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# The cleavage specificity of the aspartic protease of cocoa beans involved in the generation of the cocoa-specific aroma precursors

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ABSTRACT

Particular peptides generated from the vicilin-class(7S) globulin of the cocoa beans by acid-induced proteolysis during cocoa fermentation are essential precursors of the cocoa-specific aroma notes. As revealed by *in vitro* studies, the formation of the cocoa-specific aroma precursors depends on the particular cleavage specificity of the cocoa aspartic protease, which cannot be substituted by pepsin. Therefore, we have investigated the effects of aspartic protease inhibitors on both enzymes and comparatively studied their cleavage specificities using different protein substrates and MALDI-TOF mass spectrometric analyses of the generated oligopeptides. Three classes of cleavage sites have been identified and characterized: (I) sequences exclusively cleaved by the cocoa enzyme, (II) sequences cleaved by both pepsin and the cocoa enzyme, and (III) those cleaved exclusively by pepsin. In contrast to most aspartic proteases from other origins, basic amino acid residues, particularly lysine, were found to be abundant in the specific cleavage sites of the cocoa enzyme.



## 1. Introduction

Essential precursors of the cocoa- and chocolate-specific aroma notes developed on roasting are generated during the fermentation of the cocoa beans by acid-induced proteolysis of the storage proteins (Amin, Jinap, & Jamilah, 1997; Biehl, Brunner, Passern, Quesnel, & Adomako, 1985; De Brito et al., 2000; Rohan, 1964; Voigt & Lieberei, 2014). Mohr, Landschreiber, and Severin (1976) have reported that 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. Several authors have stressed the significance of the specific mixure of free amino acids accumulated in the cocoa beans during fermentation (Arnoldi, Arnoldi, Baldi, & Griffini, 1988; Kirchhoff, Biehl, & Crone, 1989; Rohsius, Matissek, & Lieberei, 2006). However, a synthetic mixture of free amino acids adapted to the spectrum of free amino acids present in fermented cocoa beans did not reveal cocoa or chocolate aroma when roasted in the presence of reducing sugars and deodorated cocoa butter (Mohr et al., 1976; Voigt, Biehl, et al., 1994). The conclusion that, in addition to free amino acids, specific peptides are essential precursors of the typical cocoa and chocolate aroma notes was corroborated by the results of *in vitro* proteolysis and subsequent determination of the generated aroma potential (Voigt, Biehl, et al., 1994; Voigt, Heinrichs, Voigt, Wrann, & Biehl, 1994; Voigt & Lieberei, 2014). It has been shown that these particular aroma-related peptides are derived from the vicilin-class (7S) globular storage protein of the cocoa beans (Buyukpamukcu et al., 2001; Voigt, Heinrichs, et al., 1994; Voigt, Janek, Textoris-Taube, Niewienda, & Wöstemeyer, 2016; Voigt, Wrann, Heinrichs & Biehl, 1994) and that specific sequence motifs in the amino acid sequence of the cocoa vicilin-class(7S) globulin lacking in the globular storage proteins of coconuts, hazelnuts, and sunflower seeds are essential for the formation of these particular aroma-related peptides (Kratzer et al., 2009; Voigt, Biehl, & Kamaruddin, 1993; Voigt, Wrann, et al., 1994). These particular peptides are generated from the cocoa vicilin (Amin et al., 1997; Voigt, Heinrichs, et al., 1994; Voigt et al., 2016) by co-operation of the aspartic protease and a carboxypeptidase of the cocoa beans (Voigt, Biehl, et al. 1994; Voigt, Heinrichs, et al., 1994; Voigt & Lieberei, 2014).

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In addition to the particular amino acid sequence of the cocoa vicilin-class(7S) globular storage protein (Kratzer et al., 2009), the particular cleavage specificity of the aspartic protease (EC 3.4.23) proved to be important for the formation of the aroma-related peptides (Voigt & Lieberei, 2014; Voigt, Voigt, Heinrichs, Wrann & Biehl, 1994). In contrast to the carboxypeptidase of the cocoa beans, which has a specificity similar to commercial carboxypeptidase A (Bytof, Biehl, Heinrichs, & Voigt, 1995; Voigt, Biehl, et al. 1994; Voigt, Heinrichs, et al., 1994), the cleavage specificity of the cocoa aspartic protease has not yet been elucidated (Guilloteau, Laloi, Michaux, Bucheli, & McCarthy, 2005; Laloi, McCarthy, Morandi, Gysler, & Bucheli, 2002). As shown by *in vitro* studies on the proteolytic formation of the cocoa-specific aroma precursors, this particular enzyme could not be substituted by any other endoprotease studied so far (Voigt, Voigt, et al., 1994). Aroma notes far distantly related to the typical cocoa aroma were obtained, when the cocoa vicilin-class(7S) globular storage protein was digested with porcine pepsin instead of cocoa aspartic protease, post-treated with carboxypeptidase A, and the obtained proteolysis products roasted in the presence of reducing sugars and deodorated cocoa butter (Voigt, Voigt, et al., 1994). Therefore, we have comparatively investigated the cleavage specificities of the aspartic protease of the cocoa seeds and of commercial porcine pepsin.

## 2. Materials and methods

### 2.1. Materials

Cocoa beans were obtained from ripe, genetically undefined pods harvested at the Cocoa and Coconut Division of the Malaysian Agricultural Research and Development Institute (MARDI, Hilir Perak, Malaysia). Unfermented beans were taken from the pods immediately after arrival (four or five days after harvesting), shock-frozen in liquid nitrogen after removal of testae and radiculae, and freeze-dried.

Commercial porcine pepsin and carboxypeptidase A were purchased from Serva (Heidelberg, Germany). Myoglobin, Pepstatin A, and Sequinavir mesylate were obtained from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany), and Aliskiren hemifumarate was purchased from Absource Diagnostics GmbH (Munich, Germany).

### 2.2. Extraction of fat

The dry cocoa cotyledons were crushed and extracted repeatedly in a Soxhlet apparatus with petroleum ether (bp 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 partially removed by extraction of the defatted cocoa powder with chloroform for 8 h in a Soxhlet apparatus.

### 2.4. Preparation of acetone-dry powder

Acetone-dry powder (AcDP) of cocoa beans was prepared essentially as previously described (Voigt, Biehl, et al., 1994). To remove the polyphenols, the defatted cocoa powders were extracted three times with 80% (v/v) aqueous acetone containing 5 mM sodium ascorbate and subsequently with 70% (v/v) aqueous acetone. The suspensions (200 ml aqueous acetone per 10 g defatted cocoa powder) were stirred for 1 h at 4 °C and the extracts removed by centrifugation (15 min at 13,000*g*). After the final extraction step, the efficiency of polyphenol extraction was checked by heating an aliquot of the acetone-dry powder with 5 M HCl (red colour indicates the presence of residual polyphenols). After complete extraction of polyphenols, residual water was removed by extraction with 100% acetone. After final centrifugation, the sediment was evaporated under reduced pressure to remove the solvent. The AcDP was stored at −20 °C.

### 2.5. Isolation of the vicilin-class(7S) globular storage protein

The vicilin-class(7S) globulin was isolated from polyphenol-free AcDP prepared from cocoa seeds as described above essentially as previously described (Voigt et al., 1994). Polyphenol-free AcDP (50 g) was extracted with 5 L of high-salt buffer containing 1 M NaCl, 5 mM sodium ascorbate, 2 mM EDTA and 20 mM Tris-HCl, pH 7.5. The suspension was stirred at 4 °C for 1 h and centrifuged for 20 min at 20,000*g* in the Sorvall SLA1500 rotor. The pellet was resuspended in 3 L of the same buffer, stirred for at 4 °C for 1 h and centrifuged again for 20 min at 20,000×*g*. The combined supernatants were dialyzed 5-times against distilled water and finally against 20 mM sodium acetate, pH 4.3. The precipitated globular storage protein was collected by centrifugation for 20 min at 20,000×*g*, washed with distilled water and store at −18 °C until use.

### 2.6. Purification of the cocoa 21-kDa seed albumin and of the cocoa aspartic protease

Cocoa albumin and the cocoa aspartic endoprotease were prepared from the low-salt extract of polyphenol-free cocoa bean AcDP as previously described (Voigt, Heinrichs, et al., 1994). Polyphenol-free AcDP (50 g) was extracted with 5 L of low-salt buffer containing 5 mM sodium ascorbate, 2 mM EDTA and 20 mM Tris-HCl, pH 7.5. The suspension was stirred at 4 °C for 1 h and centrifuged for 20 min at 20,000×*g* in the Sorvall SLA1500 rotor. The pellet was resuspended in 3 L of the same buffer, stirred for at 4 °C for 1 h and centrifuged again for 20 min at 20,000×*g*. The combined supernatants were applied to a QAE-Sephadex A-50 column (50 × 4.6 cm; GE Healthcare Europe, Freiburg, Germany) equilibrated with 20 mM sodium phosphate, pH 7.5. The column was washed extensively with distilled water and subsequently eluted with a linear gradient from 0 to 500 mM NaCl in 20 mM sodium phosphate, pH 7.5. The eluate fractions (15 ml) were monitored for absorbance at 280 nm and measured for protease activity as described below. Furthermore, the polypeptide patterns were analyzed by SDS-PAGE according to Laemmli (1970) to detect the 21-kDa storage albumin and to minimize contamination of the aspartic protease and of the 21-kDa seed albumin with other proteins.

The 21-kDa storage albumin was found in the first peak and further purified by gel exclusion chromatography on a Sephadex G-100 column (4.6 × 90 cm; GE Healthcare Europe, Freiburg, Germany) equilibrated and eluted with a buffer containing 50 mM NaCl, 5 mM sodium ascorbate and 20 mM sodium phosphate, pH 7.0. The albumin-containing fractions were combined, concentrated against Aquacide II (Calbiochem, Darmstadt, Germany), dialysed against distilled water and stored at −18 °C until use.

The aspartic protease activity eluted between the protein peaks II and III of the QAE-Sephades A-50 column. The protease fractions were combined, concentrated against Aquacide II (Calbiochem, Darmstadt, Germany) and further purified by gel exclusion chromatography on a Sephadex G-100 column (4.6 × 90 cm; GE Healthcare Europe, Freiburg, Germany) equilibrated and eluted with a buffer containing 50 mM NaCl, 5 mM sodium ascorbate and 20 mM sodium phosphate, pH 7.0. The eluate fractions were measured for protease activity. The active fractions were com-