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Specificity and stability of the carboxypeptidase activity in ripe, ungerminated seeds of *Theobroma cacao* L.

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As previously reported the carboxypeptidase activity present in ripe, ungerminated *Theobroma cacao* seeds is involved in the proteolytic formation of the cocoa-specific aroma precursors. Therefore, we have investigated the specificity of this particular enzyme activity using crude homogenates of acetone dry powder prepared from unfermented, ripe cocoa seeds. Both oligopeptide mixtures derived from cocoa seed proteins and synthetic peptides have been found to be suitable substrates for this enzyme activity. However, peptides with carboxy-terminal arginine, lysine or proline residues are resistant against degradation by the cocoa seed carboxypeptidase. The enzyme preferentially liberates hydrophobic amino acids, whereas acidic amino acids are released very slowly. The rate of hydrolysis is not only determined by the carboxyterminal, but also affected by the neighbouring amino acid residue. Furthermore, the specificity of the enzyme is influenced by the pH-value. The specificity of the cocoa seed carboxypeptidase activity is remarkably similar to the specificity of carboxypeptidase A from porcine pancreas which (in addition to the cocoa aspartic endoprotease) has recently been successfully used for the *in vitro* formation of the cocoa-specific aroma precursors. These results are discussed in the light of the pH-dependent generation of the cocoa-specific aroma precursors during fermentation of the cocoa seeds.

INTRODUCTION

Mohr and coworkers (1971, 1976) have reported that typical cocoa and chocolate aroma was obtained when an oligopeptide fraction isolated from raw cocoa was roasted in the presence of free amino acids and reducing sugars. Since roasting of unfermented cocoa seeds did not reveal cocoa aroma (Rohan, 1964), these findings indicated that essential aroma precursors are generated by proteolytic processes during fermentation of the cocoa seeds (Ziegleder & Biehl, 1988). As shown by seed incubations under sterile laboratory conditions (Biehl *et al.*, 1985), the formation of the cocoa-specific aroma precursors does not require the presence of microorganisms, but is caused by acid-induced proteolysis of seed proteins by endogenous proteases of the cocoa seeds. Both the proteolysis of seed proteins and the generation of essential aroma precursors are strongly dependent on the degree and the time course of nib acidification (Biehl *et al.*, 1982, 1985). Although nib acidification is a prerequisite for the formation of the cocoa-specific aroma precursors, fermentations with strong nib acidification (pH 4·5–4·0) generally result in raw cocoa batches with considerably lower aroma potential than fermentations at pH 5·5–5·0 (Biehl *et al.*, 1985). The latter findings have led to the development of a procedure (post-harvest storage of cocoa pods) which has improved raw cocoa quality in Malaysia (Biehl *et al.*, 1989; Meyer *et al.*, 1989).

Recently, we have been able to produce the cocoa-specific aroma precursors by *in vitro* proteolysis of a cocoa seed protein (Voigt *et al.*, 1993*a*; 1994*a,b*). By this experimental approach, we have shown that the essential aroma precursors are derived from a globular storage protein (Voigt *et al.*, 1994*b*) belonging to the vicilin-class (7S) globulins (Voigt *et al.*, 1993*b*) and that this particular protein substrate cannot be substituted by the 12S globulins isolated from hazelnuts and sunflower seeds, respectively, or by the 7S globulin from coconuts (Voigt *et al.*, 1994*d*). Two protease activities are involved in this process: an aspartic endoprotease and a carboxypeptidase activity (Voigt *et al.*, 1993*a*; 1994*a,b*). The particular cleavage specificity of the aspartic endoprotease has been found to be essential for the formation of the cocoa-specific aroma precursors (Voigt *et al.*, 1994*c*), whereas the cocoa seed carboxypeptidase could be substituted by the commercial pancreatic carboxypeptidase A (Voigt *et al.*, 1993*a*;

1994*a,b*). Therefore, it is unclear whether the cocoa seed carboxypeptidase has a similar specificity as the carboxypeptidase A from mammalian pancreas or whether the specificity of the carboxypeptidase is not important for the generation of the cocoa-specific aroma precursors.

MATERIALS AND METHODS

Materials

N-Benzyloxycarbonyl-dipeptides (= Z-dipeptides) used as carboxypeptidase substrates were obtained from Bachem Biochemica GmbH (Heidelberg, Germany). Cocoa seeds were 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 seeds were taken from the pods immediately after arrival (4–5 days after harvesting), frozen in liquid nitrogen after removal of testae and radiculae and freeze-dried.

Extraction of fat

The dry cotyledons were crushed and portions of 10 g each were extracted repeatedly in a Soxhlet apparatus with 500 ml of petroleum ether (bp 40–70°C). After solvent evaporation, the material was powdered and extracted again for 8 h in the same manner. Finally, purine alkaloids were partially extracted with chloroform for 8 h in a Soxhlet apparatus.

Preparation of acetone dry powder (AcDP)

Acetone dry powder (AcDP) of cocoa seeds was prepared essentially as recently described (Kirchhoff *et al.*, 1989*a*). To remove the polyphenols, the defatted seed 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 seed 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, 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 acetone dry powder (AcDP) was stored at –20°C.

Autolysis

Acetone dry powder (AcDP) from ungerminated, ripe cocoa seeds (10 g) was suspended in 500 ml of sterile 0·2 M McIlvaine buffer, pH 5·5. The suspensions were incubated under sterile conditions at 45°C in a shaking water bath for 70 h.

Preparation of hydrophobic oligopeptides

To obtain the natural substrates of the cocoa seed carboxypeptidase, a mixture of hydrophobic oligopeptides was prepared by autolysis of cocoa seed AcDP at pH 3·5 as previously described (Voigt *et al.*, 1994*a*). The AcDP (10 g) was suspended in 1 litre of distilled water. The suspension was adjusted to pH 3·5 by addition of acetic acid and incubated in a shaking water bath at 50°C for 16 h. After incubation, methanol was added to a final concentration of 70% (v/v). The suspension was stirred at room temperature for 1 h and centrifuged at 20 000 × *g* for 30 min. The supernatants were collected and the methanol removed under reduced pressure at 40°C using a rotary evaporator. Finally, the aqueous solutions were freeze-dried.

Preparation of tryptic peptides

To obtain oligopeptides with carboxyterminal basic amino acid residues, 10 mg of oxidized insulin B chain (Sigma Chemie, Deisenhofen, Germany) were dissolved in 10 ml of 10 mM sodium phosphate, pH 7·5, and incubated in a shaking water bath at 30°C after addition of 100 μg trypsin (Sigma Chemie). After 3 h, another 100 μg of trypsin were added and the incubation continued for 12 h. As revealed by HPLC analysis (see below), the oxidized insulin B chain was completely transformed to the tryptic peptides after this time period. The reaction mixture was heated to 95°C for 30 min to inactivate the protease, cooled to room temperature, adjusted to pH 5·8 by addition of citric acid and stored at –20°C until use.

Determination of carboxypeptidase activity

Preparation of the enzyme solution

Polyphenol-free acetone dry powder (AcDP) was suspended in 0·02 M Na_2HPO_4 and adjusted to pH 6·8 by addition of citric acid (20 mg AcDP/ml). The suspension was homogenized for 5 min at 0–3°C using a Potter–Elvejhem homogenizer and preincubated in an ice bath in the presence of 10 μg/ml pepstatin A (Sigma Chemie, Deisenhofen, Germany) and 500 μg/ml iodoacetic acid (Merck, Darmstadt, Germany) for at least 1 h at 0°C to inhibit the endoprotease activities present in the ripe, ungerminated cocoa seeds (Biehl *et al.*, 1991, 1993).

Carboxypeptidase assay with Z-dipeptides

Aliquots (60 μl) of the homogenates prepared from cocoa seed as described above were added to 0·84 ml of McIlvaine buffer (0·2 M Na_2HPO_4 adjusted to the indicated pH-values by addition of citric acid). After addition of the Z-dipeptide (100 μl from 10 mM stock solutions), the samples were incubated for 3 h at 45°C. The reaction was stopped by addition of 0·2 ml of 25%

(w/v) trichloroacetic acid and the precipitated protein removed by centrifugation at 10000 × *g* for 15 min. Finally, the released amino acids were determined colorimetrically by the trinitrobenzene-sulfonic acid method as described by Shutov *et al.* (1982). This method could, however, not be used in case of Z-Phe-Pro, because proline does not react with trinitrobenzene-sulfonic acid. Therefore, the liberated prolin was analysed by the ninhydrin method (Biehl & Passern, 1982; Biehl *et al.*, 1993). The developed colour was quantitated by measuring the absorption at 400 nm.

Carboxypeptidase assay with peptide mixtures

Aliquots (50 μl) of the homogenates prepared from cocoa seed AcDP as described above were added to 0·45 ml of McIlvaine buffer (0·2 M Na_2HPO_4 adjusted to pH 5·8 by addition of citric acid). The reaction was started by addition of oligopeptide substrate:

— 0·5 ml of tryptic peptides derived from the insulin B chain (1 mg/ml; see above)
  or
— 0·5 ml of McIlvaine buffer (pH 5·8) containing 5 mg of hydrophobic peptides prepared from cocoa seed proteins by autolysis at pH 3·5 (see above).

The samples were incubated in a shaking water bath for 3 h at 45°C. The reaction was stopped by addition of 4 volumes of ice-cold acetone (100%). The precipitated proteins were removed by centrifugation for 20 min at 20000 × g. The supernatants were decanted and the acetone removed by a gentle stream of nitrogen. Finally, the liberated amino acids were determined by HPLC analysis (see below).

Determination of protein

Protein concentrations were determined by the dye-binding method of Bradford (1976) using Serva Blue G (Serva, Heidelberg, Germany) and bovine serum albumin as standard.

HPLC analyses of peptides

Peptide mixtures were analysed by reversed phase HPLC using the HPLC system Gold (Beckman Instruments, San Ramon, CA, USA) equipped with an Ultrasphere ODS 5μ column (4·6 mm × 25 cm). Elution of the peptides was performed at 30°C and a flow rate of 1 ml/min with 0·1% (v/v) aqueous 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; Bennett *et al.*, 1980). The eluting peptides were monitored by measuring the absorbance of the effluents at 210 nm.

Amino acid analyses

The amino acids were converted into the *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 recently described (Kirchhoff *et al.*, 1989*a*). The effluents were monitored fluorometrically with a Hitachi model F-3000 spectrofluorometer (excitation at 334 nm; emission measured at 425 nm).

RESULTS

Generation of α-NH_2 nitrogen, liberation of free amino acids and the carboxypeptidase activity were comparatively analysed during autolysis at pH 5·5 and 45°C of

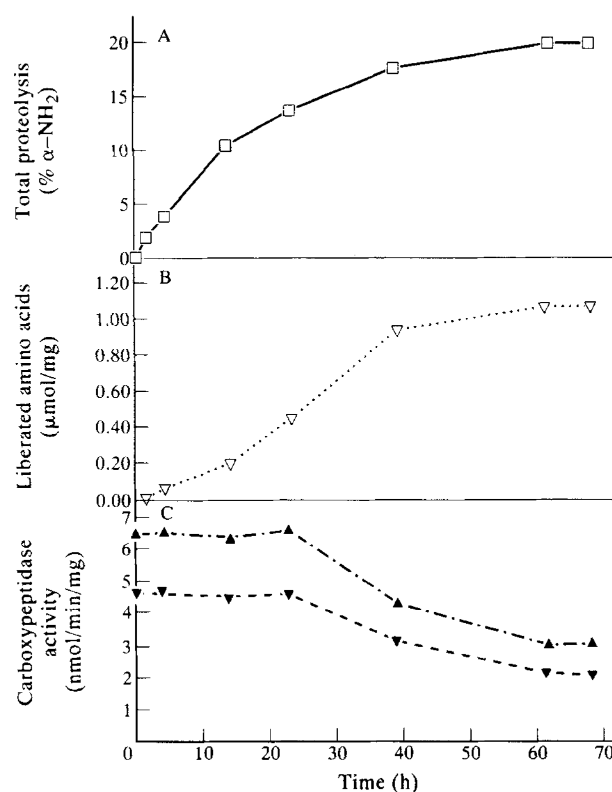

Fig. 1. Accumulation of proteolysis products and carboxypeptidase activity during long-term autolysis of cocoa seed acetone dry powder. Acetone dry powder prepared from ripe, ungerminated cocoa seeds (10 g) was suspended in 500 ml of 0·2 M McIlvaine buffer, pH 5·5, and incubated under sterile conditions in a shaking water bath at 45°C. Samples were taken after the time periods indicated in the figure and analysed for the amounts of α-NH_2 nitrogen (A), the amounts of free amino acids (B) and carboxypeptidase activity (C). The amounts of α-NH_2 nitrogen were determined by the trinitrobenzene-sulfonic acid method (Shutov *et al.*, 1982). Free amino acids were measured by HPLC analysis as described in the Materials and Methods. The amounts of α-NH_2 nitrogen (A) are expressed as percentage of total nitrogen (Kjeldahl) present in the acetone dry powder. Liberated amino acids (B) were determined by HPLC analysis and expressed in μ mol mg AcDP. Carboxypeptidase activity (C) was assayed as described in the Materials and Methods using two different substrates: a mixture of hydrophobic oligopeptides prepared by autolysis of cocoa seed AcDP at pH 3·5 (▲—·—·—▲) and Z-Phe-Leu (▼ - - - ▼), respectively.

acetone dry powder prepared from ripe, ungerminated cocoa seeds (Fig. 1). A linear increase of α-NH_2 nitrogen was observed during the first 12 h of autolysis (Fig. 1A). As revealed by HPLC analysis, accumulation of free amino acids was delayed (Fig. 1B). Therefore, the initial increase of α-NH_2 nitrogen was exclusively due to

Table 1. Effect of storage of cocoa seed AcDP on carboxypeptidase activity

Storage time[a]	Carboxypeptidase activity[b]	
	(nmol/min/mg)	(%)
0 days	9·3 ± 0·8	100 ± 9
5 days	7·9 ± 0·8	86 ± 8
4 years	6·7 ± 0·4	72 ± 4

[a]Acetone dry powder (AcDP) was stored at –20°C.
[b]Crude homogenates of cocoa seed AcDP were preincubated with pepstatin A and iodoacetic acid and subsequently assayed for carboxypeptidase as described in the Materials and Methods using Z-Phe-Ala as substrate.

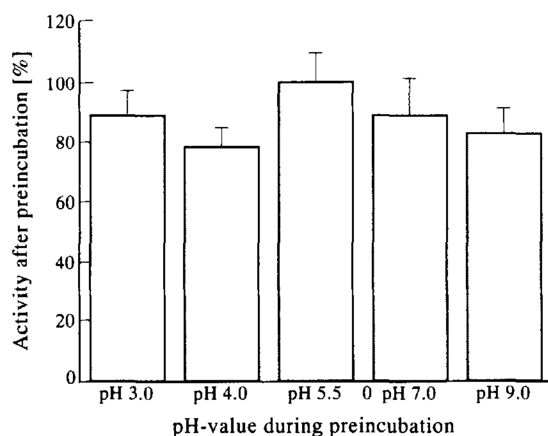

Fig. 2. pH-Stability of cocoa seed carboxypeptidase. Cocoa seed AcDP was suspended in 0·02 M McIlvaine buffer (5 mg AcDP ml) adjusted to the indicated pH-value by addition of citric acid and sodium hydroxide, respectively, and supplemented with pepstatin A (10 μg ml) and iodoacetic acid (500 μg % ml). After 2 h at 45°C, carboxypeptidase activities were determined at pH 5·5 and 45°C using Z-Phe-Leu as substrate (see Materials and Methods). Values are expressed as percentage of carboxypeptidase activity determined in a sample which was not preincubated. SD values are indicated on top of the bars.

the formation of oligopeptides, the substrates for the carboxypeptidase. Changes of the carboxypeptidase activity were investigated throughout a time period of 70 h during autolysis of cocoa seed AcDP (Fig. 1C). To this end, aliquots were taken from the reaction mixture after the indicated time periods, treated with pepstatin A and iodoacetic acid to inhibit endoprotease activities and subsequently assayed for carboxypeptidase activity. Essentially the same results were obtained with two different substrates: a synthetic, N-protected dipeptide, Z-Phe-Leu, and an oligopeptide mixture obtained by autolysis of cocoa seed AcDP at pH 3·5. No change of the carboxypeptidase activity was observed during the first 20 h of autolysis, followed by a slow decrease to about 50 % of the initial activity measured after 70 h (Fig. 1C). Comparative analysis of freshly prepared acetone dry powder (AcDP) and

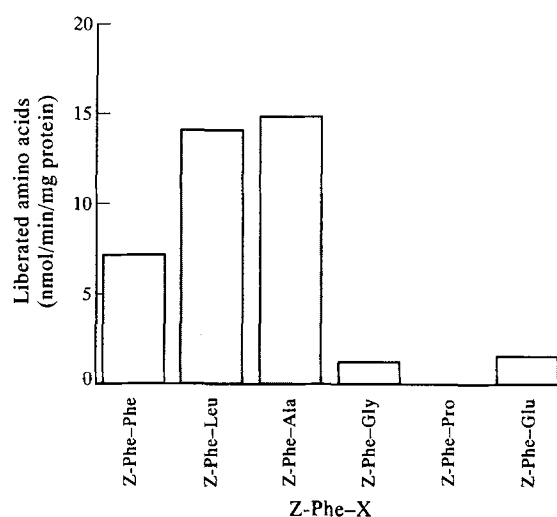

Fig. 3. Substrate specificity of the carboxypeptidase of ungerminated cocoa seeds: the specificity towards the carboxy-terminal amino acid residue. N-protected dipeptides of the type Z-Phe-X (final concentration 1 mM) were incubated at pH 5·8 and 45°C with a crude homogenate of cocoa seed AcDP preincubated with pepstatin A and iodoacetic acid as described in the Materials and Methods. After 6 h, the liberated amino acids were determined by the trinitrobenzenesulfonic acid method (Shutov *et al.*, 1982) or, in the case of Z-Phe-Pro, by the ninhydrin method (Biehl & Passern, 1982; Biehl *et al.*, 1993, 1994).

Table 2. Release of amino acids by cocoa seed carboxypeptidase activity from untreated and trypsin-digested insulin B chain

Substrate	Released amino acids[a]
Untreated insulin B chain:[b] FVDEHLCGSHLVEALYLVCGERGFFYTPKA	Ala (A)
Insulin B chain[b] digested with trypsin:[c] FVDEHLCGSHLVEALYLVCGER + GFFYTPKA	Ala (A)

[a]Untreated and trypsin-digested insulin B chain were incubated at pH 5·8 and 45°C with a crude homogenate of cocoa seed AcDP preincubated with pepstatin A and iodoacetic acid to inhibit the endopeptidase activities as described in the Materials and Methods. Released amino acids were analysed by HPLC (Kirchhoff *et al.*, 1989*a*).
[b]Commercial oxidized insulin B chain (C = $CySO_3H$).
[c]Oxidized insulin B chain was digested with trypsin as described in the Materials and Methods. Efficiency of digestion was controlled by reversed phase HPLC.

AcDP stored at -20°C for 5 days and for about 4 years, respectively, revealed that the carboxypeptidase activity was decreased by about 14% after 5 days and by only 28% after long term storage (Table 1). Furthermore, the carboxypeptidase activity present in the cocoa seeds was found to be stable over a broad range of pH-values (pH 3·0–9·0; Fig. 2).

Since the cocoa seed carboxypeptidase activity was found to be able to release leucine from the N-protected dipeptide Z-Phe-Leu (Fig. 1C; compare Voigt *et al.*, 1994*a*), the substrate specificity of this particular enzyme activity was investigated using different N-protected dipeptides of the type Z-Phe-X as substrates. As shown in Fig. 3, hydrophobic amino acids (especially Ala and Leu) were preferentially cleaved off. Z-Phe-Gly and Z-Phe-Glu were degraded very slowly, whereas Z-Phe-Pro was not cleaved at all. Oligopeptides with basic amino acid residues at their carboxyterminal ends are also resistant against degradation by the cocoa seed carboxypeptidase activity as shown by incubation with tryptic peptides derived from the oxidized insulin B chain (Table 2).

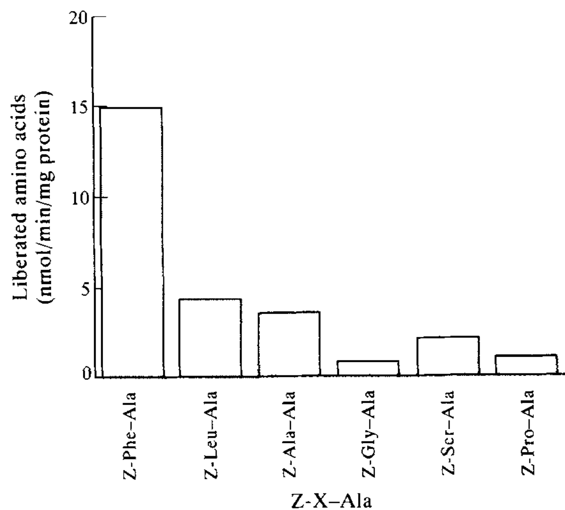

Fig. 4. Substrate specificity of the cocoa seed carboxypeptidase activity: the influence of the amino acid adjacent to the carboxyterminal amino acid residue. N-protected dipeptides of the type Z-X-Ala (final concentration 1 mM) were incubated at pH 5·8 and 45°C with a crude homogenate of cocoa seed AcDP preincubated with pepstatin A and iodoacetic acid as described in the Materials and Methods. After 6 h, the liberated amounts of alanine were determined by the trinitrobenzene-sulfonic acid method (Shutov *et al.*, 1982).

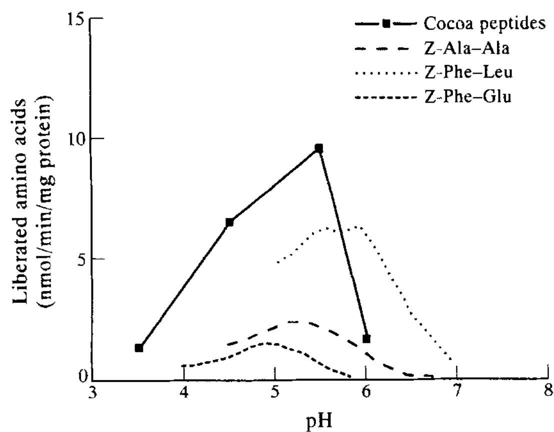

Fig. 5. pH-dependent liberation by cocoa seed carboxypeptidase of amino acids from different peptide substrates. A crude homogenate of cocoa seed AcDP preincubated with pepstatin A and iodoacetic acid as described in the 'Materials and Methods' was incubated at different pH-values (0·2 M McIlvaine buffer) and 45°C with either a mixture of hydrophobic oligopeptides prepared by autolysis of cocoa seed AcDP at pH 3·5 or the N-protected dipeptides (final concentration 1 mM) Z-Ala-Ala, Z-Phe-Leu and Z-Phe-Glu, respectively. After 6 h, the released amino acids were determined by the trinitrobenzene-sulfonic acid method (Shutov *et al.*, 1982).

Considerably different cleavage rates were observed when N-protected dipeptides of the type Z-X-Ala were used as substrates (Fig. 4). Z-Phe-Ala was found to be the most efficiently degraded peptide of this series, followed by Z-Leu-Ala and Z-Ala-Ala. Therefore, the release of the carboxyterminal amino acid is favoured by a hydrophobic side chain of the adjacent amino acid residue. This conclusion was corroborated by the finding that Z-Ser-Ala was more slowly degraded than Z-Ala-Ala (Fig. 4). Therefore, the presence of a polar OH-group in the side chain of the amino acid residue in the second position considerably reduces the cleavage rate. Z-Pro-Ala and Z-Gly-Ala were found to be very poor substrates for the cocoa seed carboxypeptidase activity (Fig. 4). These findings clearly show that specificity of the cocoa seed carboxypeptidase activity is not only dependent on the carboxyterminal amino acid of the oligopeptide substrate, but also strongly influenced by the side chain of the adjacent amino acid residue. Furthermore, the specificity of the cocoa carboxypeptidase was found to be modulated by the acidity of the reaction mixture (Fig. 5). Liberation of acidic amino acids was favoured by low pH-values.

DISCUSSION

The aroma potential of raw cocoa batches has been found to be strongly dependent on the degree and the time course of nib acidification during fermentation and fermentation-like incubations of the cocoa seeds, which considerably affects the proteolysis of seed proteins (Biehl *et al.*, 1982, 1985). A higher aroma potential is generally obtained at pH 5·5–5·0 than at pH 4·5–4·0 (Biehl *et al.*, 1985). A correlation has been observed for the aroma potential and the accumulation of hydrophobic free amino acids (Kirchhoff *et al.*, 1989*a*,*b*). Both the generation of the cocoa-specific aroma precursors and the liberation of hydrophobic amino acids are due to the cooperative action of the aspartic endoprotease and the carboxypeptidase present in the ripe cocoa seeds (Voigt *et al.*, 1993*a*; 1994*a*,*b*). Therefore, the optimum fermentation conditions (acidification to pH 5·5–5·0) are dependent on the optimum pH values of the aspartic endoprotease (pH 3·5) and of the carboxypeptidase (pH 5·8; Voigt *et al.*, 1994*a*).

The generation of the essential precursors of the cocoa-specific aroma components which are derived from the vicilin-like (7S) globulin of the cocoa seeds (Voigt *et al.*, 1993*b*, 1994*b*) is dependent on the particular chemical structure of this protein (Voigt *et al.*, 1994*d*) and on the particular specificity of the cocoa seed (aspartic) endoprotease (Voigt *et al.*, 1994*c*). A question arises as to whether or not the stability and/or the specificity of the cocoa seed carboxypeptidase activity might also contribute to the accumulation of the cocoa-specific aroma precursors.

During autolysis (at pH 5·5 and 45°C) of acetone dry powder prepared from unfermented cocoa seeds, the carboxypeptidase activity remained constant for about 20 h and subsequently decreased very slowly (Fig. 1C). After 70 h, about 50% of the initial activity remained. Furthermore, the cocoa seed carboxypeptidase activity was found to be stable over a broad pH-range (Fig. 2). Therefore, the stability of the cocoa seed carboxypeptidase is not a limiting factor for the accumulation of the cocoa-specific aroma precursors. There is, however, a pronounced specificity of the cocoa seed carboxypeptidase activity: carboxyterminal hydrophobic amino acid residues are preferentially released (Fig. 3) and the side chain of the neighbouring amino acid residue considerably affects the cleavage rate (Fig. 4), as already reported for carboxypeptidases from other organisms (Neurath, 1960; Ambler, 1972; Breddam, 1986; Breddam *et al.*, 1987). The specificity of the cocoa seed carboxypeptidase (this paper) was found to be remarkably similar to the specificity of carboxypeptidase A from porcine pancreas (Neurath, 1960; Ambler, 1972). Both enzymes preferentially liberate hydrophobic amino acids and are not able to cleave off carboxyterminal prolyl or basic amino acid residues. Glycine and acidic amino acids are released rather slowly. These results provide a reasonable explanation for our previous finding that the cocoa enzyme could be successfully substituted by carboxypeptidase A from porcine pancreas in the *in vitro* formation of the cocoa-specific aroma precursors (Voigt *et al.*, 1994*a,c*).

In addition to the released (hydrophobic) free amino acids, hydrophilic peptides have been shown to be essential precursors of the typical aroma components (Voigt *et al.*, 1993*a*, 1994*a*). These aroma-related oligopeptides must have particular amino acid sequences missing in the mixtures of proteolysis products obtained by digestion of globular storage proteins from coconuts, hazelnuts and sunflower seeds, respectively, with aspartic endoprotease and carboxypeptidase from cocoa seeds (Voigt *et al.*, 1994*d*). Therefore, the observed specificity of the cocoa seed carboxypeptidase is important with respect to formation of oligopeptides with the required carboxyterminal ends. Since the specificity of the enzyme is pH-dependent, strong acidification might impair the accumulation of the cocoa-specific aroma precursors not only by reducing the cleavage rate for hydrophobic amino acid residues, but also by increasing the release of acidic amino acids (Fig. 5).

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