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Biochemical fate of vicilin storage protein during fermentation and drying of cocoa beans

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ABSTRACT

Key cocoa-specific aroma precursors are generated during the fermentation of cocoa beans via the proteolysis of the vicilin-like globulin. Previous studies had shown that degradation of this particular 566 amino acid-long storage protein leads to three distinct subunits with different molecular masses. Although oligopeptides generated from the proteolysis of vicilin-like globulin have been studied previously, changes occurring to vicilin at different stages of fermentation have not yet been explored in detail. The aim of this study was to investigate the fate of vicilin protein from the non-fermented stage up to the dried cocoa beans. Our results showed a remarkable shift in the electrophoretic mobility of vicilin towards higher pI during the onset of fermentation. The pI-shifted subunit was found susceptible to further degradation into a lower-molecular-weight vicilin subunit. The observed pI shift correlated with, but did not depend on protein phosphorylation. Glycosylation of some but not all vicilin subunits occurred at different stages of the fermentation process. Peptides generated from vicilin throughout fermentation were analyzed by UHPLC-ESI-MS/MS revealing an initial increase and subsequent decrease in the diversity of peptides with an increasing degree of fermentation. We furthermore describe the rate of degradation of different vicilin subunits. The detected diversity and dynamics of vicilin peptides will help to define biochemical markers of distinct steps of the fermentation process.

1. Introduction

Before getting transformed into chocolate and other cocoa products, the content of cocoa beans undergoes a series of complex metabolic changes during processing steps involving fermentation, drying, roasting, and conching. The development of chocolate flavor requires that cocoa beans are fermented and dried, since during these processes essential flavor precursors are obtained, which are fully expressed later during the roasting and conching processes. These precursors form new types of molecules with other cocoa compounds as result of Maillard reactions and other biochemical processes (Lopez & Dimick, 2008). Cocoa fermentation starts with the prevalence of a succession of different microorganisms, which metabolize sugars from the mucilaginous pulp, produce ethanol, and subsequently release acetic and lactic acid (Ostovar & Keeney, 1973; Schwan & Wheals, 2004). The resulting acidification causes the death of the bean's embryo and induces several enzymatic processes resulting in the generation of essential chocolate-specific aroma precursors (Amin, Jinap, & Jamilah, 1997; Biehl, Brunner, Passern, Quesnel, & Adomako, 1985; Buyukpamukcu et al., 2001; de Brito et al., 2001; Voigt & Lieberei, 2014; Ziegleder & Biehl, 1988).

Cocoa storage proteins undergo the most intense modifications during fermentation, where microbiological fermentation products-triggered enzymatic reactions lead to their extensive breakdown yielding peptides and amino acids, which are 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 (Marseglia et al., 2014). Voigt and coworkers had reported that the total cocoa bean protein content consisted of 52% albumin and 43% vicilin-like globulin (Voigt et al., 1993). In contrast, Lerceteau et al. (1999), employing two-dimensional (2D) gel electrophoresis and silver staining, determined that vicilin-like globulin and the albumin storage protein represent 23 and 14%, respectively, of the total seed protein in addition to other abundant unknown polypeptides (Lerceteau et al., 1999). Albumin was identified as a 21-kDa protein, and its primary structure together with its trypsin inhibitory properties were previously reported (Kochhar, Gartenmann, & Juillerat, 2000). The vicilin-like globulin was originally described as a 66-kDa storage protein (Spencer & Hodge, 1992), giving rise to three distinct break-down units with molecular masses of 47 kDa, 31 kDa, and 15 kDa (Voigt et al., 1993). Recently, it was demonstrated that cocoa-specific aroma precursors can be obtained by in vitro proteolysis of vicilin-like globulin storage protein

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via addition of an aspartic endoprotease and a carboxypeptidase (Voigt, Janek, Textoris-Taube, Niewienda, & Wostemeyer, 2016). Fermentation of cocoa beans is fundamental for the activation of these two enzyme classes by microbial metabolites such as acetic acid (Schwan & Wheals, 2004). Interestingly, up to date there is no evidence that microbial enzymes might penetrate the bean's shell and create any flavor compounds (Schwan & Wheals, 2004). Furthermore, it had been shown that the formation of cocoa aroma precursors was dependent on both, the cleavage specificities of the involved proteases as well as the structure of the globular storage protein (Bytof, Biehl, Heinrichs, & Voigt, 1995; Rashidah, Jinap, Nazamid, & Jamilah, 2007; Voigt, Voigt, Heinrichs, Wrann, & Biehl, 1994a; Voigt, Wrann, Heinrichs, & Biehl, 1994b).

Although degradation of cocoa storage proteins into oligopeptides and amino acids is well-documented, the actual fate of vicilin as a main source of these compounds starting from the non-fermented cocoa beans towards different stages of fermentation, and finally towards the dried bean, remains uncharacterized. Consequently, the aim of the current work was to provide a comparative and detailed analysis of vicilin protein with respect to the molecular mass(es), isoelectric point(s) and post-translational modifications of its different subunits as well as its oligopeptide degradation products throughout the entire fermentation process.

2. Material and methods

2.1. Chemicals and reagents

Tris-HCl (Pufferan®, ≥ 99.5%), sodium dodecyl sulphate (SDS, ≥ 99.5% electrophoresis grade), HPLC-grade water (Rotisolv®), glycerol (86%), acrylamide (Rotiphorese® Gel 30:37, 5:1), acetonitrile (ACN, Rotisolv® HPLC ultra gradient grade) and 3-[(3-Cholamidopropyl)dimethyl ammonio]-1-propane sulphonate (CHAPS, Pufferan®, 98%) were purchased from Carl Roth (Karlsruhe, Germany). Dithiothreitol (DTT, Biochemica), acetone (100%, Biochemica), TEMED, ammonium persulphate (APS, analytical grade), acetic acid (100%, analytical grade) and methanol (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®), α-Cyano-4-hydroxycinnamic acid (**4-HCCA, ≥** 99%, HPLC grade), ammonium **dihydrogen phosphate ($NH_4H_2PO_4$, puriss, ≥** 99%) and formic acid (≥98% for mass spectrometry) were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Sequencing grade modified trypsin was obtained from Promega (Mannheim, Germany). 100× BioLyte® 3/10 Ampholyte and ReadyPrep™ 2-D Cleanup Kit were purchased from Bio-Rad (Munich, Germany). 2D Quant Kit was purchased from GE Healthcare Biosciences (Munich, Germany). Pierce™ Glycoprotein Staining 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. Cocoa samples

A series of cocoa beans derived from pods of the cocoa hybrid G11UTA402XT413 with different fermentation levels (fresh non-fermented cocoa beans, beans obtained at different fermentation time-points, and fully fermented dried cocoa beans) were harvested in April 2014 in the estate of Champ Semencier de Guessabo, Ivory Coast, and provided by Barry Callebaut, Belgium. The cocoa beans were spontaneously fermented, and the changes in pH and temperature were monitored continuously throughout the fermentation. Cocoa bean samples were collected at 24-h intervals of fermentation until 144 h and finally after traditional drying for 6 days. The samples were then immediately frozen at −20 °C. The bean material of different fermentation levels as well as non-fermented and fully fermented/dried beans was shipped to Bremen on dry ice. Further storage was done at −20 °C.

2.3. Protein extraction from cocoa beans

Mucilaginous pulp from the non-fermented beans was wiped off using Kim Wipes (Kimtech Science, Reigate, UK) and seed coats were removed by peeling them off with a sterile scalpel. The seed coats of fermented beans were removed using forceps. The beans were crushed into fine powder in a Retsch grindomix instrument (Chaotrope, Bremen, Germany). The finely ground powder (250 mg)was added in a 2-mL polypropylene microcentrifuge tube (Eppendorf, Hamburg, Germany) and suspended in 1 mL of protein extraction buffer (100 mM Tris HCl, 1% DTT and 1% SDS; pH adjusted to 8.1; protease inhibitor cocktail according to manufacturer's instruction (Thermo Scientific, Bremen, Germany)). The mixture was vortexed thoroughly for 60 s and then incubated for 60 min with gentle shaking at 4 °C, followed by centrifugation at 16,000 × *g* for 20 min and 4 °C in a bench top 5810R centrifuge (Eppendorf, Hamburg, Germany). The supernatant containing the protein fraction was gently transferred to a fresh cup and stored at −20 °C for further analysis.

2.4. Determination of protein concentration

Quantification of total protein content was performed according to the 2D Quant Kit protocol using 2 mg/mL bovine serum albumin as standard. Since the assay is based on the specific binding of copper ions to proteins, the amount of unbound copper ions measured with a colorimetric reagent at an absorbance at 480 nm is giving an inverse and linear relationship of color intensity and protein concentration within a range of 0 to 50 μg. All measurements were done in triplicates.

2.5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The extracted cocoa proteins were separated according to their molecular weight using SDS-PAGE. The protein samples (25 μL) were mixed with 5 μL of 6× sample buffer (Laemmli, 1970) containing bromophenol blue as tracking dye. The mixture was heated at 95 °C for 5 min and loaded on SDS–PAGE gels (83 mm × 65 mm × 1 mm) containing 12.5% (w/v) acrylamide run in the Mini-PROTEAN Tetra cell system (Bio-Rad, Munich, Germany). Electrophoresis was done at 130 V for 90 min. The resulting gel was stained with Coomassie® Blue (45% (v/v) methanol, 10% acetic acid, 2.93×10^{-3} M Coomassie® Brilliant Blue G-250) for 20 min and further treated with a destaining solution (10% (v/v) acetic acid, 5% (v/v) 2-propanol) overnight with gentle shaking.

2.6. Purification of protein samples

Before proceeding to two-dimensional gel electrophoresis, total protein extracts were purified using a Ready Prep™ 2D clean up kit as recommended by the manufacturer. This purification allowed removal of interfering substances such as ionic detergents, lipids, or phenolic compounds. After precipitation and washing in wash buffer provided with the kit, the protein sample was re-suspended in 130 μL of rehydration buffer (2 M thiourea, 6 M urea, 16.2×10^{-3} M CHAPS, 25.9×10^{-3} M DTT) and supplemented with ampholytes according to manufacturer's specification.

2.7. Two-dimensional protein gel electrophoresis

2D gel electrophoresis of cocoa proteins was performed by isoelectric focusing and subsequent SDS–PAGE. For this purpose, 80 μg of protein were applied to immobilized pH gradient (IPG) strips (7 cm, pH 3–10; Bio-Rad) by soaking for 14 h at room-temperature. Isoelectric