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Flavor precursor and volatile compounds formation of unfermented cocoa beans hydrolyzed by papain

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Abstract

Fermentation stands as a notably crucial stage in the production of cocoa flavor precursors. Nevertheless, some cocoa plantations in Indonesia forego fermentation due to the relatively small raw material quantities and the exceedingly long fermentation periods. This study aimed to improve the flavor precursors in unfermented cocoa beans (var. forastero). Unfermented cocoa beans were hydrolyzed using papain at different concentrations and incubation times. The conditions to obtain the highest degree of hydrolysis were 3.3 U/mL (papain conc.) with a 10-hour incubation. After papain treatment, the beans exhibited lower reducing sugars and polyphenol content compared to unfermented cocoa beans. Moreover, hydrophobic amino acids such as phenylalanine, valine, leucine, and isoleucine were increased. After roasting, volatile compounds for chocolate aroma were also presented. However, pyrazines, aldehydes, and esters remained less abundant than those in fermented cocoa beans. Results proved that papain hydrolysis of unfermented cocoa beans can improve their flavor precursors and volatile compounds.

Keywords: enzymatic hydrolysis; papain; flavor precursor; unfermented cocoa beans var. forastero.

Practical Application: Papain hydrolysis of unfermented cocoa improved volatile compounds for chocolate aroma production.

1 INTRODUCTION

Chocolate, a widely beloved product renowned for its distinctive aroma, undergoes a multifaceted production process crucial for the development of its desired flavor profile. Key stages in this process include hybrid selection, fermentation, and roasting of cocoa beans (Afoakwa et al., 2008; Kadow et al., 2015). Proper fermentation is a prerequisite for high-quality chocolate, as it enables the formation of specific aroma compounds during the postprocessing stage (Balcázar-Zumaeta et al., 2023; Viesser et al., 2021).

Cocoa fermentation is the process of microbial decomposition of sugars in pulps into ethanol, lactic acid, and acetic acid. During fermentation, ethanol, lactic acid, and acetic acid diffuse into the beans, resulting in a decreased cotyledon pH from 6.3–7.0 to 3.5–5 and an increase in temperature up to 50°C, leading to cocoa bean mortality. These events induce enzymatic reactions in cocoa seeds (Castro-Alayo et al., 2019). Carboxipeptidase and aspartic endoprotease break down vicilin-class globulin (VCG) into polypeptides and free amino acids (FAAs), while invertase converts sucrose into fructose and glucose (Castro-Alayo et al., 2019).

Based on data from the International Cocoa Organization (ICCO, 2022), Ivory Coast, Ghana, and Cameroon are the main cocoa producers in the world, with a total production of 3.6 million tons in 2021. Indonesia is the third largest cocoa exporter after Ghana and Ivory Coast with production of 200 thousand tons of cocoa every year and a contribution of 5.5% to the total global production. The Indonesia Ministry of Agriculture (2023) reported that most cocoa products in Indonesia are produced by smallholder plantations, with only a fraction originating from large private and state plantations. Furthermore, most dry cocoa produced by smallholder plantations in Indonesia is of low quality due to farmers skipping the fermentation process. The cocoa yield produced by each farmer is relatively small, often failing to meet the minimum weight required for fermentation, which is approximately 40 kg fresh cocoa beans (Tarigan et al., 2017).

Numerous efforts have been carried out to improve the quality of dry unfermented cocoa beans. Cocoa bean fermentation with the addition of various starters produces high-quality products, albeit woth extended fermentation durations (Assi-Clair et al., 2019; Figueroa-Hernández et al., 2019). Incubating unfermented dry cocoa bean powder in acetate buffer (pH 5.5) at 45°C increases the intensity of the yellow color and the fermentation index while reducing cocoa procyanidin levels (Misnawi et al., 2003). Hydrolyzing unfermented dry cocoa beans with Flavourzyme® and crude bromelain produced more aroma precursors, such as FAAs and reducing sugars, along with additional volatile compounds than unfermented cocoa beans (Purbaningrum et al., 2023; Tamimi et al., 2023).

Papain, a proteolytic enzyme, catalyzes the breakdown of polypeptides in protein molecules into simpler compounds like dipeptides and amino acids (Díaz & Martinez, 2013). This enzyme is often found in papaya fruit (*Carica papaya* L.) and can be easily extracted (Amri & Mamboya, 2012). However, there are no reports investigating the hydrolysis capability of papain

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in forming aroma precursors in unfermented dry cocoa beans. This research aimed to determine the effect of enzymatic hydrolysis using papain at different concentrations and hydrolysis times on the increase in flavor precursors in unfermented dry cocoa beans.

2 MATERIAL AND METHODS

2.1 Materials

Cocoa beans of the forastero variety were collected from UPH Ngudiharjo in Patuk, Gunung Kidul, and Yogyakarta, Indonesia. Papain was purchased from Chemic Lab. KP, Bogor, Indonesia. Casein from bovine milk, tyrosine standard, Folin–Ciocâlteu reagent, Na_2CO_3, hydrochloric acid, petroleum ether, trichloroacetic acid (TCA), monobasic and dibasic sodium phosphate were bought from Merck (Darmstadt, Germany).

2.2 Preparation of unfermented dry cocoa beans

Cocoa pods were split open, and the cocoa beans were separated from their shells. The cocoa beans were dried in a cabinet dryer at a temperature of 50°C until the moisture content was below 7.5%. Then, the cocoa beans were stored at 4°C until further analysis.

2.3 Hydrolysis of unfermented dry cocoa beans using papain

Prior to the hydrolysis process, an enzyme activity test was carried out using the method of Cupp-Enyard and Aldrich (2008), and an enzyme activity of 0.11 U/mL was obtained. Unfermented and fermented cocoa beans served as negative and positive controls, respectively.

2.3.1 Effect of enzyme concentrations on the degree of hydrolysis

Unfermented dry cocoa beans (± 7 g) were soaked in 10 mL of acetate buffer 0.1 M, pH 6, and then added with papain at concentrations of 0.5, 1.0, 1.5, 2.0, 2.5, 3.3, and 3.85 U/mL. The solution was then incubated in a water bath shaker (Memmert WNB14, Germany) at 50°C for 8 h. The incubated cocoa beans were sun-dried until the moisture content was under 7.5%.

2.3.2 Effect of incubation times on the degree of hydrolysis

Unfermented dry cocoa beans (± 7 g) were soaked in 10 mL of acetate buffer 0.1 M, pH 6, added with the optimal concentration of papain determined in the previous stage (30 U/mL), and incubated in a waterbath for 6, 8, 10, 12, and 14 h at 50°C. The incubated cocoa beans were sun-dried until the moisture content was under 7.5%.

2.4 Characterization of cocoa beans after enzymatic treatment

2.4.1 Degree of hydrolysis

The degree of hydrolysis (DH) was analyzed using the method of Putra et al. (2018) with slight modifications. A total of 1 g sample was diluted with 10 mL aquadest and then homogenized by vortexing for 5 min before being centrifuged at 2,800×g at 4°C for 15 min. Afterward, 5 mL of the supernatant was mixed with 5 mL TCA 20% and then centrifuged at 2,800×g at 4°C for 15 min. Total nitrogen and nitrogen soluble in the supernatant were determined using the Kjeldahl method. DH was calculated with the Equation 1:

$$DH\ (\%) = \frac{\text{Soluble nitrogen in 20\% TCA}}{\text{Total nitrogen sample}} \times 100 \quad (1)$$

2.4.2 Free amino acids

Total FAAs were measured in accordance with the work of (Selamassakul et al., 2020) with several modifications. Defatted cocoa powder (60 mg) was added with 4 mL 6 N HCl and heated for 1 h at 110 °C. Afterward, the mixture was cooled to room temperature and neutralized to pH 7 using 6 N NaOH. Next, distilled water was added to the sample up to 10 mL, and the mixture was filtered through a 0.2 µm Whatman filter paper. Subsequently, 50 µL of the sample was mixed with 300 µL of orthophthalaldehyde solution and stirred for 5 min. Finally, 10 µL of the resulting sample was injected into the high-performance liquid chromatography (HPLC) injector for further analysis. FAA analysis was carried out using an HPLC Thermo Dionex Ultimate 3000 equipped with a Thermo Ultimate 3000 RS Fluorescence Detector (ThermoFisher Scientific, Waltham, MA, USA). The analysis was carried out on a LiChrospher 100 RP-18 column (5 µm) using the mobile phase of methanol:50 mM sodium acetate: tetrahydrofuran (THF) (2:96:2) (pH 6.8) (solvent A) and 65% methanol (solvent B). The flow rate was 1.5 mL/min, and the gradient elution in the time range of 0.1–15 min was 100% A, 35% B within 15–30 min, 100% B within 30–40 min; and 100% B within 40–45 min.

2.4.3 Reducing sugars

The Nelson–Somogy method (Nelson, 1944) was used to calculate reducing sugars. A total of 1 g sample was dissolved in 50 mL warm Aquadest and then filtered. Lead acetate was added to 25 mL of the solution, filtered, and diluted to 50 mL. A 1 mL sample was placed in a test tube and added with 1 mL Nelson's reagent. The solution was heated in boiling water for 20 min. After cooling, the solution was added with 1 mL of arsenomonolydate and 7 mL of distilled water, followed by homogenization. Absorbance was measured at 540 nm using an ultraviolet-visible spectrophotometer.

2.4.4 Total polyphenols

Total polyphenols were calculated using the Folin–Ciocâlteu method (Reza et al., 2010). A 1 g sample was dissolved in 50 mL of warm Aquadest and then filtered. Exactly 1 mL of the solution was placed in a test tube and added with 1 ml Folin–Ciocalteau (10%) and 0.8 mL Na_2CO_3 (7.5%), followed by homogenization. The solution was incubated for 30 min in the dark at room temperature and absorbance was measured at 750 nm.

2.4.5 Volatile compounds

Volatile aroma compounds were analyzed based on previous method with slight modifications (Fang et al., 2020). A 3.5 g sample was added with 0.2 µL 0.001% 3,4,6-trimethylpyridine (internal standard) and extracted in a 22 ml headspace solid-phase microextraction (SPME) vial at 60°C for 50 min using SPME fibers (divinylbenzene/carboxen/polydimethylsiloxane). The extraction results were analyzed using an Agilent 7890A gas chromatograph equipped with an Agilent 5975 C Triple Axis mass spectrometer (MS) detector (USA). A splitless injector was used and set at 250°C, with MS source at 230°C, MS quad at 150°C, and interface at 250°C. Scan mass range was 29–550 amu, and the capillary column was TR-WAXMS (30 m × 0.25 mm, 0.25 µm). Oven temperature was set at 20°C for 2 min, increased to 180°C at a rate of 2°C/min for 1 min, and held at 240°C for 2 min at a rate of 10 °C/min. Helium was used as the carrier gas at a rate of 1 mL/min. Volatile components were determined using the National Institute of Standards and Technology mass spectral library (NIST14). Verification was carried out by determination of the Kovat retention Index (KI), calculated for each peak using standard n-alkane retention time data (C9–C25) injected under the same conditions as the samples. Experimental Kovat indices (KI(exp)) of the confirmed aroma compounds were calculated based on their respective retention times and compared with the KIs from literature (KI(lit)), obtained from pherobase.com, the work of Hinneh et al. (2018) and (Tuenter et al., 2020).

2.5 Statistical analysis

Data were subjected to analysis of variance (ANOVA) and further evaluated with Duncan's multiple range test if significant differences were observed in the results at a significance level of 5% ($p < 0.05$).

3 RESULTS AND DISCUSSION

3.1 Degree of hydrolysis of unfermented cocoa bean after papain treatment

3.1.1 Effect of papain concentration on the degree of hydrolysis

DH increased in accordance with the addition of papain until reaching a maximum concentration of 3.3 U/mL. As shown in Figure 1A, the rate of hydrolysis plateaued after the addition of the enzyme at concentrations above 3.3 U/mL. Noman et al. (2018) and Singh et al. (2019) stated that increasing the concentration of papain above the optimal concentration had no remarkable effect on DH. This is associated with the presence of enzyme aggregation, which causes the inhibition of substrate diffusion and thus causes a decrease in the rate of reaction. The results of this study are similar to those of Tamimi et al. (2023), who reported that the DH of dry cocoa beans incubated for 8 h increased with rising Flavourzyme® concentration. Meanwhile, different results were reported by Purbaningrum et al. (2023); the DH of dry cocoa beans did not increase considerably after enzyme addition at concentrations ranging from 3.5 U/mL to 10.5 U/mL during 8 h of incubation. In this study, the enzyme concentration of 3.3 U/mL was selected and then used to determine incubation time.

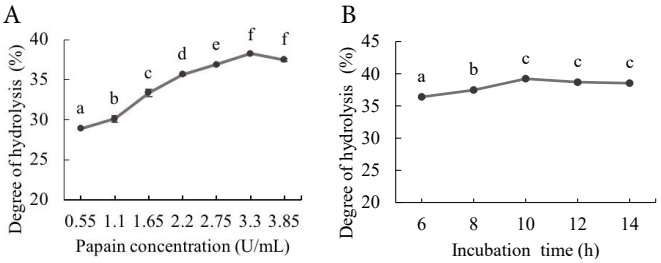

Figure 1. Degree of Hydrolysis of unfermented cocoa beans after the treatment with (A) various concentrations of papain, hydrolysis was carried out for 8 h in 10 mL of acetate buffer 0.1 M at pH 6 and a temperature of 50°C; (B) variations in incubation time, hydrolysis was carried out by the addition of 3.3 U/mL papain in 10 mL acetate buffer 0.1 M at pH 6 and a temperature of 50°C. Variations in incubation time. Values with different letters represent significant differences ($\alpha = 0.05$) in between samples.

3.1.2 Effect of incubation times on the degree of hydrolysis

DH was measured within 6–14 h of incubation. As shown in Figure 1B, the percentage of DH increased from 36.40 to 39.22% during 6–10 h of incubation. No significant increase was observed after extended incubation. Singh et al. (2019) reported that the hydrolysis rate increases until a certain optimal time and eventually reaches a stationary phase. Each type of enzyme has different hydrolytic capabilities. Bromelain can participate in hydrolysis for 6 h (Purbaningrum et al., 2023), Flavourzyme® for 8 h (Tamimi et al., 2023), and papain for 10 h. Thus, an incubation time of 10 h was selected for further hydrolysis processes.

3.2 Characterization of unfermented cocoa beans after papain treatment

3.2.1 Free amino acids

Papain-mediated hydrolysis of dry cocoa beans significantly increased the content certain hydrophobic FAAs ($p < 0.05$). Supplementary Table 1 shows the four types of hydrophobic FAAs (valine, leucine, isoleucine, and phenylalanine) whose concentrations were higher than those of unfermented cocoa beans but slightly lower than those of fermented cocoa beans. Hydrophobic FAAs, especially leucine, valine, alanine, isoleucine, and phenylalanine, act as precursors for cocoa bean flavor development (Frauendorfer & Schieberle, 2006; Ziegler, 2017). The results proved that enzymatic hydrolysis using papain increased FAA levels, augmenting flavor precursors availability in cocoa beans. The levels of valine, leucine, isoleucine, and phenylalanine increased due to wide specificity of papain in recognizing and breaking down proteins, especially the hydrophobic amino acids at the second position from the N-terminal side (Díaz & Martinez, 2013). Papain shows preference for hydrophobic amino acids, such as valine, phenylalanine, leucine, and isoleucine (Gosalia et al., 2005; Harris et al., 2000). These findings corroborate with Tamimi et al. (2023), who reported that Flavourzyme® can only increase the levels of four types of FAAs including tyrosine, valine, phenylalanine, and leucine. Meanwhile, the hydrolysis of dried cocoa beans using bromelain increased the concentrations of almost all types of FAAs detected (Purbaningrum et al., 2023).

Table 1. Free amino acid contents of cocoa powders treated with papain (concentration of 3.3 U/mL for 10 h of incubation) compared with those of unfermented and spontaneous fermented cocoa.

Free amino acid (FAA)	Sample (mg/mL)		
	Unfermented	Fermented	Papain treatment
FAA Acidic			
Aspartic acid	70.26 ± 0.67[b]	60.75 ± 0.84[a]	60.92 ± 1.33[a]
Glutamic acid	137.96 ± 0.26[b]	117.78 ± 0.43[a]	115.91 ± 1.09[a]
FAA Hydrophilic			
Serine	37.21 ± 0.18[b]	33.23 ± 0.70[a]	36.67 ± 0.24[b]
Threonine*	14.28 ± 0.05[a]	16.29 ± 0.06[c]	14.92 ± 0.07[a]
Tyrosine	45.48 ± 0.14[b]	44.48 ± 0.23[a]	43.65 ± 0.47[a]
FAA Hydrophobic			
Glisin	33.78 ± 0.28[b]	30.55 ± 0.61[a]	32.53 ± 0.07[b]
Alanin	34.58 ± 0.45[c]	31.59 ± 0.15[a]	33.40 ± 0.01[b]
Valin*	16.18 ± 0.06[a]	17.96 ± 0.51[b]	17.40 ± 0.16[b]
Leucine*	33.29 ± 0.02[a]	34.67 ± 0.43[b]	34.21 ± 0.09[b]
Iso Leucine*	8.42 ± 0.08[a]	10.13± 0.08[c]	9.39 ± 0.21[b]
Phenylalanine*	28.78 ± 0.27[a]	31.86 ± 0.31[b]	31.64 ± 0.32[b]
Metionin	1.77 ± 0.03[a]	1.74 ± 0.02[a]	1.73 ± 0.03[a]
FAA Basic			
Arginin	46.30 ± 0.11[a]	46.69 ± 0.12[a]	46.92 ± 0.05[a]
Lysin	44.64 ± 0,52[b]	41,23 ± 0,74[a]	41,60 ± 0,45[a]
Histidin	10,87 ± 0,12[b]	8,79 ± 0,63[a]	9,66 ± 0,11[a]
Total	**156,76**	**157.39**	**160.84**

Value with different letters represent significant differences (α = 0.05) between samples.

During fermentation, hydrophobic FAA contents increased significantly ($p < 0.05$), particularly valine, leucine, isoleucine, and phenylalanine. Peptides and FAAs produced during cocoa bean fermentation are flavor precursors that play a role in the development of cocoa aroma and taste. Peptides and FAAs are formed through the proteolysis of VCG induced by acetic acid and lactic acid and the joint action of aspartic endoprotease and carboxypeptidase present in cocoa beans. Hydrophobic FAAs, such as leucine, alanine, valine, isoleucine, and phenylalanine, are the main precursors that contribute to the development of cocoa aroma (Ziegler, 2017). Peptides and FAAs, which are hydrophilic and hydrophobic, react with fructose and glucose to form the desired aroma of cocoa (Afoakwa et al., 2008)

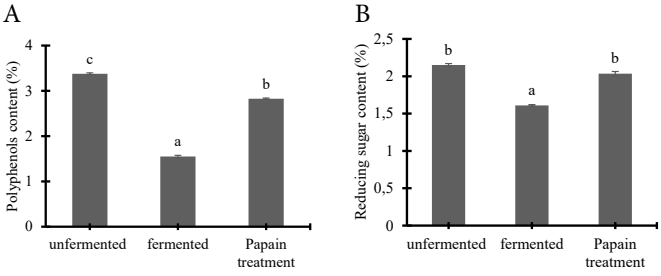

Figure 2. (A) Reducing sugar and (B) polyphenol content of cocoa powders treated with papain (concentration of 3.3 U/mL in 10 mL acetate buffer (0.1 M and pH 6) for 10 h of incubation) compared with that of unfermented and spontaneously fermented cocoa. Value with different letters represent significant differences (α = 0.05) between samples.

3.2.2 Reducing sugars content

The highest reduction in the content of reducing sugars was observed in fermented beans, followed by papain-treated and unfermented cocoa beans (Figure 2A). This research was similar to that of Calvo et al. (2021) and Viesser et al. (2021), who reported lower reducing sugar content in fermented cocoa beans compared to unfermented ones. During ermentation, microbes metabolize reducing sugars, leading to a rapid decline in their levels within 48 h, as reported by Rottiers et al. (2019). The differences in reducing sugar contents (glucose and fructose) after the fermentation process depend on the duration of the fermentation process. Glucose and fructose are consumed by microbes, such as lactic acid bacteria and yeast within 48 h to 72 and 120 h, respectively (Viesser et al., 2021).

Hydrolyzed cocoa beans did not change the reducing sugar content as papain only broke down proteins into short-chain peptides and FAAs. Meanwhile, invertase plays a role in breaking down sucrose into reducing sugars such as glucose and fructose (Castro-Alayo et al., 2019). Purbaningrum et al. (2023) and Tamimi et al. (2023) reported that the levels of reducing sugars increased after hydrolysis for 8 h. Incubation at the acetate buffer temperature of 50°C and pH 6 can activate the remaining invertase in cocoa beans. Misnawi et al. (2002) reported that 19% of invertase activity remained in unfermented dry cocoa beans. However, no invertase activity remained in the cocoa beans hydrolyzed with papain in an acetate buffer medium at pH 6 and a temperature of 50°C for 10 h. This was presumably because of the lack of interaction between invertase and the substrate contained in cocoa beans.

3.2.3 Polyphenol content

The content of total polyphenols in unfermented cocoa beans was considerably higher than those of fermented and papain-treated cocoa beans (Figure 2B). The decline in polyphenol content during the fermentation process was caused by polyphenol oxidase, which played a role in the oxidization of polyphenols in the seeds, resulting in decreased polyphenol content. A decrease in the total polyphenols was observed in cocoa beans with papain treatment, which was associated with the activation of endogenous polyphenol oxidases remaining in the beans. Misnawi et al. (2003) reported that the incubation of cocoa beans in acetate buffer at the appropriate temperature and amount of water can activate the remaining polyphenol oxidases of dry and unfermented cocoa beans.

3.2.4 Volatile compounds after roasting

A total of 26, 33, and 35 volatile compounds were identified in unfermented, enzymatically hydrolyzed, and fermented cocoa beans, respectively. The identified compounds were divided into eight groups, including pyrazines, aldehydes, esters, alcohols, ketones, acids, pyrroles, and furfural. Volatile compounds in chocolate develop during the roasting process through the Maillard reaction, which is resposible for the characteristic chocolate flavor. The Maillard reaction is responsible for the changes in FAAs, peptides, and reducing sugars and their transformation into key organic compounds in the formation of the characteristic flavor of cocoa beans (Hinneh et al., 2018). According to Aprotosoaie et al. (2016), the Maillard reaction begins when the free amino groups of amino acids attacking the carbonyl groups of glucose and fructose to form Schiff bases. The reaction continues and produces compounds, such as Amadori compounds, hydroxymethylfurfural, and furfural. At alkaline or neutral pH, compounds, such as maltol, isomaltol, and α-dicarbonyl compounds, are formed. Furthermore, the breakdown of α-dicarbonyl compounds into smaller aldehydes and ketones occurs through dehydration, fragmentation, and transamination reactions. Strecker degradation reactions also occur and produce volatile aldehydes, volatile pyrazine, and other heterocyclic compounds (Afoakwa et al., 2008).

Pyrazine is a primary group of heterocyclic volatile compounds and is the main component influencing cocoa's flavor. This compound encompasses nutty, roasted, cocoa, and chocolaty flavors (Afoakwa et al., 2009; Rottiers et al., 2019). The concentration of total pyrazine increased substantially during the hydrolysis of cocoa beans by papain (Supplementary Table 1). Eight pyrazine compounds were found in this study, with tetramethylpyrazine and trimethylpyrazine being the predominant ones. According to Bonvehí (2005) and Rodriguez-Campos et al. (2012), tetramethylpyrazine and trimethylpyrazine are pivotal pyrazine compounds as they act as chocolate flavor enhancers. The results of this study were similar to those of Purbaningrum et al. (2023) and Tamimi et al. (2023), who reported the hydrolysis of unfermented dry cocoa beans using bromelain; Flavourzyme® was proven to increase the amount of pyrazine compounds contained in the beans.

Fermented cocoa beans displayed the highest acid content, followed by enzymatically hydrolyzed and unfermented cocoa beans (Supplementary Table 1). The high acid concentration in fermented seeds was the result of microbial metabolism, which plays a role in the fermentation process (Rottiers et al., 2019). Febrianto and Zhu (2022) stated that acetic acid was highly correlated with the level of fermentation in cocoa beans. Meanwhile, the increased acid content in seeds, which resulted from enzymatic hydrolysis, was caused by the use of an acetate buffer solution to adjust pH, based on the optimal activity of papain. Acetic acid is associated with a sour taste, such as that of vinegar, and was the most active aroma compound in unroasted and roasted cocoa beans (Rodriguez-Campos et al., 2012; Rojas et al., 2022).

Alcohol is a result of microbial activity during fermentation. In addition, these compounds can be formed through the heat degradation of amino acids (Aprotosoaie et al., 2016). The alcohol compounds with the highest concentrations in fermented cocoa beans were 2-phenylethanol, 2-nonanol, and 2,3 butanediol. Meanwhile, the highest concentrations of alcohol compounds in papain treatment were 2-phenylethanol, isoamyl alcohol, and 2,3 butanediol (Supplementary Table 1). The high alcohol content of cocoa products is desirable in obtaining fruity, candy, and floral aromas (Rodriguez-Campos et al., 2012). 2-Phenylethanol is the main alcohol compound that plays a major role in creating the distinctive flavor of cocoa and has characteristic aromas, such as flowery, sweet, and bready ones (Rodriguez-Campos et al., 2012; Tuenter et al., 2020).

Aldehydes are formed during roasting through Strecker degradation of FAAs, including alanine, valine, leucine, isoleucine, and phenylalanine (Afoakwa et al., 2009; Aprotosoaie et al., 2016; Ziegler, 2017). Compounds in the aldehyde group such as 2-Methylpropanal, 2-methylbutanal, 3-methylbutanal, and 2-phenyl-2-butenal contribute to the characteristic aroma of chocolate (Rodriguez-Campos et al., 2012; Rottiers et al., 2019). In addition, benzaldehyde (sweet, almond, cherry, and bitter) and benzeneacetaldehyde (almond, fruit, and nut) compounds considerably affect the aroma characteristics of chocolate and contribute to the desired flavor profile (Rottiers et al., 2019). The fermentation process and enzymatic hydrolysis increased the amounts of 3-methylbutanal, 2-phenyl-2-butenal, benzaldehyde, and benzeneacetaldehyde in cocoa beans compared with those of unfermented cocoa beans (Supplementary Table 1). 3-Methylbutanal was the most dominant compound in fermented cocoa beans and during enzymatic hydrolysis. Meanwhile, in unfermented cocoa beans, the concentrations of aldehyde compounds, including benzaldehyde and benzeneacetaldehyde, were very low. Enzymatic hydrolysis with papain formed 3-methylbutanal in cocoa beans, while it was not formed in cocoa beans hydrolyzed by bromelain (Purbaningrum et al., 2023) and Flavourzyme® (Tamimi et al., 2023).

Ester compounds contribute to the aroma and taste profile of roasted cocoa beans. This group of volatile compounds imparts a fruity, floral, sweet, and honey-like aroma to cocoa (Rottiers et al., 2019; Utrilla-Vázquez et al., 2020). Esters are formed during the anaerobic fermentation phase, subsequent to alcohol formation, as a product of fermentation. In this stage, the reaction between organic acids and alcohols results in ester

formation (Colonges et al., 2022). The concentration of ester compounds increased considerably in cocoa beans through the fermentation and enzymatic hydrolysis processes (Supplementary Table 1). Five types of ester compounds were formed in fermented cocoa beans, whereas three types were formed in cocoa beans with papain treatment. This group of aromatic ester compounds substantially contributes to the aroma of certain products (Balcázar-Zumaeta et al., 2023). In this study, isoamyl acetate was the most dominant ester compound formed in cocoa beans under enzymatic hydrolysis and fermentation processes.

The concentration of ketone compounds in cocoa beans significantly increased during fermentation and enzymatic hydrolysis ($p < 0.05$) (Supplementary Table 1). In this study, 2-heptanone, acetoin, 2-nonanone, and acetophenone were identified. Ketone compounds contribute to the aroma and play an important role in the production of high-quality cocoa beans with a distinctive fruity and floral aroma (Assi-Clair et al., 2019; Marseglia et al., 2020). According to Rodriguez-Campos et al. (2012), ketones are produced during the fermentation process, and the ketone content in cocoa beans increases with fermentation time. 2-Acetylpyrrole and furaneol were also identified in this study. 2-Acetylpyrrole is produced from proline through Streker degradation during the Maillard reaction and imparts desired aromas, such as caramel, chocolaty, and toasted flavors (Afoakwa et al., 2009; Rodriguez-Campos et al., 2012).

4 CONCLUSION

The use of papain to hydrolyze unfermented dry cocoa beans resulted in higher concentrations of FAAs, especially hydrophobic amino acids such as phenylalanine, valine, leucine, and isoleucine, compared to unfermented cocoa beans. With the increase in aroma precursors, the amounts of desired volatile compounds in cocoa beans increased through enzymatic hydrolysis to produce a distinctive chocolate aroma. However, the amounts of main flavor compounds produced, such as pyrazines, aldehydes, and esters, were still less than those in fermented cocoa beans. The percentage of reducing sugars obtained in the enzymatic hydrolysis treatment did not change substantially. On the other hand, the total phenolic content in the enzymatically treated cocoa beans was close to that of fermented cocoa beans. Further research is needed to explore the use of other enzymes, such as invertase or polyphenol oxidase, for the creation of a complex flavor of cocoa beans.

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