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Enzymatic Extraction of Hydroxycinnamic Acids from Coffee Pulp

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Summary

Ferulic, caffeic, *p*-coumaric and chlorogenic acids are classified as hydroxycinnamic acids, presenting anticarcinogenic, anti-inflammatory and antioxidant properties. In this work, enzymatic extraction has been studied in order to extract high value-added products like hydroxycinnamic acids from coffee pulp. A commercial pectinase and enzyme extract produced by *Rhizomucor pusillus* strain 23aIV in solid-state fermentation using olive oil or coffee pulp (CP) as an inducer of the feruloyl esterase activity were evaluated separately and mixed. The total content (covalently linked and free) of ferulic, caffeic, *p*-coumaric and chlorogenic acids was 5276 mg per kg of coffee pulp. Distribution was as follows (in %): chlorogenic acid 58.7, caffeic acid 37.6, ferulic acid 2.1 and *p*-coumaric acid 1.5. Most of the hydroxycinnamic acids were covalently bound to the cell wall (in %): *p*-coumaric acid 97.2, caffeic acid 94.4, chlorogenic acid 76.9 and ferulic acid 73.4. The content of covalently linked hydroxycinnamic acid was used to calculate the enzyme extraction yield. The maximum carbon dioxide rate for the solid-state fermentation using olive oil as an inducer was higher and it was reached in a short cultivation time. Nevertheless, the feruloyl esterase (FAE) activity (units per mg of protein) obtained in the fermentation using CP as an inducer was 31.8 % higher in comparison with that obtained in the fermentation using olive oil as the inducer. To our knowledge, this is the first report indicating the composition of both esterified and free ferulic, caffeic, *p*-coumaric and chlorogenic acids in coffee pulp. The highest yield of extraction of hydroxycinnamic acids was obtained by mixing the produced enzyme extract using coffee pulp as an inducer and a commercial pectinase. Extraction yields were as follows (in %): chlorogenic acid 54.4, ferulic acid 19.8, *p*-coumaric acid 7.2 and caffeic acid 2.3. An important increase in the added value of coffee pulp was mainly due to the extraction of chlorogenic acid.

Key words: coffee pulp, olive oil, hydroxycinnamic acids, antioxidants, solid-state fermentation

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Abbreviations: FA: ferulic acid, CA: caffeic acid, *p*-CA: *p*-coumaric acid, ChA: chlorogenic acid, CP: coffee pulp, SSF: solid-state fermentation, FAE: feruloyl esterase, HAs: hydroxycinnamic acids

Introduction

Coffee pulp (CP) represents the most abundant waste obtained after wet pulping of coffee cherries. It is estimated that CP represents 40 % of several million tonnes of coffee cherries processed by the wet method in Mexico, Central America and Colombia. In Mexico, a production of 3760 million tonnes was reported in 2008 (*1*). This by-product contains 23–27 % fermentable sugars on a dry mass basis. CP treatment should be carried out in order to avoid serious problems of pollution in rivers and soil close to the industrial coffee processing facilities (beneficios). Several alternatives have been suggested for the utilisation of CP for fuel production, furfural extraction and as a fermentable substrate (*1*). However, it is a material that could be used for the extraction of high-value compounds such as hydroxycinnamic acids (ferulic acid, caffeic acid and *p*-coumaric acid) (*2*). The hydroxycinnamic acids (HAs) are usually covalently linked to polysaccharides of plant cell walls through ester linkage. They exhibit anticarcinogenic, anti-inflammatory and antioxidant properties. These properties are of great interest for the food, pharmaceutical and cosmetology industries (*3*). Ferulic acid (FA) is found in a wide variety of plant cell walls and can be used as an additive due to its ability to inhibit peroxidation of fatty acids and constitutes the active ingredient in many skin lotions and sunscreens. Commercial utilisation of natural FA has been limited by its availability and cost (*2*). In plant material, HAs are also found as a soluble conjugate of quinic acid named chlorogenic acid (*4*). As a consequence, enzymes allowing the release of these aromatic compounds are of special interest due to their potential industrial applications.

Feruloyl esterases (FAE; E.C. 3.1.1.73), belonging to a subclass of the carboxylic acid esterases (E.C. 3.1.1.1), have been found to cleave the ester linkage between FA and plant cell wall polysaccharides. They are organised into four functional classes named types A, B, C and D according to their ability to hydrolyse the four methyl esters of HAs, methyl ferulate, methyl caffeate, methyl sinapate and methyl *p*-coumarate. Types A and B feruloyl esterases hydrolyse methyl ferulate and methyl *p*-coumarate. Type A feruloyl esterase also hydrolyses methyl sinapate and type B also hydrolyses methyl caffeate. Types C and D feruloyl esterases hydrolyse the four methyl esters of HAs. Only types A and D release diferulic compounds (*5*). On the other hand, hydrolytic enzyme lipases are glycerol ester hydrolases (E.C. 3.1.1.3), which hydrolyse ester linkages of glycerides at the water-oil interface. There are three amino acid residues in the FAE active site: serine (Ser), aspartic acid (Asp) and histidine (His) (*5*,*6*). FAE presents a protein sequence homology belonging to the same family as the serine proteases, esterases and lipases, with a serine residue acting as the nucleophile in a catalytic region comprising the hydroxyl group of the active serine, the imidazole side chain of histidine and a buried carboxylic acid chain, a similar catalytic mechanism to the fungal lipases. Therefore, the lipases can exhibit FAE activity (*5*,*7*,*8*). The use of single enzymatic activities is not recommended for the release of HAs from natural plant polymers, so the use of suitable enzyme mixtures including pectinase, xylanase and amylase has been reported (*4*,*5*).

FAE production by microorganisms has become important, especially in biotechnological processes for many industries and medicinal applications (*9*). Thus the discovery of new FAEs with novel properties continues to be an important research area. FAE is produced from *Aspergillus* sp. using either solid-state fermentation (SSF) or submerged fermentation, along with pectinases, cellulases and xylanases, and can release HAs from wheat bran, rice bran, sugar cane, bamboos and sugar beet pulp, among others. SSF is attractive because it presents many advantages for fungal cultivation. The solid supports used in the SSF process can be inert materials such as perlite or natural materials such as agricultural, forestry and food processing residues and wastes, used as inducers and carbon sources for the production of FAEs. *Penicillium* sp. and the thermophilic fungus *Sporotrichum thermophile* are capable of producing FAEs when grown on agricultural residues such as sugar beet pulp and brewer's spent grain under SSF (*9*). An esterase from *Fusarium oxysporum* plays a significant role in the production of flavouring and fragrance compounds from geraniol and fatty acids. Pentylferulate ester, a flavour precursor in food processing and also in cosmetics, is a product of FAE using water-in-oil microemulsions (*10*). Currently, there are no reports of FAE production using SSF by *Rhizomucor pusillus*. The aim of this work is to evaluate the release of the HAs from the cell wall of coffee pulp (CP) by enzymatic treatment of commercial pectinase and enzyme extract produced in SSF by *R. pusillus* using olive oil or CP as inducers.

Material and Methods

Strain, conservation and propagation

Rhizomucor pusillus strain 23aIV was obtained from the microorganism collection of Dr. Jesús A. Córdova López (University of Guadalajara, Jalisco State, Mexico). The strain was maintained on agar plates at 4 °C. The inoculum was prepared by culturing *R. pusillus* in 500-mL Erlenmeyer flasks containing 60 mL of potato dextrose agar (PDA) at 30 °C for 5 days. Spores were harvested with 30 mL of Tween 80 solution (0.01 %) and recovered with a magnetic stirrer. Spores were counted using a Neubauer hemocytometer (Blaubrand, Germany).

Agro-industrial substrate

Coffee pulp was obtained from a commercial co-operative enterprise, Beneficios de café (Veracruz, Mexico). For conservation, CP was dried at 60 °C for 24 h until it reached a moisture content close to 5 % and it was ground and sieved (particle sizes 0.8 mm and 1.12 cm). Distilled water was added to CP to reach a moisture content of 30 %, and then it was autoclaved for 10 min at 103.42 kPa.

Solid-state fermentation conditions

R. pusillus 23aIV was cultivated in SSF under two conditions: (*i*) perlite was used as an inert support impregnated with a culture broth reported by Rodríguez *et al.* (*11*), and olive oil as the inducer, and (*ii*) CP as the substrate, which was impregnated with the medium reported by Asther *et al.* (*12*) and diluted to 50 %. Glass columns (20×2.5 cm of diameter) were packed with per-

lite (particle sizes 0.8 and 1.19 mm) or CP (1.12 cm) impregnated with the inoculated culture medium. The medium was inoculated with $3 \cdot 10^7$ spores per g of dry substrate and the moisture content was adjusted to 60 %. Columns were incubated at 40 °C. Fungal growth was determined by monitoring the production of CO_2 (*13*). Results were expressed as mg of CO_2 per g of dry matter (DM) per hour.

Collection of the enzyme extract

At the end of culture fermentation, the solids were dried by passing through an air flow of 10 L/min at 30 °C for less than 20 min, until a moisture content close to 5 % was reached. FAE crude enzyme extracts were obtained in a 1:5 (by mass per volume) mixture of fermented solids (dry basis) and 50 mM citrate buffer at pH=6. The mixture was stirred using a vortex mixer for 5 min and was centrifuged at 5 000 rpm for 15 min in a centrifuge (AllegraTH 25R; Beckman Coulter Inc., Mexico City, Mexico), at 4 °C. The crude enzyme extracts were stored at 4 °C until use.

Enzymatic extraction

Enzymatic extraction of HAs from CP without free HAs was performed as follows: dry CP (10 g) was mixed with 90 mL of citrate buffer (50 mM, pH=6). Five enzymatic treatments were performed as indicated in Table 1.

Table 1. Enzymatic treatments

Enzyme extracts	Inducer	Treatments				
		A	B	C	D	E
Commercial pectinase*		X			X	X
FAE by *R. pusillus***	olive oil		X		X	
	coffee pulp			X		X

*10 U of commercial pectinase from *A. niger* (Fluka, USA)
**10 U of FAE activity produced in this work by SSF

The enzymatic extraction was performed in darkness at 30 and 40 °C under continuous stirring (100 rpm). Samples were taken at 0, 1, 3, 6 and 12 h of the reaction. A selective extraction of HAs was carried out with a ratio of sample to ethyl acetate of 1:1 (by volume). Then, HA levels were determined by HPLC.

Determination of HAs in CP

Total content of FA, CA, *p*-CA and ChA was determined by alkaline hydrolysis as follows: a CP sample of 10 g (particle size 0.8 mm) was mixed with 50 mL of 2 M NaOH (pH=12), and hydrolysis was carried out at 50 °C and 100 rpm for 2 h. Then, the supernatant was acidified to pH=3 with HCl (J.T. Baker, Mexico City, Mexico) and the extraction of HAs with ethyl acetate (J.T. Baker) was carried out as indicated above, followed by HPLC analysis. Free (non-covalently linked) FA, CA, *p*-CA and ChA were extracted with aqueous methanol (80 %) as follows: a sample of CP of 10 g (particle size 0.8 cm) was mixed with 50 mL of aqueous methanol (80 %) at 40 °C and 100 rpm for 30 min. The supernatant was evaporated (50 °C) under reduced pressure in a rotavapor (R-200; Büchi Labortechnik AG, Flawil, Switzerland) and then analysed by HPLC (*14*). Exhausted solids of CP without free HAs were dried to moisture content close to 5 % and stored until use.

Enzyme assay

FAE activity was measured using a titrimetric technique. The reaction was performed in a mixture of 4.5 mM methyl ferulate (Extrasynthese, Genay Cedex, France) and the enzyme extract in a ratio of 12:1 (by volume) at 45 °C, pH=6, for 15 min. The volume of NaOH added to maintain the pH constant (6.0) was registered continuously for 15 min using a DL 21 titrator (Mettler Toledo, Greifensee, Switzerland). Activities were expressed in enzyme units (U). One U was defined as the amount of enzyme required to release 1 μmol of product per minute under the assay conditions. Protein concentration was determined according to Lowry *et al.* (*15*).

HPLC analysis

HPLC was performed using an LC 250 binary LC pump (PerkinElmer, Inc., Waltham, MA, USA), coupled with a variable UV/VIS detector and a Varian Polaris 5 amide C-18 (Agilent Technologies, Santa Clara, CA, USA) reversed phase column (200×4.6 mm) at 36 °C and with a mobile phase at a flow rate of 1 mL/min. The mobile phase was a mixture of two solvents: solvent A was an aqueous solution of acetic acid (0.5 %), and solvent B was methanol. Separation was achieved by an elution gradient using an initial composition of 0 to 12 min of 80 % solvent A. Solvent A was then decreased to 10 % in 5 min and it was increased to 80 % in 10 min. Then, the column was washed with 80 % solvent A for 5 min (*4*).

Results and Discussion

Content of HAs in CP

The content of esterified and free FA, CA, *p*-CA and ChA in CP was 4416.7 and 856 mg of HAs per kg of CP, respectively. Most HAs in the CP were covalently bound to the cell wall and were probably esterified. The distribution of linked *p*-CA, CA, ChA and FA was 97.2, 94.4, 76.9 and 73.4 %, respectively (Table 2). The total mass of these HAs per mass (kg) of CP was in agreement with that reported by other authors: 3600–27000 mg of ChA, 1600–3100 mg of CA, 100–240 mg of FA and 80 mg of *p*-CA (*4,16,17*). CP was rich in ChA and CA. The ChA covalently bound to the cell wall was probably responsible for the resistance of cell wall to the degradation by the microorganism (*18*). The conditions used during the alkaline hydrolysis allowed for the determination the content of ChA, without hydrolysis into CA and quinic acid. This paper reports for the first time the esterified and free amounts of FA, CA, *p*-CA and ChA in coffee pulp.

Enzyme production in SSF

Fig. 1 shows the CO_2 production rate (CPR) by *R. pusillus* under the two conditions used. The maximum value of CPR using CP as the substrate of (5.9±0.05) mg of CO_2 per h per g of DM was reached after 31 h of cultivation. This value was increased by a factor of 2.3 after

Table 2. Hydroxycinnamic acid composition of coffee pulp on a dry basis

	w(chlorogenic acid)	*w*(caffeic acid)	*w*(ferulic acid)	*w*(*p*-coumaric acid)
	mg/kg	mg/kg	mg/kg	mg/kg
Esterified+free HAs*	3097±3.5	1985±3.6	113±4.7	81±6.0
Esterified HAs**	2382.8±4.5	1873.1±4.5	83±5.8	78.7±5.9
Free HAs***	714.2±4.7	111.9±4.9	30±7.0	traces

Data are mean values of triplicate analyses; *alkaline hydrolysis: 2 M NaOH, 2 h, 50 °C, 100 rpm; **calculated as the difference between total HAs and free HAs; ***extraction with 80 % methanol, 30 min, 40 °C, 100 rpm; traces: not detected by HPLC

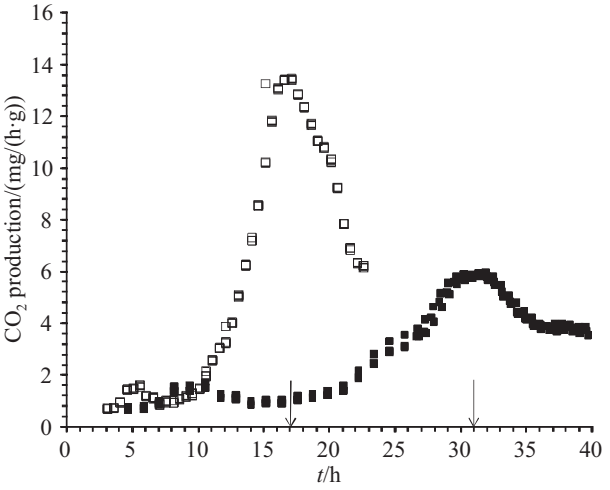

Fig. 1. Production of CO_2 by *R. pusillus* in SSF expressed as mass (mg) of CO_2 per time (h) per mass (g) of dry matter (DM) using agrolite as an inert support and olive oil as an inducer (□) and CP as both support and inducer (■). Arrows indicate the fermentation time for the collection of the enzyme extract

17 h of cultivation when olive oil was used as an inducer. These differences were probably due to the type of inducer and culture medium composition used in the SSF (*12*).

The enzyme extract was obtained when the maximum CPR in the SSF was reached. In previous studies (data not shown), we had demonstrated that the maximum FAE activity is well correlated with the maximum CPR. After this event, the FAE activity decreased, probably due to the presence of proteases. The FAE activity found in the extract from commercial pectinase was 5.3 and 4.3 times greater than that found in the enzymatic extract produced by *R. pusillus* using olive oil or coffee pulp as inducers, respectively (Table 3). The FAE activities of both enzymatic extracts per DM were higher than previously reported (*12*) for FAE by *Aspergillus niger* (0.03 U/g). There are no reports of FAE production using SSF by *Rhizomucor pusillus*.

Enzymatic extraction of HAs

The enzymatic extraction of HAs was performed with a mixture of enzymatic extracts as shown Table 1. The best conditions (data shown) for enzymatic extraction of FA, CA, *p*-CA and ChA were determined to be 40 °C for 6 h at 100 rpm.

Table 3. FAE activity in enzymatic extract

Support/ inducer	Enzymatic extract	FAE activity	
		U/mg	U/g
Agrolite/ olive oil	Commercial pectinase	31.28±0.05*	52.13±0.03**
	Esterase (23aIV)	0.85±0.13*	9.89±0.03***
CP/CP	Esterase (23aIV)	1.12±0.10*	12.11±0.05***

Data are mean values of triplicate analyses; *U per mass (mg) of protein, **U per mass (g) of dry solid, ***U per mass (g) of fermented dry substrate; pectinase was from *Aspergillus niger* (1.41 U/mg)

The two enzymatic extracts produced by *R. pusillus* released HAs from CP and showed different enzyme activities. CP was a better inducer than olive oil for specific FAE production. The amount of free ChA, CA, FA and *p*-CA released by the esterases increased in the presence of pectinase (Table 4).

The commercial pectinase extract exhibited both de-esterifying and depolymerising activities. These enzyme activities were responsible for the increase in the extraction yield of HAs from CP. These results suggest that the pectinase activity solubilised part of the cell wall structure of CP. Table 4 shows the results of HA extraction using five different treatments. It is worth noting that the extraction yields of the combined treatments (D and E) were higher in comparison with those obtained using individual enzyme extracts (A, B, C).

Asther *et al.* (*12*) reported an extraction yield of 68 % for FA in the treatment of CP with FAE type A by *A. niger* with xylanase. This value was higher than the extraction yield obtained in this study. However, there are no data that mention the origin of the extracted FA, either covalently bound to the cell wall or free. Furthermore, no data were provided about the extraction of other HAs such as ChA, CA and *p*-CA.

The enzymatic extract produced by SSF using CP as an inducer had the ability to release *p*-CA from the cell wall of CP. These results strongly suggest that the production of different types of FAE depends on the kind of inducer used in the SSF. Further studies on purification and characterisation of different FAE types could help to establish a detailed relationship with the inducer used. The HA that was released from CP in the highest amount was ChA. The commercial value of the HAs produced in this work was calculated, with the added value of CP close to 4846 US$/tonne. The main compound extracted

Table 4. Hydroxycinnamic acids extracted using different treatments of coffee pulp without free HAs

	Treatments	Hydroxycinnamic acids			
		Chlorogenic acid	Caffeic acid	Ferulic acid	*p*-Coumaric acid
A	*w*(HA)/(mg/kg)	940.30±4.8	34.33±4.9	15.12±6.3	traces
	Y/%	39.5	1.8	18.2	
B	*w*(HA)/(mg/kg)	105.72±4.7	9.65±4.9	3.21±2.7	traces
	Y/%	4.4	0.5	3.9	
C	*w*(HA)/(mg/kg)	331.03±5.7	19.17±2.4	3.17±2.4	5.15±1.3
	Y/%	13.9	1.0	3.8	6.5
D	*w*(HA)/(mg/kg)	1072.8±4.9	49.8±4.9	21.2±6.4	traces
	Y/%	45.0	2.7	25.5	
E	*w*(HA)/(mg/kg)	1296.24±3.3	43.99±3.2	16.43±3.6	5.67±1.2
	Y/%	54.4	7.2	19.8	2.3

Data are mean values of triplicate analyses; values were expressed on dry basis; extraction yield (Y) corresponded to the HAs covalently bonded to the cell wall of CP; traces: not detected by HPLC

was CA, and its highest commercial value represents 94.3 % of the total value-added CP.

Conclusion

To our knowledge, this is the first report on the esterified and free FA, CA, *p*-CA and ChA content in CP. The SSF with *R. pusillus* using olive oil as an inducer showed an earlier CO_2 production rate, compared to CP used as the substrate. Only the enzymatic extract produced by SSF using CP as the inducer had the ability to release *p*-CA from the cell wall of CP. The commercial pectinase extract exhibited both de-esterifying and depolymerising activities, which could be responsible for the increase in the extraction yield of HAs studied in this work. This study shows the potential of the enzymatic release of antioxidants such as FA, CA, *p*-CA and ChA from CP using commercial esterases produced by SSF.

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