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Partial purification and characterisation of the peptide precursors of the cocoa-specific aroma components

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ABSTRACT

Essential precursors of the cocoa-specific aroma notes are formed during fermentation of the cocoa beans by acid-induced proteolysis. It has been shown that, in addition to free amino acids, hydrophilic peptides derived from the vicilin-class(7S) globular storage protein are required for the generation of the cocoa-specific aroma notes during the roasting process. To identify those peptides responsible for the generation of the cocoa-specific aroma components, we have developed a procedure for the fractionation of the aroma precursor extract from well-fermented cocoa beans by ligand-exchange and subsequent Sephadex-LH20 chromatography. The cocoa-specific aroma precursor fractions were characterised by matrix-assisted laser-desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF) and the determination of their amino acid sequences by electrospray ionisation mass spectrometry (ESI-MS/MS).

1. Introduction

Cocoa aroma is developed on roasting of fermented but not of unfermented cocoa beans (Rohan, 1964). Cocoa fermentation starts with a succession of microorganisms which metabolise the sugars from the mucilaginous pulp and produce acetic and lactic acid (Ostovar & Keeney, 1973; Schwan & Wheals, 2004). This acidification causes the death of the seeds and induces proteolytic processes resulting in the generation of essential cocoa- and chocolate-specific aroma precursors (Biehl, Brunner, Passern, Quesnel, & Adomako, 1985; Amin, Jinap & Jamilah, 1997; Buyukpamukcu et al., 2001; De Brito et al., 2000; Voigt & Lieberei, 2014; Ziegleder & Biehl, 1988). Mohr, Landschreiber, and Severin (1976) have reported that a typical cocoa aroma was obtained when a peptide fraction isolated from fermented cocoa beans was roasted in the presence of free amino acids and reducing sugars. 20 years ago, it has been shown that the cocoa-specific aroma precursors can be obtained by *in vitro* proteolysis of the vicilin-class(7S) globular storage protein and that this process requires the co-operation of an aspartic endoprotease and a carboxypeptidase of the cocoa beans (Voigt, Biehl, et al., 1994; Voigt, Heinrichs, Voigt, Wrann, & Biehl, 1994). Furthermore, it has been shown that the formation of the cocoa-specific aroma precursors is dependent both on the cleavage specificities of the involved aspartic endoprotease and carboxypeptidase (Bytof, Biehl, Heinrichs, & Voigt, 1995; Voigt, Voigt, Heinrichs, Wrann, & Biehl, 1994) and on the particular structure of the globular storage protein of the cocoa beans (Rashidah, Jinap, Nazamid, & Jamilah, 2007; Voigt, Wrann, Heinrichs, & Biehl, 1994). Based on the amino acid sequence of their common precursor derived from the nucleotide sequence of the corresponding cDNA (McHenry & Fritz, 1992; Spencer & Hodge, 1992), the amino acid sequences of the different subunits of the cocoa vicilin-class(7S) globulin have been previously determined by MALDI-TOF analysis (Kratzer et al., 2009). Recently, about 40 peptides present in well-fermented cocoa beans from different origins have been isolated and characterised (Marseglia et al., 2014). Up to now, however, there are no data concerning the number and structures of the particular peptides required for the formation of the cocoa-specific aroma notes during the roasting process. In the present communication, we describe the results of our efforts to isolate and to characterise these particular peptides.

2. Materials and methods

2.1. Materials

Well-fermented cocoa beans (Ivory Coast) were obtained from Jacobs-Suchard Research and Development (Neuchatel,

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Switzerland). Deodorised cocoa butter was provided by Gustav F. W. Hamester GmbH (Hamburg, Germany).

2.2. Extraction of fat

The dry cocoa cotyledons were crushed and extracted repeatedly in a Soxhlet apparatus with petroleum ether (b.p. 40–70 °C). After solvent evaporation, the material was powdered and extracted again for 8 h in the same manner.

2.3. Removal of purine alkaloids

Purine alkaloids were removed from the defatted cocoa bean powder by extraction with chloroform for 8 h in a Soxhlet apparatus.

2.4. Aroma precursor extract

After removal of purine alkaloids, the defatted cocoa powder was extracted four times with 70% (v/v) aqueous methanol (100 mL per g of cocoa powder) for 2 h at 4 °C. The filtered extracts were passed through a polyamide CC 6 column (80 cm × 5.6 cm; Macherey–Nagel, Düren, Germany) to remove the polyphenols and the solvent was removed at 40 °C by use of a rotary evaporator. Finally, the remaining aqueous solutions were freeze-dried.

2.5. Ligand-exchange chromatography

Group separation of peptides by ligand-exchange chromatography has been described in the literature (Monjon & Solms, 1987). Therefore, we have used this method as a first step in the purification of the aroma-relevant cocoa peptides. The aroma precursor extract was dissolved in distilled water and subjected to a copper-Chelex 100 column (5.6 × 15 cm). The column matrix was prepared by treatment of Chelex 100 (Bio-Rad, Munich, Germany) with an excess of saturated aqueous $CuSO_4$ solution, followed by extensive washing with distilled water. After application of the precursor extract, the column was washed with distilled water. The flow-through fractions were combined and freeze-dried to get the LEC1 fraction. The bound material was eluted with 50 mM aqueous NH_3, concentrated using a rotary evaporator and finally freeze-dried to get the LEC2 fraction.

2.6. Sephadex LH20 chromatography

The freeze-dried LEC2 fraction was dissolved in distilled water and subjected to a Sephadex LH20 column (4.6 × 80 cm; GE Healthcare Europe, Freiburg, Germany) equilibrated and eluted with 25 mM aqueous NH_3. Fractions of 10 mL were collected and the absorbance measured at 280 nm. The different peak fractions (LH1, LH2, LH3, and LH4) were combined and freeze-dried.

2.7. Sensory evaluation

Aliquots (0.75 g each) of total precursor extract or of the obtained precursor fractions to be analysed for their aroma potential or the synthetic mixture of free amino acids (composition: see Supplementary Table S1) were mixed with 0.25 g glucose, 0.75 g fructose, and 0.3 g water. After addition of 8.25 g deodorised cocoa butter (commercial product obtained from Gustav F. W. Hamester GmbH, Hamburg, Germany), the mixture was formulated either with mortar and pestle or by ultrasonication for 30 s by using a Branson Sonifier B12 at maximum power. Finally, the samples were filled into glass Petri dishes as thin layers (2–3 mm) and roasted for 10–15 min in an oven preheated to 120 °C.

The aroma notes generated during the roasting process were analysed by sniffing analyses using 10–12 trained panellists. The panellists were trained by evaluating the aroma notes of standard samples: rye bread roasted in deodorised cocoa butter for "bread-like" aroma, roasted sugar for caramel aroma, coarse meal heated in deodorised cocoa butter for "cereal" aroma, cocoa liquor (prepared from roasted cocoa beans) for "cocoa" aroma, coffee powder heated in deodorised cocoa butter for "coffee-like" aroma, different commercial dark chocolates for "dark chocolate" aroma, fresh oleander flowers for "flowery" aroma, apple peel for "fruity" aroma, heated aqueous honey solution for "honey-like" aroma, malt extract for "malty" aroma, mixed nuts powder heated in deodorised cocoa butter for "nutty" aroma, roasted peanut powder for "roasty" aroma, gammon for "smoky" aroma, vegetable stock for "vegetable-like" aroma, and 7% acetic acid for "acidic" aroma. For the dark chocolate and cocoa liquor samples, best results were obtained when the aroma notes were evaluated at temperatures between 35 and 40 °C. Therefore, sniffing analyses of the unknown samples were always performed at this temperature. Each aroma analysis of the unknown samples was repeated at least three times with different preparations of the same type. The order of the various aroma notes was random in the forms. Evaluation was performed using a scale from 0 (not detectable) to 3 (very strong) for each attribute. Mean values are shown: "−" = not detectable (<0.2); "+" = weak (0.2–1.4); "++" = strong (1.5–2.2): "+++" = very strong (>2.2).

2.8. Amino-acid analyses

The amino acids were converted into their *o*-phthalaldehyde (OPA) derivatives, separated by reversed-phase HPLC using a Shandon Hypersil ODS 5 (240 mm × 4.6 mm) column and a Shandon Hypersil ODS 10 (20 mm × 4.6 mm) precolumn as previously described (Voigt, Biehl, et al., 1994; Voigt, Heinrichs, et al., 1994). The effluents were monitored fluorometrically with a Hitachi model F-3000 spectrofluorometer (excitation at 334 nm; emission at 425 nm).

2.9. HPLC analyses of peptides

Peptide mixtures were analysed by reversed-phase HPLC using the Gold HPLC system (Beckman Instruments, San Ramon, CA) equipped with an Ultrasphere ODS 5 µ column (250 mm × 4.6 mm; Hichrom Ltd, Reading, UK). Elution of the peptides was performed at 30 °C and a flow rate of 1 mL/min with 0.1% (v/v) trifluoroacetic acid (7 min) and subsequently with a linear gradient from 0% to 50% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid (Mahoney & Hermodson, 1980). The eluting peptides were monitored by measuring the absorbance of the effluents at 210 nm.

2.10. MALDI-TOF analyses

Samples were dissolved in 0.1% (v/v) trifluoroacetic acid and concentrated using µziptip C18 pipette tips (Millipore, Billerica, MA). Peptide solution (1.0 µL) was mixed with the same volume of α-cyano-4-hydroxycinnamic acid matrix and spotted on a MALDI plate. MALDI-MS and MALDI-MS/MS were performed on an Applied Biosystems 4700 Proteomics Analyzer with TOF/TOF ion optics (Applied Systems, Foster City, CA). The mass spectrometer was operated in positive ion reflector mode with five spots of standard (ABI 4700 calibration mixture) for calibration. Mass spectra were obtained from each spot using 500 shots per spectrum. Tandem mass spectra were acquired by accelerating the precursor ions to 8 keV, selecting them with the timed gate set to a window of 3 Da, and performing CID at 1 keV. Gas pressure (air) in the CID