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Origin and varietal based proteomic and peptidomic fingerprinting of *Theobroma cacao* in non-fermented and fermented cocoa beans

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ABSTRACT

It is well known that the development of chocolate flavor is initiated during cocoa bean fermentation. Storage proteins undergo the most intensive breakdown yielding peptides and free amino acids, which both serve as flavor precursors. A comprehensive analysis of cocoa proteins and oligopeptides of non-fermented and fermented beans from various geographic origins allows the assessment of systematic differences with respect to their origin as well as fermentation status. Protein quantities as well as their profiles derived from two-dimensional gel electrophoresis, showed striking differences for non-fermented beans depending on their geographical origin. From fermented beans, oligopeptides were relatively quantified by utilizing UHPLC-ESI-Q-q-TOF and annotated based on their characteristic fragmentation pattern in the positive-ion mode. With > 800 unique oligopeptides, excluding di- and tri-peptides, across 25 different samples, we are herein reporting on the largest collection of cocoa oligopeptides ever observed and identified. The detected diversity of peptides could not be correlated to the geographical origin but rather to the degree of fermentation. Our findings suggest that the variability in peptide patterns depends on the fermentation method applied in the country of origin ultimately indicating diversified proteolytic activities. Furthermore, our results showed that well-fermented and fair-fermented beans can be differentiated from partially fermented and under-fermented ones by higher numbers and total amounts of oligopeptides.

1. Introduction

The principal ingredient of chocolate and its related products' is the cocoa bean - the seed from fruit pods of the tree, *Theobroma cacao* Linné (*T. cocoa*). Although *T. cacao* is native to Central and northern South America, it grows in tropical regions throughout the world and nearly 70% of the world crop is currently produced in West Africa. Presently, Ivory Coast is leading in cocoa production with 1.65 million tons per year, followed by Ghana, Nigeria, Cameroon, and Togo, producing additional 1.55 million tons (Nkamleu & Kielland, 2006). Raw cocoa has an astringent, unpleasant taste and flavor. In order to obtain the characteristic cocoa taste, raw cocoa is fermented, dried and roasted (Doyle, Beuchat, & Montville, 2001). Non-fermented or under-fermented cocoa beans that were dried without fermentation or that were improperly fermented were shown to not develop any chocolate flavor when roasted and remained astringent and bitter (Hurst et al., 2011).

Cocoa storage proteins undergo the most intensive modifications during fermentation, where microbiological fermentation triggers extensive proteolytic breakdown yielding peptides and free amino acids, which were suggested to serve as important flavor precursors (Zak & Keeney, 1976). Proteins account for 10–15% of the dry weight of cocoa seeds, the second most abundant constituent after cocoa fat and superior to polyphenolics (D'Souza et al., 2017). Cocoa proteins are cleaved to yield hydrophilic and hydrophobic peptides as well as amino acids mainly through the activity of two endogenous enzyme groups, which have been suggested to be aspartic endoproteases and carboxypeptidases (Amin, Jinap, Jamilah, Harikrisna, & Biehl, 2002). Fermentation of cocoa beans is fundamental for the activation of these two enzymes by microbial metabolites (such as acetic acid), while there is no evidence that microbial enzymes penetrate the beans and create peptide flavor precursors (Schwan & Wheals, 2004). Previous studies have shown that proteolysis of the globulin fraction, produced peptides and amino acids that contributed directly to the flavor of cocoa (Voigt, Heinrichs, & Voigt, 1994) and to so-called Maillard reactions during roasting. Interestingly, the profile and the process conditions of roasting turned out to be more responsible for a good flavor than the total

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Table 1
Sample overview and accessory information for the cocoa beans.

Code	Country of origin	Hybrid	GPS Co-ordinates	Fermentation type	Fermentation duration (d)	Drying duration (d)
MA01	Malaysia	PBC-140	4°15′0″N 101°58′59″E	Spontaneous	6	9
MA02	Malaysia	PBC-123	4°15′0″N 101°58′59″E	Spontaneous	6	9
MA03	Malaysia	PBC-159	4°15′0″N 101°58′59″E	Spontaneous	6	9
IA01	Indonesia	ICS-60	6°55′40″S 106°55′48″E	Spontaneous	6	7
IA03	Indonesia	TSH-858	6°55′40″S 106°55′48″E	Spontaneous	6	7
IA09	Indonesia	Sulawesi 1	8°15′24″S 113°36′40″E	Spontaneous	5	10
IA12	Indonesia	PA191	8°23′45″S 114°0′11″E	Spontaneous	6	16
BB01	Brazil	Comum	14°46′39″S 39°03′22″W	Spontaneous	6	–
BC01	Brazil	CCN51	14°2′53″S 39°23′2″W	Spontaneous	6	16
BC02	Brazil	Comum	14°43′10″S 39°11′56″W	Controlled	7	14
BC04	Brazil	CCN51	–	–	–	8
EB03	Ecuador	EET-103	0°47′60″S 80°8′8″W	Spontaneous	4	10
EB05	Ecuador	EET-544	2°19′47″S 80°12′54″W	Spontaneous	5	9
EB06	Ecuador	EET-558	2°19′47″S 80°12′54″W	Spontaneous	5	9
EC05	Ecuador	CCN51	2°20′48″S 80°13′19″W	–	–	–
EC06	Ecuador	CCN51	2°22′27″S 80°14′10″W	–	–	–
EC07	Ecuador	CCN51	2°21′48″S 80°13′2″W	–	–	–
VA01	Ivory Coast	UPA405 × C412	5°30′12″N 3°0′19″W	Spontaneous	6	8
VA05	Ivory Coast	UPA409 × C501	6°48′20″N 6°59′22″W	Spontaneous	7	8
VB04	Ivory Coast	C308 × C5	5°29′14″N 2°59′52″W	Spontaneous	6	11
TA04	Tanzania	Unknown	9°36′27″S 33°54′57″E	Controlled	4	–
TA07	Tanzania	Unknown	9°22′29″S 33°49′22″E	Controlled	4	–
TA09	Tanzania	Unknown	9°36′27″S 33°54′57″E	Controlled	4	–
CD03	Cameroon	German (Forastero)	4°17′34″N 11°44′6″E	Spontaneous	6	10
CD04	Cameroon	Criollo	4°17′34″N 11°44′6″E	Spontaneous	6	10

amount of free amino-acids (Rohan & Stewart, 1967). Currently around 50 oligopeptides generated during fermentation-based proteolysis have been reported and were suggested as key cocoa quality markers (Voigt, Janek, Textoris-Taube, Niewienda, & Wostemeyer, 2016).

Flavor quality of cocoa beans is believed to depend on the genotype and the origin of the cocoa tree that produced the beans (Kongor et al., 2016). Both, fermentation and drying, are usually conducted as traditional, indigenous processes (Ho, Zhao, & Fleet, 2014), the details of which depend on the country of origin. However, both processes additionally influence the taste and flavor of the cocoa products. Characteristics of geographical origins of the fermented cocoa beans could thus be linked to both, the fermentation and drying procedure as well as to the cocoa genetic varieties. Since the quality of the resulting beans represents their economic values, there is a strong need for adequate methods for quality assessment.

Variations in bean characteristics based on genotype and geographic origins are not restricted to fermented beans but also occur with non-fermented cocoa beans (also frequently referred to as raw or wet cocoa beans) from different genotypes and origins (Kongor et al., 2016). Their particular flavor differences has been attributed to the inherent genetic composition of the bean, its location of growth, growing conditions such as the amount and time of sunshine and rainfall, soil conditions, ripening status, time of harvesting, and the time between harvesting and bean fermentation (Kongor et al., 2016).

Although protein and peptide profiles of cocoa beans based on their genotypes have been studied previously (Afoakwa, Paterson, Fowler, & Ryan, 2008; Gu et al., 2013), the combined impact of genetic diversity among cocoa beans and extrinsic factors such as geographic origin, harvest season, fermentation methods as well as subsequent processing steps remains under-explored. Consequently, the aim of this study was to provide a comprehensive analysis of the most diverse sample set of cocoa beans yet reported, paired in their non-fermented and fermented form, from seven different countries of origin, namely Malaysia, Indonesia, Brazil, Ecuador, Ivory Coast, Tanzania and Cameroon. Apart from the geographical diversity, these countries were chosen for their contribution to global cocoa production as well as sourcing opportunities. As part of our analysis, we present a detailed comparison of the protein content and profiles of 25 non-fermented bean samples as well as the peptide profiles of their dried and fermented equivalent, respectively.

2. Material and methods

2.1. Chemicals and reagents

Tris-HCl (Pufferan®, ≥99.5%), sodium dodecyl sulphate (SDS, ≥99.5% electrophoresis grade), High performance liquid chromatography (HPLC)-grade water (Rotisolv®), glycerol (86%), acrylamide (Rotiphorese® Gel 30:37, 5:1), acetonitrile (ACN, Rotisolv® HPLC ultra gradient grade), sodium acetate, HPLC-grade methanol and 3-[(3-Cholamidopropyl)dimethyl ammonio]-1-propane sulphonate (CHAPS, Pufferan®, 98%) were purchased from Carl Roth (Karlsruhe, Germany). Acetic acid, sodium carbonate and hesperetin were obtained from Sigma-Aldrich (Germany). Dithiothreitol (DTT, Biochemica), acetone (100%, Biochemica), TEMED, ammonium persulphate (APS, analytical grade) and acetic acid (100%, analytical grade) were purchased from Applichem (Darmstadt, Germany). Isopropanol (100%, analytical grade) and bromophenol blue sodium salt (research grade) were purchased from Serva Electrophoresis (Heidelberg, Germany). Urea (≥99.5%, BioScience-Grade), thiourea (ACS reagent, ≥99%), 2,5-Dihydroxybenzoic acid (> 99%, HPLC grade), trifluoroacetic acid (TFA, 99%, ReagentPlus®), 100 × BioLyte® 3/10 Ampholyte and ReadyPrep™ 2D Cleanup Kit were purchased from Bio-Rad (Munich, Germany). Pierce™ Coomassie (Bradford) Protein Assay kit and protease inhibitor cocktails were purchased from ThermoFisher Scientific (Bremen, Germany). Coomassie® Brilliant blue G-250 (electrophoresis grade) was purchased from Merck (Darmstadt, Germany).

2.2. Sample collection and preparation

Cocoa bean samples were collected from seven countries, i.e., Malaysia, Indonesia, Ecuador, Brazil, Ivory Coast, Tanzania and Cameroon, by Barry Callebaut AG, in non-fermented and fermented form. Information on fermentation and drying times is presented in Table 1. The bean samples were processed to a fine powder using a knife mill as described in our previous study (Kumari et al., 2016). Peptide samples were further processed by defatting around 5 g of fine powder using dichloromethane in an automated Soxhlet extraction