Enzymatic Synthesis of the Delicious Peptide Fragments in Eutectic Mixtures

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The feasibility of flavor peptide production has been investigated using proteases in liquid/semiliquid eutectic mixtures comprised of the substrates together with small amounts of water and alcohols (19–24% w/w). The three functional fragments of the so-called "Delicious Octapeptide", L-Lys-Gly, L-Asp-Glu-Glu, and L-Ser-Leu-Ala, were obtained in good yields without any requirement for the regioselective side-chain protection of multifunctional amino acids. The peptides were synthesized on a preparative scale in overall yields of 29–77% and were fully characterized.

Keywords: *Delicious Peptide; food peptides; flavor peptides; enzymatic peptide synthesis; eutectic mixtures; proteases; adjuvants*

INTRODUCTION

There is increasing evidence that relatively short oligopeptides, derived from the hydrolysis of proteins, play an important role in the appreciation of food (Gill et al., 1995; Nishimura and Kato, 1988). In addition to imparting a particular taste and flavor to products such as meat and cheese (Cliffe and Law, 1991; McGugan et al., 1979; Nishimura and Kato, 1988; Nishimura et al., 1988; Spanier and Miller, 1993), food peptides also display a wide range of physiological activities (Aas, 1978; Ariyoshi, 1993; Gill et al., 1995; Meisel and Frister, 1989; Meisel and Schlimme, 1990; Messina and Messina, 1991; Mills et al., 1992) and are also believed to exert psychological effects, such as influencing the appetite and mood (Meisel and Schlimme, 1990; Mills et al., 1992). Consequently, the preparative synthesis of such peptides, for the purposes of investigating their structure–taste relationships and with a view to their commercialization as new flavors or food additives, is currently a topic of great interest.

Enzymes have recently emerged as powerful alternatives to conventional chemical catalysts for the synthesis of peptides (Andersen et al., 1991a,b; Bongors and Heimer, 1994; Jakubke, 1987; Kullmann, 1987; Morihara, 1987; Sinisterra and Alcántara, 1993), and the commercial feasibility of the enzymatic approach has been established with the use of proteases in the large scale manufacture of aspartame and N-malyltyrosine (Auriol et al., 1990; Cheetham, 1994; Gross, 1991). The use of enzymes for peptide synthesis typically dispenses with the requirement for side-chain protection and also avoids racemization. In addition, syntheses are performed under very mild reaction conditions, and attendant chemical and operational hazards are greatly reduced. This aspect is clearly of great importance in the production of food ingredients. On the other hand, the relatively low productivity of enzymatic methods and the limited choice of suitable solvents have to a certain extent restricted the wider use of proteases in preparative peptide synthesis.

Recently we have shown that these problems can be circumvented by performing the required reactions in (semi)liquid eutectic mixtures of substrates rather than in aqueous or organic solutions (Gill and Vulfson, 1993, 1994). Eutectic mixtures were readily formed by a wide range of amino acid derivatives bearing various protecting groups, either in the total absence of added solvent or in the presence of small amounts of eutectic modifiers (such as water, alcohols, and ethers), termed adjuvants (López-Fandiño et al., 1994a,b). This approach offers the possibility of carrying out peptide synthesis at very high overall substrate concentrations even when the selection of a suitable common solvent for the substrates is problematic (López-Fandiño et al., 1994b).

This paper demonstrates the usefulness of this methodology for the preparative synthesis of flavor peptides containing acidic, basic, and hydroxy amino acids. The three functional fragments (L-Lys-Gly, L-Asp-Glu-Glu, and L-Ser-Leu-Ala) of the so-called "Delicious Octapeptide" L-Lys-Gly-Asp-Glu-Glu-Ser-Leu-Ala (Tamura et al., 1989; Yamasaki and Maekawa, 1978) were synthesized in good yields on a gram scale without any requirement for the regioselective protection of the side chains of multifunctional amino acids.

MATERIALS AND METHODS

Subtilisin (EC 3.4.21.14, 10 units mg^{-1} of solid), thermolysin (EC 3.4.24.4, 48 units mg^{-1} of solid), papain (EC 3.4.22.2, ca. 11 units mg^{-1} of solid) and chymopapain (EC 3.4.22.6, ca. 1.0 units mg^{-1} of solid) were purchased from Sigma Chemical Co. (Dorset, England). Mercaptoethanol, palladium on alumina catalyst (5% w/w palladium content), and formic acid were obtained from Aldrich Chemical Co. (Dorset, England). N_α,N_ζ-Di-Cbz-L-lysine, N-Cbz-L-aspartic acid, N-Cbz-L-serine, glycine ethyl ester hydrochloride, glycinamide hydrochloride, L-glutamic acid diallyl ester tosylate salt, L-leucine ethyl ester hydrochloride, L-alaninamide hydrochloride, CHES, TAPS, and diisopropylethylamine were supplied by Sigma. Celite (GLC grade, 30–80 mesh) was obtained from BDH Ltd. (Dorset, England). Sorbsil RP18/C200/40–60 μm was obtained from Crossfields Chemicals Ltd. (Berkshire, England). All of the solvents used were of the highest available purity. All of the other reagents and chemicals were of analytical grade.

Preparation of the Substrates. N_α,N_ζ-Di-Cbz-L-lysine ethyl ester was synthesized using the thionyl chloride method, and N-Cbz-L-aspartic acid diallyl ester was prepared via the cesium salt route (Xaus et al., 1989). The free bases of glycinamide, alaninamide, and leucine ethyl ester were obtained by dissolving the hydrochloride salts in ethanol and neutralizing with Amberlite IRA400-OH or 0.5 M ethanolic sodium hydroxide, followed by filtering and rotary evaporating to dryness at 30 °C. Glycine ethyl ester was used as a concentrated ethanolic solution (ca. 60–70% w/w), since the free base could not be isolated due to extensive decomposition

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to the diketopiperazine product. The free base of L-glutamic acid diallyl ester was obtained by neutralizing an aqueous solution of the tosylate salt with an aqueous solution of sodium hydroxide, followed by extraction with ethyl acetate and rotary evaporation at 30 °C.

Preparation of Immobilized and Lyophilized Enzymes. For immobilization purposes, a solution of the enzyme in the appropriate buffer was added dropwise to Celite at 0.9 mL g^{-1} while mixing thoroughly with a stainless steel or Teflon spatula. The immobilized enzyme was then dried in a fluidized bed drier at room temperature until the water content was reduced to below 0.1% w/w. The following enzyme loadings and buffers were employed: subtilisin, 40 mg g^{-1} (enzyme dissolved in 100 mM CHES, pH 9, containing 50 mM sodium sulfate, 5 mM calcium acetate, and 1 mM EDTA); papain, 30 mg g^{-1} (enzyme dissolved in 100 mM TAPS, pH 8.5, containing 2 mM EDTA); chymopapain, 40 mg g^{-1} (enzyme dissolved in 200 mM CHES, pH 9, containing 2 mM EDTA); thermolysin, 30 mg g^{-1} (enzyme dissolved in 100 mM TAPS, pH 8, containing 10 mM calcium acetate and 1 mM zinc acetate). Lyophilized chymopapain was prepared by dissolving the enzyme (100 mg) in 1.0 mL of 400 mM CHES, pH 9, containing 100 mM sodium sulfate, followed by freeze-drying.

General Procedure for Enzymatic Reactions. The substrates and adjuvants were mixed together at room temperature and gently warmed to 50–60 °C for 0.5–1 min in a sealed flask or vial, and the mixture was allowed to cool to room temperature. Immobilized or lyophilized enzyme was then added with thorough mixing to give a slurry or paste. The open flask or vial was then transferred to a heating block maintained at 37 °C, and samples were taken from the reaction mixture at the appropriate time intervals for HPLC analysis. When thermolysin was used as the catalyst, and *N*-Cbz-L-serine as the acyl donor, the free acid was warmed with 1 molar equiv of diisopropylethylamine and the required adjuvants, prior to addition of the second substrate. When papain and chymopapain were employed as catalysts, 0.5 or 1% v/w of mercaptoethanol was included in the reaction mixture.

General Procedure for Transfer Hydrogenation. The *N*-Cbz peptides were deprotected by transfer hydrogenation with formic acid in methanol. In a typical procedure, the *N*-Cbz-protected peptide (2.0 mmol) was dissolved in 50 mL of methanol containing 5% v/v formic acid. Palladium on alumina (500–600 mg; 5% w/w loading) was then added and the mixture stirred at 20 °C for 30 min, by which time deprotection was complete. The reaction mixture was then filtered through a pad of diatomaceous earth, and the solution was concentrated by rotary evaporation at 30 °C. The concentrate was then diluted with water (50 mL) and freeze-dried to give the formate salt in almost quantitative yield.

Analysis, Purification, and Characterization of Products. Samples of the reaction mixtures were taken at the required time intervals, dissolved in pure methanol, and then centrifuged at 12000*g* for 2 min. These were then analyzed by reversed phase HPLC using a Gilson 305/306 quaternary pump system connected to a Gilson 231 autosampler, an ACS 750/12 UV–vis detector, and a Hewlett-Packard HP 35900 Chemstation for data acquisition and integration. Analysis was carried out on a 0.46 × 15 cm Hichrom RPB/5 μm column, maintained at 45 °C, using a flow rate of 1.0 mL min^{-1} and with detection at 220 or 257 nm. Methanol and 8:2 water/methanol, both containing 0.05% v/v of phosphoric acid, were used as the mobile phases.

Products were typically purified by solvent/acid/base extraction procedures, as described in detail below. *N*-Cbz-L-aspartyl-L-glutamyl-L-glutamic acid tetraallyl ester was purified by reversed phase medium-pressure liquid chromatography (RP-MPLC). This was carried using a 2.5 × 40 cm Buchi jacketed column, a Buchi 688 preparative chromatography pump fitted with a 30 mL injection loop, and an LDC Milton Roy Spectromonitor D. The column was slurry packed with Sorbil RP18/C200/40–60 μm and maintained at 20 °C. The sample was injected as a solution in methanol and purified using isocratic elution with 7:3 methanol/water, at a flow rate of 12 mL min^{-1} and with detection at 220 nm.

The purified products were fully characterized by NMR, MS, and FTIR. ^1H and ^{13}C DEPT, $^1H-^1H$ COSY, and $^1H-^{13}C$ COSY spectra were recorded on a JEOL EX270 FT spectrometer at 35 °C, using DMSO-d_6 as solvent. Proton spectra were recorded at 270.0 MHz and carbon-13 spectra at 67.8 MHz with broadband proton decoupling. IR spectra were recorded on a Perkin-Elmer 1600 FTIR spectrometer using a diffuse reflectance cell (64 scans, 0.1 cm^{-1} resolution) using potassium bromide as the matrix. FAB-MS was carried out on a Kratos MS9/50TC spectrometer, using xenon at 5–7 kV and a source potential of 5.33 kV. Glycerol was used as the matrix, and spectra were recorded at 0.1 mmu in positive ionization mode, using polyglycerol ions as reference. Melting points were recorded on a Stuart SMP-1 apparatus and are uncorrected.

N_α,N_ζ-Di-Cbz-L-lysylglycine Ethyl Ester (1). N_α,N_ζ-Di-Cbz-L-lysine ethyl ester (3.5 g, 8.3 mmol), glycine ethyl ester (3.9 g of 67% w/w solution in ethanol, 25 mmol), water (0.30 mL), mercaptoethanol (39 μL), and lyophilized or immobilized chymopapain (1.5 or 3.0 g, respectively) were mixed together in an open beaker, and the mixture was incubated at 37 °C for 25 h. The reaction mixture was then mixed with 5 g of diatomaceous earth and washed successively with 3 × 30 mL of 1:1 petroleum ether/ether, 3 × 30 mL of 1:3 petroleum ether/ether, and finally 3 × 15 mL of 5% w/v aqueous sodium bicarbonate saturated with sodium chloride. Finally, the product was extracted with 3 × 20 mL of hot ethanol, and the solution was rotary evaporated to dryness at 35 °C. The product was thus obtained as a white solid (2.61 g, 63% yield using lyophilized enzyme, and 2.28 g, 55% yield using immobilized enzyme, >96% pure): mp 68–70 °C; FTIR (cm^{-1}) 3310.2, 3065.7, 3033.4, 2942.0, 2867.4, 1950.6, 1742.2, 1692.0, 1649.8, 1548.0, 1443.3, 1258.2, 1133.9, 1038.1, 739.5, 696.6; ^1H NMR (δ) 1.26 (t, 3H, J = 7.3 Hz, OCH_2CH_3), 1.29–1.52 (m, 4H, Lys-H_δ + Lys-H_γ), 1.62 (m, 1H, Lys-$H_{\beta 1}$), 1.69 (m, 1H, Lys-$H_{\beta 2}$), 3.05 (dt, 2H, Lys-H_ζ), 3.87 (m, 1H, Gly-$H_{\alpha 1}$), 3.91 (m, 1H, Gly-$H_{\alpha 2}$), 4.09 (dt, 1H, Lys-H_α), 4.16 (q, 2H, J = 7.3 Hz, OCH_2CH_3), 5.08 (s, 2H, Z_ζ-CH_2), 5.11 (s, 2H, Z_α-CH_2), 7.26 (d broad, Lys-NH-Z_ζ), 7.30–7.51 (m, 10H, Arom), 7.40 (d, 1H, Z_α-NH-Lys), 8.46 (dd broad, 1H, Lys-NH-Gly); ^{13}C NMR (δ) 13.912 (OCH_2CH_3), 22.537 (Lys-γ), 28.952 (Lys-δ), 31.486 (Lys-β), 40.416 (Lys-ζ), 40.596 (Gly-α), 54.468 (Lys-α), 64.998/65.286 (Z_α-CH_2 + Z_ζ-CH_2), 127.567/128.196/136.928/137.198 (Arom), 155.832/155.976/169.542/172.417 (carbonyl); FAB-MS (M + H) calcd for $C_{26}H_{34}N_3O_7$ 500.2396, obsvd 500.2385.

N_α,N_ζ-Di-Cbz-L-lysylglycinamide (2). N_α,N_ζ-Di-Cbz-L-lysine ethyl ester (4.42 g, 10 mmol), glycinamide (1.50 g, 20 mmol), water (0.59 mL), ethanol (1.13 mL), and immobilized subtilisin (1.18 g) were thoroughly mixed together, and the mixture was incubated at 37 °C in an open beaker for 48 h. The reaction mixture was then thoroughly mixed with 5 g of diatomaceous earth and washed successively with 3 × 100 mL of 1:1 dichloromethane/ether, 2 × 100 mL of 0.2 M aqueous hydrochloric acid, and finally 2 × 50 mL of water. The residue was then extracted with hot ethanol and the solution rotary evaporated to dryness at 35 °C. The title compound was thereby obtained as a white product (3.71 g, 79% yield, >99% pure): mp 134–136 °C; FTIR (cm^{-1}) 3424.2, 3318.0, 3068.5, 3033.1, 2947.3, 1950.2, 1874.3, 1691.6, 1641.0, 1552.8, 1443.9, 1264.4, 1149.0, 1076.0, 1037.7, 977.1, 910.2, 860.8, 841.9, 745.8, 693.0, 656.9; ^1H NMR (δ) 1.24–1.39 (m, 4H, Lys-H_γ + Lys-H_δ), 1.50 (m, 1H, Lys-$H_{\beta 1}$), 1.62 (m, 1H, Lys-$H_{\beta 2}$), 2.96 (dt, 2H, $J_{\delta,\epsilon}$ = 6.3 Hz, Lys-H_ϵ), 3.62 (m, 1H, Gly-$H_{\alpha 1}$), 3.64 (m, 1H, Gly-$H_{\alpha 2}$), 3.95 (m, 1H, Lys-H_α), 5.00 (2s, 4H, Z_α-CH_2 + Z_ζ-CH_2), 6.81 (s, 1H, Gly-NH_2), 7.14 (s, 1H, Gly-NH_2), 7.16 (d broad, 1H, Z_ζ-NH-Lys), 7.31–7.36 (m, 5H, Arom-H), 7.40 (d broad, 1H, Z_α-NH-Lys), 8.03 (t broad, 1H, Lys-NH-Gly); ^{13}C NMR (δ) 22.627 (Lys-γ), 28.970 (Lys-δ), 28.970 (Lys-β), 39.195 (Lys-ζ), 41.836 (Gly-α), 54.792 (Lys-α), 65.016/65.411 (Z_α-CH_2 + Z_ζ-CH_2), 127.585/128.231/136.857/137.216 (Arom), 155.994/156.066/170.674/172.022 (carbonyl); FAB-MS (M + H) calcd for $C_{24}H_{31}N_4O_6$ 471.2243, obsvd 471.2245.

***N*-Cbz-L-aspartyl-α-L-glutamyl-α-L-glutamic Acid Tetraallyl Ester (3).** *N*-Cbz-L-aspartic acid diallyl ester (3.34 g, 10 mmol), L-glutamic acid diallyl ester (6.84 g, 30 mmol), water (1.03 mL), glycerol (1.23 mL), immobilized papain (3.1 g), and mercaptoethanol (96 μL) were thoroughly mixed together in a beaker, and the mixture was incubated at 37 °C

for 13 h. The reaction mixture was then extracted with methanol and the solution concentrated by rotary evaporation at 35 °C. Purification by RP-MPLC gave the pure product as a white solid (2.04 g, 30% yield, >98% pure): mp 79−81 °C; FTIR (cm^{-1}) 3290.0, 3069.9, 2944.7, 2886.9, 2759.7, 1728.4, 1697.9, 1642.8, 1547.1, 1466.0, 1390.5, 1312.0, 1262.2, 1218.3, 1176.4, 1054.4, 991.4, 936.2, 817.8, 776.0, 698.5; ^{1}H NMR(δ) 1.78−1.96 (m, 2H, Glu$_2$-H$_\beta$), 1.96−2.11 (m, 2H, Glu$_3$-H$_\beta$), 2.34−2.46 (m, 4H, Glu-H$_\gamma$), 2.62 (dd, 1H, $J_{\alpha,\beta 1}$ = 8.6 Hz, $J_{\beta 1,\beta 2}$ = 13.6 Hz, Asp-H$_{\beta 1}$), 2.77 (dd, 1H, $J_{\alpha\beta,2}$ = 5.2 Hz, $J_{\beta 1,\beta 2}$ = 13.6 Hz, Asp-H$_{\beta 2}$), 4.26−4.36 (m, 2H, $J_{\alpha 1,NH}$ = 7.3 Hz, $J_{\alpha 2,NH}$ = 7.6 Hz, Glu$_2$-H$_\alpha$ + Glu$_3$-H$_\alpha$), 4.42 (m, 1H, $J_{\alpha,NH}$ = 7.9 Hz, $J_{\alpha,\beta 1}$ = 8.6 Hz, $J_{\alpha,\beta 2}$ = 5.2 Hz, Asp-H$_\alpha$), 4.52−4.57 (m, 8H, OCH_2CH=CH$_2$), 5.03 (s, 2H, Z-CH_2), 5.16−5.33 (m, 8H, OCH$_2$CH=CH_2), 5.80−5.97 (m, 4H, OCH$_2$CH=CH$_2$), 7.29−7.39 (m, 5H, Arom-H), 7.63 (d, 1H, $J_{\alpha,NH}$ = 7.9 Hz, Asp-NH-Z), 8.01 (d, 1H, $J_{\alpha,NH}$ = 7.3 Hz, Glu$_2$-NH-Glu$_3$), 8.28 (d, 1H, $J_{\alpha,NH}$ = 7.6 Hz, Asp-NH-Glu$_2$); ^{13}C NMR (δ) 25.754 (Glu$_3$-β), 27.029 (Glu$_2$-β), 29.581 (Glu$_2$-γ + Glu$_3$-γ), 35.996 (Asp-β), 51.180 (Asp-α + Glu$_3$-α), 51.557 (Glu$_2$-α), 64.261/64.333/64.477/64.890 (OCH$_2$CH=CH$_2$), 65.483 (Z-CH$_2$), 117.522/117.594/117.684/117.791 (OCH$_2$CH=CH$_2$), 127.567/127.674/127.836/127.908/128.196 (Arom), 132.131/132.382/132.562/132.598 (OCH$_2$$C$H=CH$_2$), 157.742/169.758/170.297/170.872/170.908/171.645/171.860 (carbonyl); FAB-MS (M + H) calcd for $C_{34}H_{44}N_3O_{12}$ 686.2925, obsvd 686.2953.

***N*-Cbz-L-seryl-L-leucine Ethyl Ester (4).** *N*-Cbz-L-serine (2.39 g, 10 mmol), diisopropylethylamine (1.75 mL, 10 mmol), water (0.56 mL), and glycerol (0.88 mL) were thoroughly mixed together in an open beaker until a homogeneous syrup was obtained. L-Leucine ethyl ester (3.18 g, 20 mmol) and immobilized thermolysin (4.18 g) were then added, and the mixture was incubated at 37 °C for 60 h. The reaction mixture was then extracted with 2 × 100 mL of ethyl acetate, and the solution was washed with 3 × 100 mL of 5% w/v aqueous citric acid, followed by 3 × 100 mL of 5% w/v aqueous sodium bicarbonate. After drying over anhydrous magnesium sulfate, the organic layer was rotary evaporated to dryness at 35 °C. The product was thus obtained as a white solid (3.21 g, 84% yield, >98% pure): mp 61−63 °C; FTIR (cm^{-1}) 3294.6, 3070.8, 2950.4, 2076.5, 1951.9, 1881.1, 1805.8, 1722.6, 1691.7, 1657.5, 1548.1, 1535.9, 1468.2, 1451.6, 1389.5, 1293.9, 1250.5, 1209.8, 1130.9, 1111.6, 1020.0, 913.1, 888.4, 826.1, 753.1, 698.1, 668.0; ^{1}H NMR (δ) 0.84 (d, 3H, $J_{\gamma,\delta 1}$ = 6.3 Hz, Leu-H$_{\delta 1}$), 0.88 (d, 3H, $J_{\gamma,\delta 2}$ = 6.3 Hz, Leu-H$_{\delta 2}$), 1.17 (t, 3H, J = 7.1 Hz, OCH$_2$CH_3), 1.52 (m, 2H, $J_{\alpha,\beta}$ = 8.7 Hz, Leu-H$_\beta$), 1.60 (m, 1H, Leu-H$_\gamma$), 3.62 (m, 2H, Ser-H$_{\beta 1}$ + Ser-H$_{\beta 2}$), 4.06 (q, 2H, J = 7.1 Hz, OCH_2-CH$_3$), 4.12 (m, 1H, Ser-H$_\alpha$), 4.28 (dt, 1H, $J_{\alpha,NH}$ = 7.6 Hz, $J_{\alpha,\beta}$ = 8.7 Hz, Leu-H$_\alpha$), 5.03 (s, 2H, Z-CH_2), 7.13 (d, 1H, $J_{\alpha,NH}$ = 8.2 Hz, Ser-NH-Z), 7.30−7.35 (m, 5H, Arom-H), 8.11 (d, 1H, $J_{\alpha,NH}$ = 7.6 Hz, Ser-NH-Leu); ^{13}C NMR (δ) 13.858 (OCH$_2$$CH_3$), 21.297 (Leu-$\delta_1$), 22.573 (Leu-$\delta_2$), 24.047 (Leu-$\gamma$), 39.805 (Leu-$\beta$), 50.263 (Leu-α), 57.056 (Ser-α), 60.290 (OCH$_2$CH$_3$), 61.674 (Ser-β), 65.286 (Z-CH$_2$), 127.387/127.585/127.962/128.178/136.893 (Arom), 155.742/170.064/172.130 (carbonyl); FAB-MS (M + H) calcd for $C_{19}H_{29}N_2O_6$ 381.2025, obsvd 381.2035.

***N*-Cbz-L-seryl-L-leucyl-L-alaninamide (5).** *N*-Cbz-seryl-L-leucine ethyl ester (4, 3.04 g, 8 mmol), alaninamide (1.43 g, 16 mmol), water (1.34 mL), and immobilized subtilisin (1.56 g) were thoroughly mixed together, and the mixture was incubated at 37 °C for 4 h. The reaction mixture was then gently triturated and washed with 2 × 100 mL of ether, followed by 2 × 100 mL of 5% w/v aqueous sodium bicarbonate. The residue was extracted with 3 × 100 mL of hot dimethylformamide and the solution concentrated by rotary evaporation at 35 °C. Water (50 mL) was then added and the solution freeze-dried to give the title compound as a white solid (2.23g, 66% yield, >98% pure): mp 196−197 °C; FTIR (cm^{-1}) 3382.9, 3356.6, 3325.4, 3232.2, 2947.2, 2866.0, 1960.6, 1880.2, 1721.6, 1688.4, 1666.0, 1642.3, 1529.9, 1451.2, 1390.1, 1352.2, 1291.1, 1253.3, 1235.9, 1168.9, 1100.0, 1073.6, 1026.3, 996.0, 963.6, 786.0, 739.8; ^{1}H NMR (δ) 0.83 (d, 3H, $J_{\gamma,\delta 1}$ = 6.3 Hz, Leu-H$_{\delta 1}$), 0.88 (d, 3H, $J_{\gamma,\delta 2}$ = 6.3 Hz, Leu-H$_{\delta 2}$), 1.19 (d, 3H, $J_{\alpha,\beta}$ = 6.9 Hz, Ala-H$_\beta$), 1.49−1.51 (m, 2H, Leu-H$_{\beta 1}$ + Leu-H$_{\beta 2}$), 1.60 (m, 1H, Leu-H$_\gamma$), 3.59 (dd, 2H, Ser-H$_\beta$), 4.10 (m, 1H, $J_{\alpha,NH}$ = 7.6 Hz, $J_{\alpha,\beta}$ = 6.9 Hz, Ala-H$_\alpha$), 4.13 (m, 1H, Ser-H$_\alpha$), 4.26 (dt, 1H, $J_{\alpha,NH}$ = 7.6 Hz, $J_{\alpha,\beta}$ = 6.6 Hz, Leu-H$_\alpha$), 5.03 (s, 2H, Z-CH_2), 6.95 (s, 1H, Ala-NH_2), 7.06 (s, 1H, Ala-NH_2), 7.23 (d, 1H, $J_{\alpha,NH}$ = 7.2 Hz, Ser-NH-Z), 7.32−7.35 (m, 5H, Arom-H), 7.79 (d, 1H, $J_{\alpha,NH}$ = 7.6 Hz, Leu-NH-Ala), 8.06 (d, 1H, $J_{\alpha,NH}$ = 7.6 Hz, Ser-NH-Leu); ^{13}C NMR (δ) 17.919 (Ala-β), 21.333 (Leu-δ_1), 22.986 (Leu-δ_2), 24.029 (Leu-γ), 39.805 (Leu-β), 47.999 (Ala-α), 51.288 (Leu-α), 56.804 (Ser-α), 61.782 (Ser-β), 65.429 (Z-CH$_2$), 127.585/127.656/128.213/136.857 (Arom), 155.778/170.441/171.339/173.981 (carbonyl); FAB-MS (M + H) calcd for $C_{20}H_{31}N_4O_6$ 423.2243, obsvd 423.2232.

L-Lysylglycine Dihydrobromide Salt (6). All of the following operations were performed under nitrogen, in the absence of light, and using oven-dried glassware. N_α,N_ζ-Di-Cbz-L-lysylglycine ethyl ester (1, 0.60 g, 1.2 mmol) was dissolved in dichloromethane (10 mL) and the solution cooled in an ice−salt bath to −10 °C. Boron tribromide (10 mL of 1.0 M in dichloromethane, 10 mmol) was then added (in four portions of 2.5 mL) to the stirred solution over a period of 1 h. The mixture was stirred at −10 °C for a further 1 h and then allowed to warm up to 20 °C. After stirring for 10 h at 20 °C, the mixture was cooled in ice and then pressure filtered under nitrogen, and the residue was washed thoroughly with ice cold 1:1 dichloromethane/ether. The residue was then treated with water (20 mL) and filtered, and the filtrate was freeze-dried. After crystallization from 1:5 ethanol/dichloromethane, the title compound was obtained as a highly hygroscopic microcrystalline white solid (0.40 g, 92% yield, >96% pure): mp not determined, due to highly hygroscopic nature of salt; FTIR (cm^{-1}) 3387.2 (broad), 3076.8 (broad), 1733.3, 1717.0, 1683.8, 1653.2, 1558.3, 1506.4, 1457.0, 1196.6, 1024.0, 830.6; ^{1}H NMR (δ) 1.50 (m, 2H, Lys-H$_\gamma$), 1.64 (m, 2H, Lys-H$_\delta$), 1.84 (m, 2H, Lys-H$_\beta$), 2.81 (m, 2H, Lys-H$_\zeta$), 3.86−4.01 (3m, 3H, Lys-H$_\alpha$ + Gly-H$_{\alpha 1}$ + Gly-H$_{\alpha 2}$), 7.90 (s broad, 3H, Lys-NH_3^+), 8.26 (s broad, 3H, Lys-NH_3^+), 8.88 (t, 1H, $J_{\alpha,NH}$ = 5.6 Hz, Lys-NH-Gly); ^{13}C NMR (δ) 21.172 (Lys-γ), 26.580 (Lys-δ), 30.623 (Lys-β), 37.991 (Lys-ζ), 40.938 (Gly-α), 52.060 (Lys-α), 169.075/170.854 (carbonyl); FAB-MS (M + H) calcd for $C_8H_{19}N_3O_3Br_2$ 204.1348, obsvd 204.1354.

L-Lysylglycinamide Diformate Salt (7). N_α,N_ζ-Di-Cbz-L-lysylglycinamide (2, 0.94 g, 2 mmol) was deprotected as described above. The product was obtained as a white semisolid (0.57 g, 97% yield, >97% pure): mp semisolid; FTIR (cm^{-1}) 2944.4 (broad), 1679.1, 1582.3, 1380.8, 1344.4, 1255.9, 1144.7, 1020.2, 765.1; ^{1}H NMR (δ) 1.46 (m, 2H, Lys-H$_\gamma$), 1.63 (m, 2H, Lys-H$_\delta$), 1.74 (m, 2H, Lys-H$_\beta$), 2.83 (m, 2H, Lys-H$_\zeta$), 3.72 (m, 1H, Lys-H$_\alpha$), 3.77 (d broad, 2H, Gly-H$_\alpha$), 7.17 (s, 1H, Gly-NH_2), 7.61 (s, 1H, Gly-NH_2), 8.20 (s, 2H, formate), 8.47 (s, 6H, Lys-NH$_3^+$), 8.89 (t broad, 1H, Lys-NH-Gly); ^{13}C NMR (δ) 21.459 (Lys-γ), 26.598 (Lys-δ), 31.432 (Lys-β), 38.314 (Lys-ζ), 41.980 (Gly-α), 52.689 (Lys-α), 166.272/170.890/171.034 (carbonyl); FAB-MS (M + H) calcd for $C_8H_{19}N_4O_2$ 203.1508, obsvd 203.1518.

L-Aspartyl-α-L-glutamyl-α-L-glutamic Acid Formate Salt (8). *N*-Cbz-L-aspartyl-α-L-glutamyl-α-L-glutamic acid tetraallyl ester (3, 0.68 g, 1 mmol) was dissolved in 80 mL of 3:1 ethanol/acetonitrile; a solution of sodium hydroxide (60 mL of 0.1 M in 2:1 ethanol/water, 6 mmol) was added dropwise to the stirred solution over a period of 1 h at 5 °C, and stirring was continued for a further 1 h. Sulfuric acid (3.0 mL of 1.0 M solution, 3 mmol) was then added, and the solution was rotary evaporated to dryness with ethanol at 35 °C. The residue was extracted with 2:1 acetonitrile/ethanol and filtered, and the filtrate was rotary evaporated to dryness at 35 °C. The solid obtained was then deprotected by transfer hydrogenation as described above to give the product as a white solid (0.42 g, 96% yield, >95% pure): mp 102−105 °C; FTIR (KBr) (cm^{-1}) 3092.0 (broad), 1547.4, 864.5, 704.0, 661.3, 584.6; ^{1}H NMR (δ) 1.67−2.12 (m, 4H, Glu$_2$-H$_\beta$ + Glu$_3$-H$_\beta$), 2.19−2.28 (m, 4H, Glu$_2$-H$_\gamma$ + Glu$_3$-H$_\gamma$), 2.48 (m, 1H, Asp-H$_{\beta 1}$), 2.75 (dd, 1H, $J_{\alpha,\beta 2}$ = 6.4 Hz, $J_{\beta 1,\beta 2}$ = 15.0 Hz, Asp-H$_{\beta 2}$), 3.66 (m, 1H, Asp-H$_\alpha$), 4.03 (m, 1H, Glu$_3$-H$_\alpha$), 4.20 (m, 1H, Glu$_2$-H$_\alpha$), 7.95 (d, 1H, $J_{\alpha,NH}$ = 7.6 Hz, Glu-NH-Glu), 8.26 (s, 1H, formate), 8.51 (d broad, 1H, Asp-NH-Glu); ^{13}C NMR (δ) 26.688 (Glu$_3$-β), 27.047 (Glu$_2$-β), 30.497 (Glu$_3$-γ), 31.072 (Glu$_2$-γ), 36.301 (Asp-β), 50.839 (Asp-α), 52.132 (Glu$_3$-α), 52.384 (Glu$_2$-

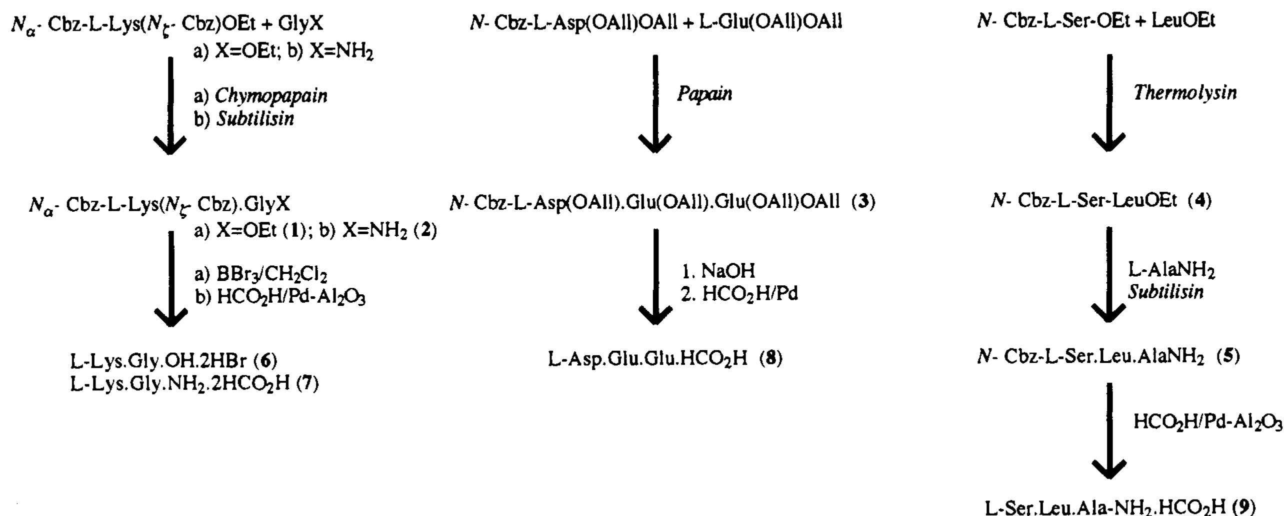

Figure 1. Synthesis of Delicious Peptide fragments in eutectic media.

α), 170.064/170.639/173.280/174.089/174.143/174.376 (carbonyl); FAB-MS (M + H) calcd for $C_{14}H_{22}N_3O_{10}$ 392.1305, obsvd 392.1385.

L-Seryl-L-leucyl-L-alaninamide Formate Salt (9). *N*-Cbz-L-seryl-L-leucyl-L-alaninamide (**5**, 0.85 g, 2 mmol) was deprotected as described above to give the product as a white hygroscopic solid (0.64 g, 97% yield, >96% pure): mp 164–166 °C; FTIR (cm^{-1}) 3401.8, 3195.1, 3070.6, 2965.2, 1981.8, 1645.1, 1586.1, 1480.0, 1365.9, 1335.8, 1252.4, 1143.1, 1018.6, 981.1, 935.8, 798.0, 767.4, 633.5; ^1H NMR (δ) 0.85 (d, 3H, $J_{\gamma,\delta 1}$ = 6.3 Hz, Leu-$H_{\delta 1}$), 0.88 (d, 3H, $J_{\gamma,\delta 2}$ = 6.3 Hz, Leu-$H_{\delta 2}$), 1.20 (d, 3H, $J_{\alpha,\beta}$ = 6.9 Hz, Ala-H_β), 1.44–1.51 (m, 2H, Leu-$H_{\beta 1}$ + Leu-$H_{\beta 2}$), 1.53–1.66 (m, 1H, Leu-H_γ), 3.33 (t, 1H, $J_{\alpha,\beta}$ = 5.9 Hz ,Ser-H_α), 3.47 (m, 2H, Ser-H_β), 4.16 (m, 1H, $J_{\alpha,NH}$ = 7.6 Hz, $J_{\alpha,\beta}$ = 6.9 Hz, Ala-H_α), 4.26 (m, 1H, Leu-H_α), 6.93 (s, 1H, Ala-NH_2), 7.09 (s, 1H, Ala-NH_2), 7.94 (d, 1H, $J_{\alpha,NH}$ = 7.6 Hz, Leu-*NH*-Ala), 8.15 (d broad, 1H, Ser-*NH*-Leu), 8.29 (s, 1H, formate); ^{13}C NMR (δ) 18.243 (Ala-β), 21.747 (Leu-δ_1), 23.328 (Leu-δ_2), 24.370 (Leu-γ), 40.884 (Leu-β), 48.341 (Ala-α), 51.557 (Leu-α), 55.834 (Ser-α), 63.255 (Ser-β), 165.158/171.465/171.627/174.358 (carbonyl); FAB-MS (M + H) calcd for $C_{12}H_{25}N_4O_4$ 289.1876, obsvd 289.1869.

RESULTS AND DISCUSSION

The Delicious Octapeptide, L-Lys-Gly-Asp-Glu-Glu-Ser-Leu-Ala, was first isolated from meat samples treated with papain (Yamasaki and Maekawa, 1978). This peptide has been implicated in the development of beef soup flavor and has been shown to impart a so-called "delicious" taste, corresponding to a compound *Umami* type sensation (Tamura et al., 1989; Yamasaki and Maekawa, 1980). Furthermore, it has been shown that the three constituent fragments L-Lys-Gly, L-Asp-Glu-Glu, and L-Ser-Leu-Ala themselves possess distinct sensory activities, and this together with the demonstration that a mixture of these peptides can be used to fully reconstruct the taste profile of the complete octapeptide (Yamasaki and Maekawa, 1980; Tamura et al., 1989) has led to considerable interest in their synthesis for possible application as flavorants and/or flavor enhancers. However, due to the presence of four different multifunctional amino acids in the sequence, the conventional chemical synthesis of these fragments requires an extensive use of regioselective protection and employs rather expensive precursors (Tamura et al., 1989; Yamasaki and Maekawa, 1980). We felt therefore that a significant simplification of the synthetic protocol could be achieved through the application of enzymes and that it should be possible to prepare the products in multigram quantities by performing the reactions in eutectic mixtures of substrates.

The precursors of all three fragments were indeed readily obtained in one or two steps using this methodology. In the case of the first dipeptide, L-Lys-Gly, precursors of both the free acid and the amide were synthesized (Figure 1 and Table 1), using N_α-N_ζ-di-Cbz-L-lysine ethyl ester as the acyl donor and GlyNH_2 or GlyOEt as the acyl acceptor. Chymopapain and subtilisin readily catalyzed the synthesis of the dipeptide ethyl ester (**1**, 63%) and amide (**2**, 79%), respectively. The lower yield obtained with GlyOEt was probably due to the lower nucleophilicity of amino acid esters as compared with the corresponding amides in protease-catalyzed reactions (Kullmann, 1987). The two dipeptides were easily deprotected to give L-Lys-GlyOH·2HBr (**6**) and L-Lys-GlyNH_2·$2HCO_2H$ (**7**) in overall yields of 58 and 77%, respectively.

The synthesis of the first fragment clearly demonstrates the potential benefits gained by performing the reactions in eutectic substrate mixtures. Thus, while the acyl donor was very hydrophobic, the acyl acceptors were highly polar, and finding a good common solvent for the substrates would present a problem if the reactions were to be performed in a conventional solvent system. In contrast, liquid eutectics were readily formed when the substrates were mixed together in the presence of appropriate adjuvants (Table 1). Generally, high concentrations (up to 1.3 M for the acyl donor and up to 3.3 M for the acyl acceptor) were readily acheived in the reactions studied, and this resulted in the accumulation of up to 0.52 g of product/g of reaction mixture.

A linear N to C strategy was employed for the synthesis of the fragments L-Asp-Glu-Glu and L-Ser-Leu-Ala, in which suitable amino acid esters were added in a stepwise manner to an *N*-protected substrate (Figure 1 and Table 1). This allowed the direct elongation of the respective peptide chains without the need for intermediate C-deprotection/activation steps. Where the acidic fragment L-Asp-Glu-Glu was concerned, reactions were performed using both the ethyl and allyl esters as donors/acceptors (Aso et al., 1988; Xaus et al., 1989; Uemura et al., 1990), and the results obtained are shown in Table 2. The allyl esters of aspartic and glutamic acid were found to be good substrates for papain, and it proved possible to perform a one-pot synthesis using exclusively the allyl esters throughout

Table 1. Preparative Synthesis of the Delicious Peptide Fragments in Eutectic Mixtures of Substrates

product	enzyme[a]	adjuvant[b] (% w/w)	concn[c] (M)		scale (mmol)	time (h)	yield[d] (%)	[product][e] (g/g)
			acyl donor	acyl acceptor				
1	chymopapain	5.0% water 18.6% ethanol	1.07	3.33	8.3	25	63 (55)[f]	0.34 (0.30)[f]
2	subtilisin	8.0% water 12.0% ethanol	1.31	2.62	10.0	48	79	0.52
3	papain	8.1% water 12.1% glycerol	0.80	2.40	10.0	13	30	0.16
4	thermolysin	6.6% water[g] 13.0% glycerol	1.14	2.28	10.0	60[h]	92	0.35
5	subtilisin	23.1% water	1.38	2.75	8.0	4	66	0.38

[a] All of the enzymes employed were immobilized on Celite. Chymopapain was also used as a lyophilized powder. [b] The percentage of adjuvant is given on the basis of the reaction mixture excluding the biocatalyst. [c] The concentrations are calculated on the basis of the reaction mixture excluding the biocatalyst. [d] The preparative yields are given. [e] This is calculated on the basis of the reaction mixture excluding the biocatalyst. [f] The figure in parentheses refers to the immobilized enzyme. [g] One molar equivalent of diisopropylethylamine was included in the reaction mixture. [h] A yield of 47% was obtained after 24 h. However, as the reaction was under thermodynamic control, an extended incubation period was used to ensure that equilibrium was reached.

Table 2. Synthesis of Precursors of the Acidic Tripeptide L-Asp-Glu-Glu in Eutectic Mixtures of Substrates[a]

acyl donor	acyl acceptor	enzyme[b]	adjuvant[c] (% w/w)	yield[d] (%)
N-Cbz-L-Asp(OAll)OAll	L-Glu(OAll)OAll	α-chymotrypsin	20% water	56
N-Cbz-L-Asp(OEt)OEt	L-Glu(OEt)OEt	α-chymotrypsin	30% water	70
N-Cbz-L-Asp(OAll)OAll	L-Glu(OEt)OEt	α-chymotrypsin	20% water	78
N-Cbz-L-Asp(OAll)-Glu(OAll)OAll	L-Glu(OAll)OAll	papain	30% water	37
N-Cbz-L-Asp(OAll)-Glu(OAll)OAll	L-Glu(OAll)OAll	chymopapain	20% water	26
N-Cbz-L-Asp(OEt)-Glu(OEt)OEt	L-Glu(OEt)OEt	chymopapain	30% water 20% glycerol	39
N-Cbz-L-Asp(OAll)-Glu(OEt)OEt	L-Glu(OEt)OEt	chymopapain	30% water 20% glycerol	40

[a] Reactions were conducted on a 0.2 mmol scale using a 50% molar excess of the acyl acceptor, 37 °C, 17 h. [b] All of the enzymes employed were immobilized on Celite. [c] The percentage of adjuvant is given on the basis of the reaction mixture excluding the biocatalyst. [d] The analytical yields as determined by HPLC are reported.

the reaction sequence, thereby providing the tripeptide triallyl ester (**3**) with a yield of 30% (product concentration of 0.16 g g^{-1}) (Table 1). This compares very favorably with the yields of 21–31% obtained for the two-step syntheses. After almost quantitative deprotection by hydrolysis followed by transfer hydrogenation, the free acid L-Asp-Glu-Glu·HCO_2H (**8**) was obtained with an overall yield of 29%.

The third fragment, Ser-Leu-Ala, was synthesized with a yield of 54%, and a product concentration of 0.38 g g^{-1}, by the consecutive addition of L-LeuOEt and L-AlaNH_2 to the starting material *N*-Cbz-L-SerOH, using thermolysin and subtilisin as the respective catalysts.

It should be mentioned that the Celite immobilized enzymes employed for the syntheses displayed good operational stabilities under the conditions used, and a mild solvent extraction step was sufficient to allow their recovery in a highly active form ready for reuse. For example, the immobilized subtilisin used for the synthesis of dipeptide **1** was recycled four times (after extraction of the reaction mixture with ethanol, followed by filtration), giving consecutive product yields of 79, 75, 73, and 72%. The good stability of these biocatalysts suggests that, if required, it should be possible to employ them for semicontinuous syntheses carried out in stirred-tank or other suitable reactor configurations.

CONCLUSION

This work further demonstrates that protease catalysis in eutectic mixtures of substrates is an attractive and useful technique for the preparative synthesis of short peptides on a multigram scale. Eutectics were readily obtained by using amino acid derivatives commonly employed in peptide synthesis, and the inclusion of hydrophilic adjuvants such as water, ethanol, and glycerol was found to promote eutectic formation. In addition, the use of eutectic media allowed reactions to be performed at high substrate concentrations of 0.8–2.8 M, which are considerably in excess of those normally attainable in organic solvents. The use of eutectic mixtures as reaction media allowed us to obtain 0.16–0.52 g of the di- and tripeptide products/g of reaction mixture.

The described approach allows the preparation of Delicious Peptide fragments in good yields and in quantities sufficient for structure–taste relationship studies. The simplicity of the overall protocol and the fact that only small quantities of water or alcohol were used at the coupling stages makes this strategy rather attractive for the large scale synthesis of these and other flavor peptides.

ACKNOWLEDGMENT

We extend our thanks to Dr. R. López-Fandiño, Dr. R. Valivety, and Dr. A. O'L. Richards for helpful discussions.

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Received for review March 7, 1995. Accepted July 24, 1995.[⊗] We are grateful to the BBSRC for financial support and to the EC for providing a FLAIR Postdoctoral Fellowship to X.J.

JF950134+

[⊗] Abstract published in *Advance ACS Abstracts*, September 1, 1995.