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Variations of peroxidase activity in cocoa (*Theobroma cacao* L.) beans during their ripening, fermentation and drying

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Abstract

An increase of peroxidase activity in the seeds of cocoa (*Theobroma cacao* L.) during their ripening has been determined. An additional increase of the peroxidase activity (about 10 times) is observed during the fermentation and drying of the beans. The residual activity determined in the cocoa beans after sun-drying was higher than that in unfermented ripe seeds. The major cocoa isoperoxidase was shown to be an acidic enzyme with pI 4.7. Using isoelectrofocusing, the appearance of two basic isoenzymes of the peroxidase with pI 8.6 and 9.0 during the process of the fermentation has been detected.

Keywords: Cocoa; Fermentation; Flavour; Isoenzymes; Peroxidase

1. Introduction

The production of flavour is important in the manufacturing of cocoa powder. This organoleptic characteristic is developed in the course of the ripening and then the fermentation and drying of the beans. It is well-known that there are two basic groups of compounds, which essentially influence this parameter. The first group consists of peptides and aminoacids. These substances are formed in the enzymic hydrolysis of storage proteins (vicilin-class globulins) and after reaction with reducing sugars (on a roasting of beans) they generate the typical cocoa flavour. This process has been intensively studied by Voigt, Biehl, Heinrichs, Kamaruddin, Gaim, Marsoner, and Hugi (1994a); Voigt, Heinrichs, Voigt, and Biehl (1994b); Voigt, Voigt, Heinrichs, Wrann, and Biehl (1994c); and Voigt, Wrann, Heinrichs, and Biehl (1994d).

The other compounds, contributing to flavour of cocoa, are the phenols. These substances cause an astringent and early-developing bitter taste. After fermentation and drying, astringency is reduced by oxidation of flavonoid tannins and subsequent polymerization. Earlier it was shown that this process proceeds under the control of polyphenol oxidase (EC 1.14.18.1) in the presence of oxygen (Wong, Dimick, & Hammerstedt, 1990; Hammer, 1993).

However, the cocoa beans also contain peroxidase (EC 1.11.1.7; Ndoumou, Djocgoue, Nana, & Debost, 1995). This heme-containing oxidoreductase also oxidizes efficiently the phenolic molecules using H_2O_2 as a co-substrate. Although the role of this enzyme in the catabolism of cocoa phenols may be very important, there is very limited information about the peroxidase of the cocoa seeds. In the present paper we demonstrate the variations of the activity of the peroxidase and the composition of its isoenzymes which appear in cocoa beans during their ripening, fermentation and drying.

2. Materials and methods

2.1. Materials

Ripe cocoa pods were harvested in the territory of the state of Santander, Colombia. Guaiacol, $(NH_4)_2S_2O_8$, acrylamide were obtained from J. T. Baker; H_2O_2, $(NH_4)_2SO_4$ and ascorbic acid from Merck; EDTA from Aldrich; N,N′-methylene-bis-acrylamide, TEMED from Sigma, ampholines Bio-Lyte 3/10 from BioRad; pI protein markers (mixture 9; amyloglucosidase (pI 3.5), ferritin (4.4), bovine albumin (4.7), beta-lactoglobulin (5.34), conalbumin (5.9), horse myoglobin (7.3), whale

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myoglobin (8.3), ribonuclease (9.45), cytochrome c (10.65) from Serva.

2.2. Enzyme extraction

At the examination of the variation of the activity in beans during their ripening, 30 seed samples, at different stages of ripening, were analyzed. The experiment was repeated three times.

To examine the change of the peroxidase activity on cocoa beans as a function of the fermentation and drying time, at different intervals, 30 seeds were removed and used in the analysis. The cocoa was fermented in the wood tambour (fermenter) at ambient temperature for 4 days. The weight of the beans in each fermentation was typically about 200 kg ($2–3\times10^5$ beans). All cocoa was sun-dried for 4 days. The fermentation of cocoa was repeated three times during 3 months.

The samples of the cocoa beans were macerated using a blender. Humidity was determined in each sample of the powder obtained by drying to constant weight at 100°C. Then 10 g of the wet cocoa powder were homogenized in a blender with 100 ml of buffer whose composition was as follows: 0.1 M phosphate buffer; 28 mM ascorbic acid; 5 mM EDTA; 5% NaCl; pH 7.0. The homogenate was incubated for 1 h at ambient temperature and then centrifuged at $29\,000\times\boldsymbol{g}$ for 15 min at 20°C. The solid residue was discarded and the extract obtained was used in the following experiments.

The samples for the determination of the total activity were prepared as follows: 1.2 g of solid $(NH_4)_2SO_4$ was dissolved in 2 ml of the extract of the cocoa seeds and then the solution was incubated at 4°C. One hour later the precipitate formed was separated from the supernatant by centrifugation ($37\,000\times\boldsymbol{g}$; 20 min; 4°C). It was collected and dissolved in 2 ml of 10 mM phosphate buffer, pH 6.0. The peroxidase activity in the samples was measured on the same day.

The samples for the determination of the isoperoxidase composition were prepared as follows: 80 ml of the extract were dialyzed against 50 mM phosphate buffer, pH 7.3, and then applied onto a DEAE-Toyopearl (2.5×8.5 cm) column equilibrated with 150 mM phosphate buffer, pH 7.3, for the separation of the peroxidase from pigments. All isoenzymes of the cocoa peroxidase were eluted using 150 mM phosphate buffer, pH 7.3. The fractions containing the activity were pooled and concentrated (100 times) using a YM-10 membrane. Before isoelectrofocusing, the samples were dialyzed against 10 mM phosphate buffer, pH 7.0

2.3. Enzyme assay

The peroxidase activity was determined using guaiacol and o-phenylenediamine as substrates. The oxidation of guaiacol was measured spectrophotometrically at 470 nm, 25°C in 3 ml of 10 mM phosphate buffer, pH 6.0, containing 20 mM guaiacol and 4.4 mM H_2O_2. The oxidation of o-phenylenediamine was determined spectrophotometrically at 450 nm, 25°C in 3 ml of 50 mM citrate buffer, pH 4.75, containing 2.8 mM o-phenylenediamine and 4.4 mM H_2O_2. One unit of the activity (U) was defined as the amount of enzyme that caused an absorbance change of 0.001 per min under standard conditions. The specific activity was expressed as units of activity per gram of dry powder of the cocoa seeds.

2.4. Isoelectrofocusing

The isoelectric point (pI) values of peroxidase in plant extracts were determined in PAAG using a horizontal isoelectrofocusing cell (BioRad). The gel composition was as follows: 4.8% w/v acrylamide; 0.15% w/v bis(acrylamide); 5% w/v glycerol; 0.25 ml of ampholines (range 3–10); 0.04% w/v $(NH_4)_2S_2O_8$, 0.1% v/v TEMED. The samples (2 μl) were applied on the gel surface. The run was performed by keeping the voltage constant initially at 100 V for 15 min, then at 200 V for a subsequent 15 min, and finally at 450 V for 45 min. The isoenzymes of peroxidase were stained to immerse the gel in 50 ml of a solution of 20 mM guaiacol in 0.01 M of phosphate buffer, pH 6.0 containing 4.4 mM H_2O_2 at ambient temperature.

3. Results and discussion

Recently it was demonstrated that cocoa beans contain peroxidase activity (Ndoumou et al., 1995). Both the polyphenol oxidase, which oxidized phenols in the cocoa beans in the presence of oxygen (Hammer, 1993; Wong et al., 1990) and the peroxidase, which can catalyze the oxidation of phenolic compounds, may be considered to be important enzymes controlling the specific flavour of cocoa powder. Because the degree of participation of the peroxidase in this process depends upon its concentration, we have first determined its activity in the ripe seeds of cocoa. This value, measured toward guaiacol, was equal to 5700 U g^{-1} of seed. A similar value of the peroxidase activity was observed in the peel of the ripe pod while, in the pulp, the activity was absent. Comparison of the total activity of peroxidase in cocoa beans, at different ripening stages, showed that, at the wax stage, the seeds have low activity which increased significantly in the process of ripening (Fig. 1).

A further increase of the POD activity was registered during the fermentation and drying of cocoa beans. In this case the variations of the peroxidase activity had more complex character (Fig. 2(a)). So, after the first day of the fermentation, the activity remained invariable. However, the next day when, under the conditions of fermentation, the cocoa seeds die, according to

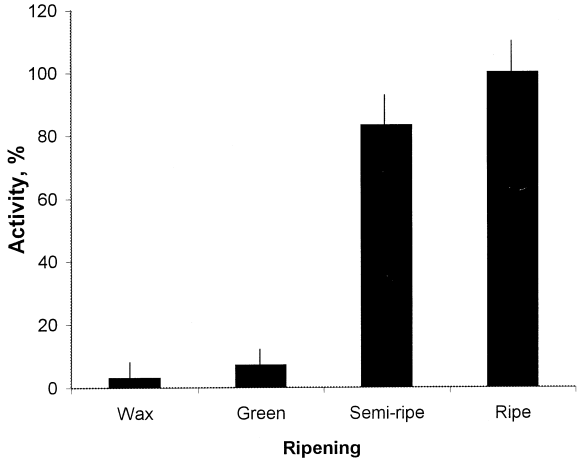

Fig. 1. Variation of the peroxidase activity in seeds in the course of the ripening of the cocoa pods. The ripening stage of pods was defined using their weight and colour: wax 39 ± 3 g and green; green 250 ± 10 g and green; semi-ripe 467 ± 11 g and green-yellow; ripe 501 ± 10 g and yellow, respectively. Each point represents the mean of three determinations; bars are standard deviations.

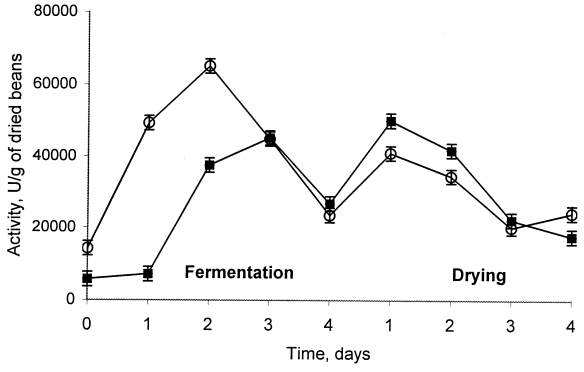

Fig. 2. Variation of the activity of the peroxidase during the fermentation and sun-drying of cocoa beans. The specific activity was determined toward (a) guaiacol (■—■) and (b) o-phenylenediamine (○—○). Each point represents the mean of three determinations; bars are standard deviations.

Ziegleder and Biehl (1988), a sharp increase of the specific activity was observed. This high level of the activity also remained at day 3, but then the activity decreased a little.

The next increase of the activity was registered in the course of the cocoa drying. The increased of the peroxidase activity detected on drying seems to be related to the stress reaction, which resulted from the change of conditions from fermentation to drying. Earlier it was shown in numerous works (Edreva, Salcheva, & Georgieva, 1993; Valpuesta, Quesada, Pilego, Hasegave, & Botella, 1993; Bakardjieva, Chrislova, & Christova, 1996; Kristensen & Rasmussen, 1996) that stress stimulated biosynthesis of plant peroxidases. In this case we have to assume that the 'dead' cocoa beans, which after their fermentation are already incapable of germination, retain the capacity to produce the peroxidase.

Thus, in the course of the fementation and drying, when the content of phenols in cocoa beans is decreased, the peroxidase activity increases significantly. The data obtained demonstrate that the oxidation of phenols may be catalysed, not only by polyphenol oxidase, but also by peroxidase. The second substrate of the peroxidase, H_2O_2, is generated in plants by autooxidation and polyphenol oxidase-catalyzed oxidation of (+)catechin. In this case a co-operative effect of the polyphenol oxidase and the peroxidase is observed. A similar cooperation between these oxidases of peach and pear has previously been reported (Alba, De Forchetti, & Tigier, 1996; Richard-Forget & Gauillard, 1996).

It should also be noted that, during the entiure process, the peroxidase activity in the beans was higher than that in unfermented ripe seeds (Fig. 2). Moreover, the cocoa beans, after finishing their fermentation and drying, retained high peroxidase activity. The presence of the active enzyme in the final product can vary the cocoa quality during its storage. In order to prevent these possible changes, according to the recommendations developed for long time storage of fruits and vegetables (Mueftuegil, 1985; Pizzocaro, Ricci, & Zanetti, 1988; Hammer, 1993), it is necessary to completely denature the peroxidase. In a separate experiment it was shown that, for 100% inactivation of the peroxidase, the fermented and dried beans have to be incubated for more than 3 days at 30°C.

It is well-known that plant peroxidases have numerous isoenzymes (Shannon, Kay, & Lew, 1966; Shannon, 1968; Powell, Pickering, Wander, & Smith, 1975). Their isoenzymes composition in tissues can be changed for their ripening, growth, differentiation or other physiological processes (Gaspar, Penel, Hageye, & Greppini, 1991; Valpuesta, Quesada, Sánchez-Roldán, Tigier, Heredia, & Bukovac, 1991). Moreover, the isoenzymes of peroxidase possess different substrate specificities and, hence, different physiological functions. So, the basic isoenzymes have higher specific activities toward guaiacol, phenolantipyrine, pyrogallol, while the acidic peroxidases prefer to oxidize o-phenylenediamine, ABTS, luminol, diaminobenzidine (Gazaryan, 1992). The peroxidase activity variations during fermentation and drying (Fig. 2(a)) were measured by use of guaiacol as a substrate. Later the peroxidase activity toward o-phenylenediamine (Fig. 2(b)) was also determined. Comparison of the data obtained showed that both curves are similar, but not identical. Presumably, during the fermentation and drying of the cocoa beans, there is not only an increase of the total activity of the peroxidase, but also a variation in the isoenzyme composition of the peroxidase.

To examine this hypothesis, an isoelectrofocusing technique was used. Thus, the peroxidase samples, obtained from ripe unfermented beans of cocoa and beans fermented for 1 day, contained only acidic

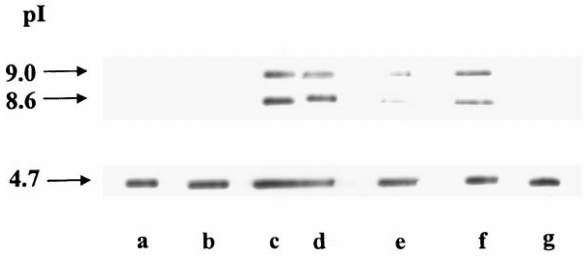

Fig. 3. Isoelectrofocusing of the peroxidase samples obtained from the cocoa beans at different stages of their fermentation and sun-drying: (a) unfermented ripe beans; (b)–(e) beans after 1st, 2nd, 3rd and 4th day of the fermentation, respectively; (f) and (g) beans after 1st and 3rd day of drying, respectively.

isoenzyme with pI 4.7 (Fig. 3(a) and (b)). However, already at the end of the second day of fermentation the two basic isoenzymes with pI 8.6 and 9.0 appeared (Fig. 3(c)). These basic isoenzymes were present at all following stages of the fermentation and drying of the beans although their concentration in the seeds varied (Fig. 3(d)–(g)). At the present time it is difficult to suggest any role for the minor isoperoxidases registered because the properties of these enzymes are unknown. The analysis of the catalytic properties of different isoperoxidases will be the subject of further studies.

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