante Braga et al., 2013). It is also important to analyze the estimated thermodynamic parameters, since they are helpful to understand the probable denaturation mechanism, which is very important in practical enzymatic processing (Ustok et al., 2010). In this paper, we describe the characterization of crude CHase from *A. niger* AKU 3302 and give some comparisons with other CHases.

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. The yerba mate sample, named as concentrated aqueous yerba mate extract (YME), was obtained from a local yerba mate processing company (La Cachuera S.A., Misiones Province, Argentina). YME was prepared as it was described in our previous studies (Butiuk et al., 2015).

2.2. Microbial strain, culture conditions, and preparation of a crude CHase

A. niger AKU 3302, used in this study was kindly provided by Prof. J. Ogawa, Kyoto University, Japan (Butiuk et al., 2015). The fungus was grown in Erlenmeyer flasks containing modified Czapek medium, at 30 °C, for 48 h under agitation at 180 rpm. YME (0.125% w/v, dry basis) was added to the culture medium after 24 h of culture. According to our previous results it provided a final concentration of 0.14 g CGA L^{-1} of medium (Butiuk et al., 2015). The mycelia grown in the shape of pellets ($\approx$ 4.5–5 $g_{biomass}$ L^{-1}) were separated by paper filtration (0.45 μm pore size, E04WP04700, MSI, USA), thoroughly washed with distilled water and kept at 5 °C in 2 mM sodium phosphate buffer (NPB), pH 7.0, until use (Butiuk et al., 2015).

The mycelia were milled in a mortar to homogenous gruel in 2 mM NPB, pH 7.0, at the rate of 0.5 g wet biomass mL^{-1} buffer. The homogenate was centrifuged at 10,000 × *g* for 25 min and the supernatant, designated as crude enzyme extract, was kept at − 20 °C.

CHase activity was measured according to the method of Okamura and Watanabe (1982a). The reaction was carried out at 30 °C for 10 min using 1.5 μmol of CGA in 50 mM NPB, pH 6.5. The relative difference in absorbance at 350 nm was read and the rate of CGA hydrolysis was calculated. An optical difference of 1.0 at A_{350} was equivalent to the consumption of 0.14 μmol of CGA based on the molecular extinction coefficient (ε_{CGA} = 7200 mol^{-1} L^{-1} cm^{-1}). CA showed no appreciable absorbance at A_{350}. One unit of CHase activity was defined as the amount of enzyme that hydrolyzed 1 μmol of CGA per minute under the conditions used. All assays were performed in triplicate and standard deviation was less than 5% of the mean.

2.3. Catalytic properties and optimization by the Response Surface Methodology

The optimal pH of CHase activity was determined over a pH range from 4.0 to 10.0 in 50 mM NPB and 20 mM Tris-MES-glycine (TMGB) buffers, under the standard assay conditions. The pH stability of CHase was determined by incubating the crude enzyme extract crude enzyme extract at 5 °C for 24 or 48 h in either NPB or TMGB. After incubation, the residual enzyme activity was assayed by the addition of substrate under the standard assay conditions. The optimal temperature of CHase activity was assayed at pH 7.5 under different temperatures from 30 °C to 80 °C under the standard conditions. For the determination of the thermal stability, the enzyme was pre-incubated under different temperatures from 30 to 60 °C in NPB, pH 7.5. Residual enzyme activity was measured after incubation for under the standard enzyme assay conditions. Kinetic behavior of CHase was determined from initial velocity measurements using GCA as substrate in concentration range from 0.025 to 1.12 g L^{-1}. The kinetic constants (V_{max} and K_m) were estimated by linear regression (Lineweaver-Burk). The Response Surface Methodology (RSM) was applied to optimize the effect of pH and temperature on CHase activity in NPB according to Doehlert experimental design (Doehlert, 1970). The number of experiments required in this design was of 9 runs, including three central points for experimental error detection. The central values, zero level, for the experimental designs were 40 °C and pH 7.0. According to the experimental design, temperature varied from 30 to 60 °C and pH from 5.0 to 8.0. Regression linear analysis was used to eliminate terms of $p > 0.05$.

2.4. Enzymatic hydrolysis of CGA from yerba mate

Enzymatic hydrolysis of CGA was performed incubating 990 μl of YME, which was diluted 50 times in 50 mM NPB, pH 7.5, with 10 μl of CHase. The incubation was carried out for 8 h at 45 °C. Quantitative measurement of CGA, CA and QA in the reaction mixture (20 μl) was analyzed by HPLC method, adopted from Adachi et al. (2008). An HPLC Model 1233 (Waters) equipped with a refractive index detector, which allowed possible to detect any compounds having no UV intensity, was used. Separation was achieved on a C_{18} RP Phenomenex Prodigy ODS3 column (5 μm, 250 mm × 4.6 mm I.D.) and the temperature was

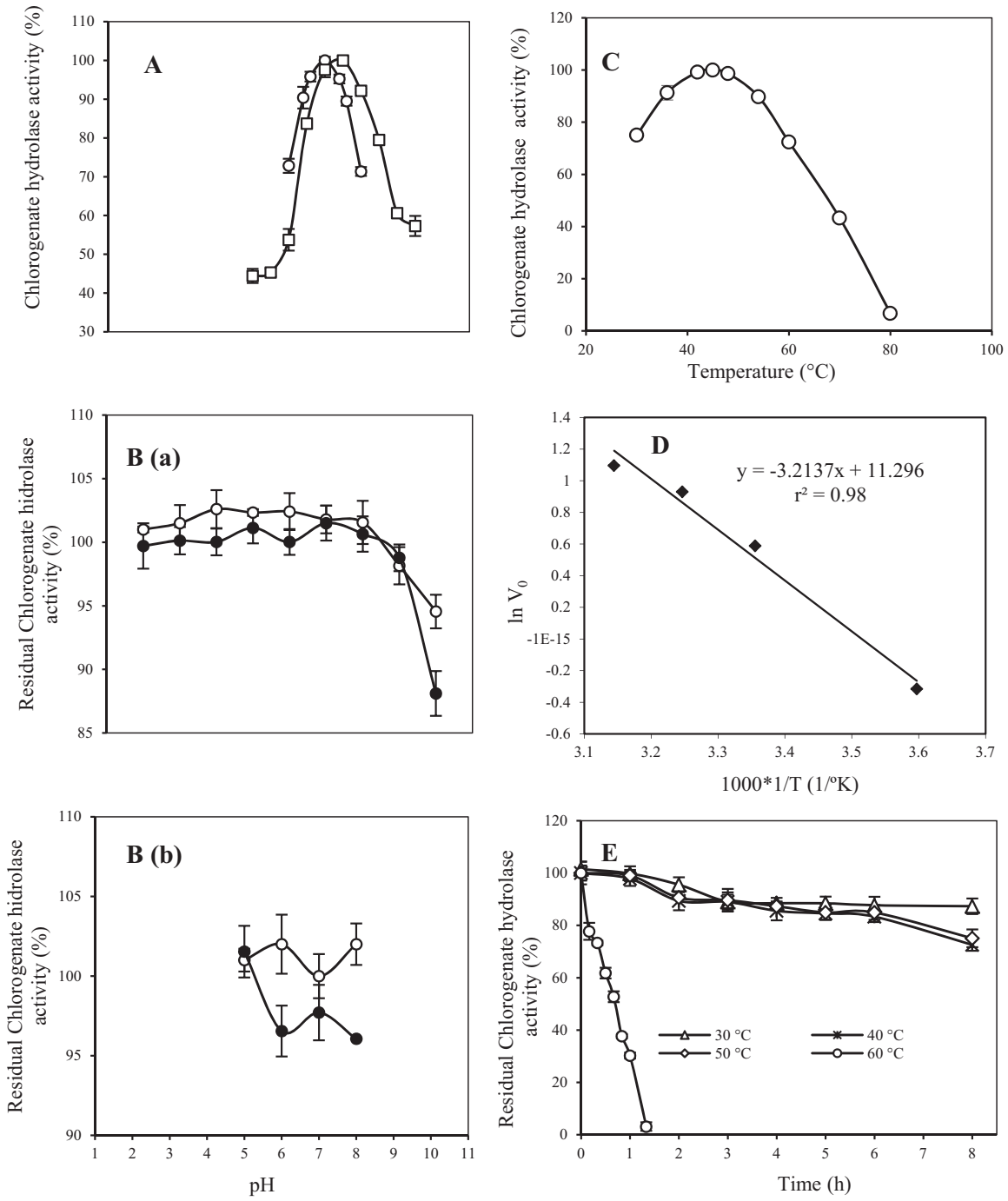

Fig. 1. A. Effect of pH on CHase activity. CHase activity was tested with various pHs: (○) 50 mM sodium phosphate (pH range of 6.0–8.0). (□) 20 mM Tris-MES-glycine buffers (pH range of 5.0–9.5). Fig. 1**-B.** pH stability of crude CHase. CHase activity was examined with various pHs: (a), 20 mM Tris-MES-glycine (pH range of 2.0–10.0). (b), 50 mM sodium phosphate (pH range of 5.0–8.0) buffers. Residual enzyme activity was determined after incubation for 24 h (○) and 48 h (•). Fig. 1**-C.** Effect of temperature on CHase activity. CHase activity was shown by the relative enzyme activity observed at 45 °C assayed under the standard conditions. Fig. 1**-D.** Arrhenius plots for measuring activation energy of CHase. Fig. 1**-E.** Thermostability of CHase. CHase activity was determined relative to the initial enzyme activity under the standard assay conditions.

maintained at 30 °C. The column was eluted with 50 mM acetic acid/CH_3CN = 8:2 at a flow rate of 0.8 mL min^{-1}. The standard *Rt* for the authentic compounds was 3.98 min for QA, 6.92 min for CGA, and 9.22 min for CA under these conditions.

3. Results and discussion

3.1. Effect of pH on CHase activity and stability

The effect of pH to CHase reaction and pH stability are shown in Figs. 1-A and 1-B. When TMGB was used as the reaction buffer, CHase activity exhibited the maximal at pH 7.5. In NPB, the optimum pH of CHase was shifted by 0.5 pH unit to neutral region (Fig. 1). The species of buffers, NPB or TMGB, did not affect the CHase stability ($p \leq 0.05$).

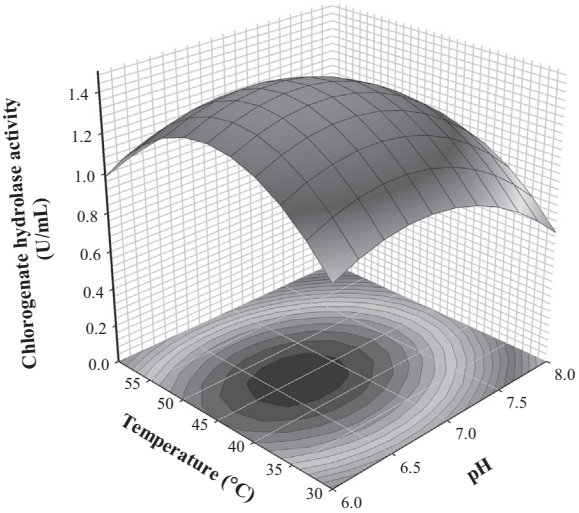

Fig. 2. Response surface and contour plots showing the effect of pH and temperature on CHase activity.

Table 1
Experimental data and enzyme activity attained in Doehlert's design for the effect of pH and temperature on enzyme activity.

Exp.	Temperature (°C)	pH_{NPB}	CHase activity (U/mL)
1	45	8,0	1105
2	30	7,5	1016
3	30	6,5	1123
4	45	6,0	1340
5	60	6,5	1094
6	60	7,5	0893
7	45	7,0	1484
8	45	7,0	1443
9	45	7,0	1424

NPB: sodium phosphate buffer.

CHase remained stable at the pH range from 2.0 to 8.0 in NPB or TMGB, after being incubated for 48 h at 5 °C (Fig. 2). About 90% of the initial enzyme activity remained at pH 10.0 after 48 h of incubation in TMGB buffer (Fig. 2a). Schöbel and Pollmann (1980a), Okamura and Watanabe (1982b) and Adachi et al. (2008) reported similar data for the optimum pH and pH stability with CHase from *A. niger*, *A. japonicus* ATCC 20236 and *A. sojae* AKU 3312, respectively. These fungal CHases exhibited the optimum pH in the neutral pH region between 6.5 and 7.5 and were stable in the pH range over 3.0–8.0. The basic information indicated that CHase activity was very stable and not so serious in which pH the enzyme was imposed the CHase activity. In addition, the species of buffer did not affect significantly. Since CHase activity was almost constant over the reaction conditions employed, the buffer species were not important factor to enzyme activity and stability. It is rather important to allow CHase to work well and the working pH should be kept under neutral pH to get the optimum condition hydrolyzing CGA into QA and CA. These data looked advantageous to apply CHase to the practical CGA catalysis where the reaction condition was difficult to control strictly.

3.2. Effect of temperature on CHase activity

The effect of temperature on CHase activity was studied in NPB buffer (Fig. 1-C). It showed relatively broad temperature ranges. Considerable enzyme activity was evident even at room temperature, whereas a rapid heat inactivation of the enzyme was observed beyond 55 °C. The maximum enzyme activity was found at around 45–50 °C. Schöbel and Pollmann (1980a) reported the optimum temperature of 45 °C for CHase produced by *A. niger*. Okamura and Watanabe (1982b) and Asther et al. (2005) observed the optimum temperature at 55 °C with CHase from *A. japonicus* ATCC 20236 and *A. niger* BRFM 131, respectively. Data of optimal temperature revealed that CHase activity of our enzyme was in the same range of those of related enzymes. Thermal activation of CHase was measured by the Arrhenius equation (Bragger et al., 2000; Kuz'mina et al., 2003):

$$V_0 = A \exp\left(\frac{-E_a}{RT}\right)$$

where V_o was enzyme activity, A was the pre-exponential factor, E_a was the activation energy, R was the gas constant and T was the absolute temperature in Kelvin. Plotting $\ln V_o$ versus the reciprocal of the absolute temperature ($1/T$), in a temperature range from 30 °C to the optimum temperature of the enzyme (45 °C) gave a straight line with a slope of E_a/R. The E_a value of the enzyme was calculated to be 26 kJ mol^{-1} with a correlation coefficient of $r_f^2 = 0.981$ (Fig. 1-D). Schöbel and Pollmann (1980b) reported a similar value of E_a of 25 kJ mol^{-1} for CHase from *A. niger*. These results showed similar activation energy values for CHases from *A. niger*, indicating that these CHases were sensitive to the temperature and consequently readily activated with slight temperature changes.

3.3. Effect of temperature on CHase stability

The effect of temperature on CHase stability is shown in Fig. 1-E. In the absence of substrate, CHase activity decreased slowly after incubation at 30 °C, 40 °C and 50 °C for 6 h. In any cases, more than 80% of the initial enzyme activity survived, while CHase activity was lost rapidly at 60 °C after 80 min of incubation. Crude CHase from *A. niger* AKU 3302 was more thermostable than those purified CHases reported so far. Purified CHase from *A. sojae* AKU 3312 remained stable up to 50 °C for 30 min (Adachi et al., 2008). Purified CHases from *A. niger* and *A. japonicus* ATCC 20236 were stable up to heating for 10 min at 55 °C and 60 °C, respectively (Schöbel and Pollmann, 1980b; Okamura and Watanabe, 1982b). Purified CHase from *A. niger* BRFM 131 was more heat labile and inactivated when heated at 40 °C for 10 min (Asther et al., 2005).

These results are according with the data described in literatures that proteins are less stable as they are purified. This is related to the protective effect of contaminating proteins and other components present in crude enzyme preparations. It is also said that thermostability of CHase was highly related to degree of glycosylation (Chu et al., 1978; Wyss and Wagner, 1996; Benoit et al., 2006). Benoit et al. (2007) reported that CHase obtained from a transformant of *A. niger* accommodating overexpressed CHase gene of *A. niger* BRFM 131 showed higher thermostability by higher degree of glycosylation of the recombinant CHase. Since it was not touched here, CHase may still active at nearly 0 °C and show a considerable enzyme activity, as judged from a similar enzyme, fungal tannase (Yamada et al., 1968). The fungal tannase exerted the enzyme activity more than 50% to the maximum enzyme activity at 0 °C. The tannase is a glycosylated protein containing 25.4% hexose and shows the isoelectric point in the vicinity of pH 4.0 (Adachi et al., 1968). There are not so many examples of highly purified CHase of which carbohydrate content was estimated. As an indirect evidence proving that CHase is a glycolated protein, a clear mobility difference in SDS-PAGE between a purified CHase and the deglycosylated one was shown by Benoit et al. (2007).

Kinetic parameters such as V_{max} and K_m were determined from the regression lines of Lineweaver-Burk plots. The K_m and V_{max} values obtained for the CHase were 0.72 mM, and 1.996 unit mg^{-1} (r^2: 0.99), respectively. Similar K_m values, have been described for CHase enzymes from others fungal strains, such as 0.74 and 0.70 mM for CHase produced by *A. sojae* AKU 3312 (Adachi et al., 2008) and *A. niger* (Schöbel and Pollmann, 1980a), respectively.

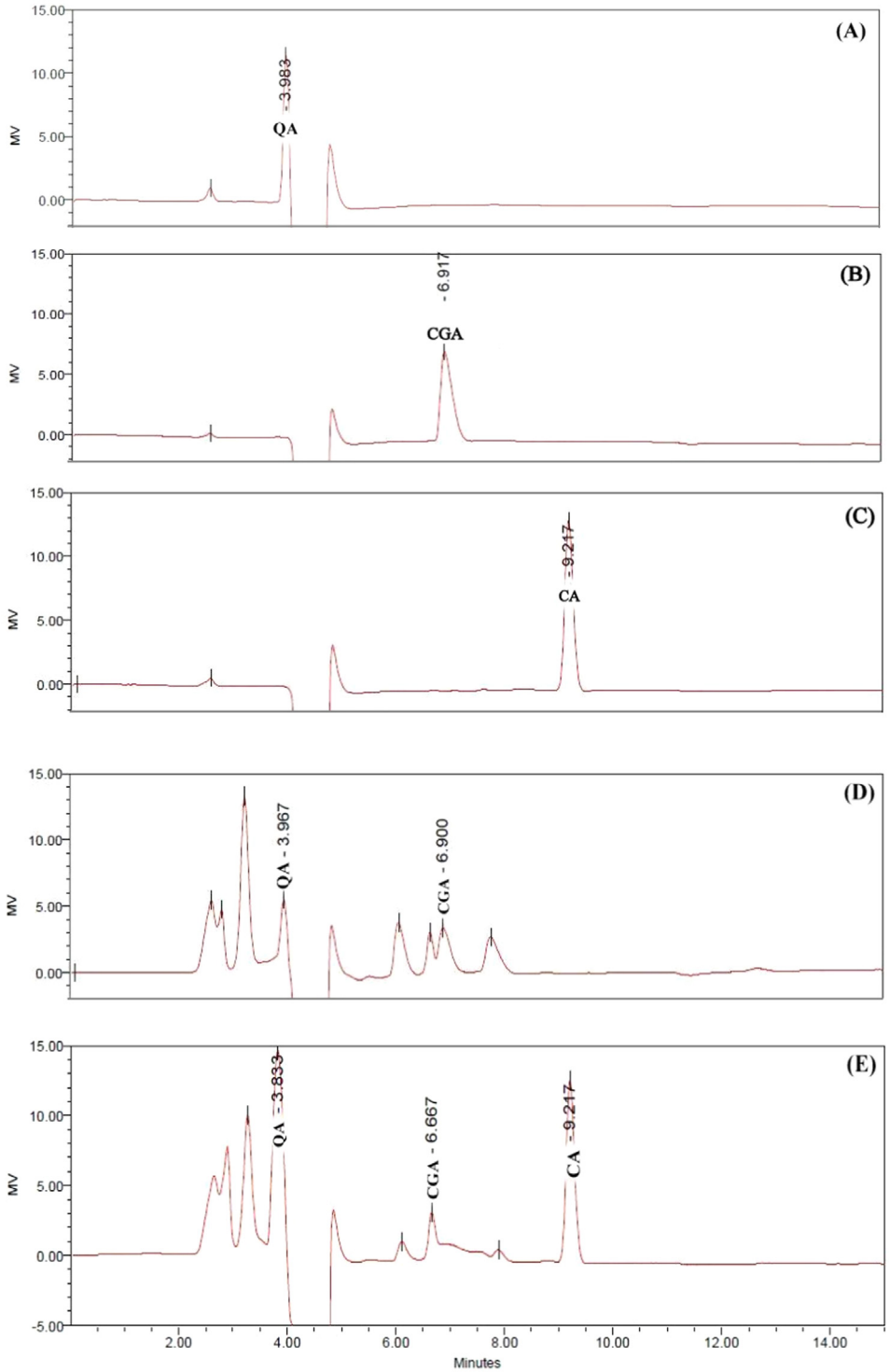

Fig. 3. HPLC analysis of authentic samples and reaction products of CGA hydrolysis with CHase. CHase (10 µl) was incubated with YME of 50 times diluted with 50 mM of NPB, pH 6.5, at 30 °C for 8 h. An aliquot of the reaction mixture (20 µl) was analyzed as before reaction (**D**) and after reaction (**E**). (**A**), Authentic QA; (**B**), authentic CGA; (**C**), authentic CA.

3.4. Optimization of CHase activity by the Response Surface Methodology

Table 1 presents the treatment combinations according to Doehlert's experimental design and the corresponding responses. The data were converted into second-order polynomial equation, where pH and T are given as codified data (Eq. (1)).

Since the r^2 value was equal to 0.98, they indicated that the equation had a very good fit to the experimental data.

$$CHase(U/ml)_{NPB} = 1.45 - 0.142*pH - 0.228*pH^2 - 0.508*T^2 \quad (1)$$

Statistical analysis of the results revealed that, the pH had a significant linear effect on CHase activity. The negative values of quadratic effects for pH and temperature indicated the existence of a maximum as a function of these variables. No significant interactions were observed among the variables (Eq. (1)). Fig. 2 shows the response surface and contour plot for CHase activity as a function of the two independent variables studied. As it is shown here, the optimal temperature was

Table 2
CGA, CA and QA contents in YME before and after enzymatic hydrolysis with CHase from *A. niger* AKU 3302.

Compound	Before treatment (μmol/g_{ms})	After treatment (μmol/g_{ms})	Difference
CGA	2.98	0.74	− 2.24
CA	nd	3.86	+ 3.86
QA	1.54	6.85	+ 5.31

nd: not detected. CGA: chlorogenic acid; CA: caffeic acid; QA: quinic acid.

45 °C and the optimum pH was in the range of 6.5–7.0. At 30 °C and pH 6.5, CHase shows more than 80% of the optimal enzyme activity. Similar results were reported by other authors. Schöbel and Pollmann (1980b) reported values of optimum pH and temperature of 6.5 and 45 °C, respectively, for a tetrameric CHase produced by *A. niger*. The optimum temperature and pH values for CHase produced by *A. japonicus* ATCC 20236 were 45 °C and 6.5, respectively (Okamura and Watanabe, 1982b). Asther et al. (2005) have obtained the optimum temperature of 55 °C and the optimum pH of 6.0 for CHase produced by *A. niger* BRFM 131. CHase from *A. niger* BRFM 131 was cloned and characterized giving the same value for the optimum pH, but a higher optimum temperature of 60 °C (Benoit et al., 2007). Adachi et al. (2008) reported the optimum pH in the neutral region between 7.0 and 7.5 for CHase produced by *A. sojae* AKU 3312.

3.5. Enzymatic hydrolysis of CGA from yerba mate

Enzymatic hydrolysis of CGA in YME was performed in 50 mM NPB, pH 6.5 and at 30 °C, instead of 45 °C, in order to reduce the costs inherent to warming in future stages of scaling up. The identification of CGA, CA and QA in the YME before and after enzymatic hydrolysis with CHase was monitoring by HPLC (Fig. 3). Table 2 shows the corresponding concentrations of all compounds, expressed as $\mu mol\, g^{-1}$ of dry matter.

Before enzymatic hydrolysis, YME contained 2.98 $\mu mol\, g^{-1}$ and 1.54 $\mu mol\, g^{-1}$ of CGA and QA, respectively. No significant amounts of CA were detected (Table 2).

More than 75% of CGA was hydrolyzed for 8 h incubation accompanying increase in the amount of QA (5.31 $\mu mol\, g^{-1}$) and the formation of CA (3.86 $\mu mol\, g^{-1}$). The large amounts of these compounds produced indicated that at least 58% of QA and 42% of CA, were probably released from other esters than CGA. These results suggest that CHase is capable of acting on other QA esters present in YME solution, such as, dicaffeoylquinic, feruloylquinic and caffeoylquinic acids (Clifford, 1999, 2000).

Similar results were obtained by Asther et al. (2005), who reported that a purified CHase from *A. niger* BRFM 131 was able to hydrolyze 100% of CGA (12 $\mu mol\, g^{-1}$) content in coffee pulp simultaneously produced larger amount of CA (21 $\mu mol\, g^{-1}$). The same authors also studied the enzymatic hydrolysis of CGA content in apple marc, reaching yields close to 86% (1.9 $\mu mol\, g^{-1}$). Adachi et al. (2008) obtained yields of 100% (37.5–140 mg/2 g) after CGA hydrolysis content in different extracts of instant coffee by an immobilized CHase in coffee pulp by a *koji* fermentation type. Similarly, there was a noticeable increase in the CA content (98–110 $\mu mol\, g^{-1}$) after enzymatic hydrolysis.

4. Conclusions

It was extremely important to acquire knowledge of the properties of crude CHase in order to determine the optimal conditions for CGA hydrolysis extracted from plant resources, such as yerba mate. More than 75% of CGA in YME solution was hydrolyzed by CHase for 8 h incubation. The amounts of CA and QA released from YME were stoichiometrically higher than the amounts of hydrolyzed CGA suggesting that CHase extended the catalytic activity to other esters containing QA and CA found in the YME. The use of crude enzyme extract showed a considerable CHase activity that was strong enough catalyzing enzymatic release of important compounds, CA and QA. They are highly attractive as antioxidants as well as key intermediates for new drug synthesis. On the other hand, the direct use of crude CHase without any previous purification step is very useful for future scaling up the process in order to reduce the cost performance of CGA technology.

However, intact fungal mycelia themselves could exert the similar results like *koji* catalyst, when they are used instead of a crude solution of CHase. Thus, we believe that most of the data presented could be directly transferred to the data expected with *koji* mold catalyst, which are grown to induce CHase on powdered yerba mate bed. It can be employed for mass production of enzymes and thus the fungal mycelia can be directly used as the enzyme required instead of purified or partially purified enzymes. It can be applied to large scale production of enzymes and thus used for industrial scale production of sugars, amino acids, and other hydrolyzed products from agricultural low materials, as Ichishima stated, 2012.

Many investigators reported purification of CHase of which purity and purification methods were variable by case, a purified CHase show similar enzymatic properties (Adachi et al., 2008; Asther et al., 2005; Okamura and Watanabe, 1982a; Schöbel and Pollmann, 1980b; Nieter et al., 2015). However, a purified enzyme is intolerant to treat huge amounts of YME to produce CA and QA in industrial scale, although CHase in fungal mycelia is stable against prepared reaction conditions. From these results and those of other investigators, it could be reasonably concluded that utilization efficiency of a crude enzyme preparation of CHase is excellent, with various merits as indicated: use of a crude enzyme reduce the cost of enzyme production. According to our data, the use of a crude enzyme preparation does not result in the formation of undesirable by-products. Thus, in our next study, we should like to report several practical aspects of yerba mate *koji* catalyst will be conducted.

Acknowledgments

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