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The stability of peptides in nasal enzymic systems

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Abstract

Nasal homogenates, cellular fractions and nasal wash preparations were used to study the peptidase activity of sheep nasal mucosa. Three commercially available proteases, carboxypeptidase A, cytosolic leucine aminopeptidase and microsomal leucine aminopeptidase, were also investigated for comparison. It was found that sheep nasal mucosa possesses significant peptide hydrolase activity with a variety of exopeptidases and an endopeptidase which were capable of hydrolysing di- (FG, GF, WG), tri- (FGG, WGG, GGF) and tetrapeptides (FGGF). A large variation in the stabilities of the substrates in nasal homogenate was found, with half-lives ranging from ~ 1 min for the disappearance of GF to ~ 140 min for the loss of WG. Enzyme activity was mediated by both aminopeptidase and carboxypeptidase with some specificity for aromatic amino acids with degradation being favoured at WG, FG and GF in preference to GG. Despite the complexity of the enzyme preparation, Michaelis-Menten kinetics were followed and apparent K_m and V_{max} values were calculated. The aminopeptidase activity per mg of protein was greater in the microsomal preparation than that in the cytosolic preparation towards GGF. Greater microsomal activity per mg of protein was also observed when the activities of the purified cytosolic and microsomal aminopeptidases against FGG, WGG, GGF and FG were compared.

Keywords: Aminopeptidase; Carboxypeptidase; Nasal delivery; Peptide; Protease; Stability

1. Introduction

The numerous proteolytic enzymes in the gastrointestinal tract provide a formidable barrier to the oral delivery of peptide drugs (Lee, 1988, 1991; Audus and Raub, 1993). Degradation can occur luminally, at the brush border or intracellularly and the site and extent of degradation are dependent upon the size and amino acid composition of the peptide (Lee and Yamamoto, 1990). To overcome stability and absorption problems, other delivery sites are under intensive investigation although the proteolytic activities, subcellular distribution and substrate specificities of enzymes at other potential sites of drug administration are less well-characterised. Nasal delivery has shown much promise in this regard and, although it was originally believed that the nasal mucosa was deficient in enzymic activity, in vitro

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studies have shown that this tissue possesses many enzymes including monooxygenases (Longo et al., 1989), reductases (Larsson et al., 1989), transferases (Aceto et al., 1989), esterases (Zhou and Li Wan Po, 1990, 1991), a variety of proteases (Dodda Kashi and Lee, 1986; Stratford and Lee, 1986) and is a site of steroid metabolism for hormones such as oestradiol, progesterone and testosterone (Barwashi-Nassar, 1989). Much work has been undertaken to assess the ability of nasal tissue to activate or detoxify carcinogenic compounds implicated in nasal and respiratory cancer. These include aflatoxin B (Larsson et al., 1989), nitrosamines (Longo et al., 1989), polycyclic amines (Bond, 1983) and benz[*a*]pyrene (Matsubara et al., 1974). Such experiments have demonstrated the presence of cytochrome P_{450}-dependent monooxygenase in rat, hamster (Longo et al., 1986) and human nasal microsomes (Longo et al., 1989). Moreover, the concentrations of the cytochrome P_{450} enzymes are second only to those of the liver as determined by the activity per mg of microsomal protein (Dahl et al., 1982).

Although the efficiency of nasal delivery is highly dependent upon residence time, it was the lack of effective absorption at this site which prompted assessment of its proteolytic activity in both in vivo (Hussain et al., 1985) and in vitro (Hirai et al., 1981) systems. The presence of both endo- and exopeptidases has been demonstrated and enzymes include aminopeptidases A, B, N, leucine aminopeptidase and microsomal aminopeptidase (Stratford and Lee, 1986; Audus and Tavakoli-Saberi, 1991; Sarkar, 1992). The presence of dipeptidylpeptidase, diaminopeptidase, post-prolyl cleaving-enzyme, angiotensin-converting enzyme and endopeptidase (Dodda Kashi and Lee, 1986; Hayakawa et al., 1987; Lee and Dodda Kashi, 1987; Lee et al., 1987a,b) has also been indicated. Conclusions on the subcellular distribution of enzymic activity (Choi and Lee, 1986; Yamamoto et al., 1988) and the activity of nasal mucosa compared to other potential sites of delivery (Dodda Kashi and Lee, 1986; Lee and Dodda Kashi, 1987; Lee et al., 1987a,b; Zhou and Li Wan Po, 1990) appear dependent upon the peptide structure and mucosal source.

The most commonly employed species are dogs, rats, rabbits and sheep (Chien et al., 1989). The relevance of such models to the human situation and their anatomical and physiological variations have been reviewed (Gizurarson, 1990, 1993) with dog, monkey, sheep and rabbit being considered particularly useful for pharmacokinetic and formulation studies. In the present study, sheep nasal mucosa was employed as this is an established in vivo (Farraj et al., 1990) and in vitro (Wheatley et al., 1988) model for nasal delivery which offers a good correlation with the intranasal absorption of insulin in man (Longenecker et al., 1987).

2. Theoretical treatment of results

The rate of an enzyme-catalysed reaction is dependent upon the concentration of both enzyme and substrate. If the concentration of substrate is in excess, the reaction rate increases linearly with increasing enzyme concentration. For a fixed concentration of enzyme, increasing substrate concentration increases the rate of the reaction in a non-linear relationship until the maximum rate occurs. This relationship is described by the Michaelis-Menten equation (Eq. 1):

$$v = \frac{V_{max}[S]}{K_m + [S]} \tag{1}$$

where v is the initial rate of the reaction $(-dS/dt)$, [S] denotes the substrate concentration, V_{max} is the maximum reaction rate and K_m represents the Michaelis constant, equal to the substrate concentration at which the reaction rate is half its maximum value. The parameters V_{max} and K_m are determined to indicate substrate capacity and affinity often by using a range of substrate concentrations at one concentration of enzyme. An alternative method uses half-time analysis (Wharton and Szawelski, 1982; Nichols and Hewinson, 1987), based upon the integrated Michaelis-Menten (Eq. 2):

$$\ln(S_t) + \frac{S_t}{K_m} = \ln(S_0) + \frac{S_0}{K_m} - \frac{V_{max}t}{K_m} \tag{2}$$

This non-linear equation approximates to the zero-order case when $S_0 \gg K_m$ ($v = V_{max}$) and to

the first-order case when $K_m \gg S_0$ ($v = k \cdot [S]$; where the first-order rate constant $k = V_{max}/K_m$). Assuming that the products exert no effect on the rate of the reaction, rearrangement yields a linear relationship between the fractional lifetime (e.g., t_x, the time taken for the substrate concentration to fall from S_0 to $x \cdot S_0$) and the substrate concentration (Eq. 3):

$$t_x = \frac{(1-x) \cdot S_0'}{K_m} + \frac{\ln\frac{1}{x} \cdot K_m}{V_{max}} \quad (3)$$

where S_0' is the instantaneous substrate concentration at any time on the reaction progress curve and t_x denotes the time taken for any value of S_0' to fall to $x \cdot S_0'$. For the special case when half of the substrate has disappeared, this becomes (Eq. 4):

$$t_{\frac{1}{2}} = \frac{S_0'}{2V_{max}} + \frac{\ln(2) K_m}{V_{max}} \quad (4)$$

where $t_{\frac{1}{2}}$ is the time taken for any value of S_0' to fall to $S_0'/2$.

Using half-life analysis, the complete time profile for the hydrolysis of a substrate is recorded and successive $t_{\frac{1}{2}}$ values are measured for a series of S_0' values. A plot of $t_{\frac{1}{2}}$ vs S_0 yields V_{max} ($0.5 \times \text{gradient}^{-1}$) and K_m ($2.8854 \times$ intercept on the ordinate axis $\times$ gradient^{-1}). To obtain reliable estimates, the initial concentration (S_0) should be within the range $0.5–5K_m$. The validity of the estimated values may be determined by comparison of the theoretical and experimental profiles using computer modelling of the Michaelis-Menten equation.

3. Materials and methods

3.1. High-performance liquid chromatography

System A consisted of an Altex model 110A pump, a Rheodyne 7120 or 7125 injection port fitted with a 100 μl loop and a Pye Unicam LC3 variable-wavelength ultraviolet detector. Chromatography was performed on a 100×4.6 mm column packed with 5 μm ODS-Hypersil reversed-phase material. A guard column (10×4.6 mm) packed with the same material was used to increase the lifetime of the column. Chromatograms were recorded on either a JJ Instruments recorder or an Omniscribe D500 chart recorder. A Hewlett Packard 3390A recording integrator permitted the peak areas to be determined. System B (used for the majority of the metabolism experiments) consisted of a Waters Chromatography station comprising a Waters 600E system controller, a WISP 712 autoinjector and a Waters 484 ultraviolet variable-wavelength detector. Chromatography was performed on a 250×4 mm Lichrospher 100 RP-18 end-capped column packed with 5 μm particles protected by a guard column packed with the same material. Chromatograms were collected and integrated on

Table 1
HPLC conditions and retention times for peptides

Mobile Phase	F	FG	GF	FGG	GGF	FGGF	W	WG	WGG
1	2.0						5.5		
2							5.0	8.0	7.5
3							13.8	22.2	20.2
4	8.1	13.0	14.5	11.4	17.8				
5						4.6			

Aqueous mobile phases contain: 1, CH_3CN (5%)/Et_2N (0.1%)/H_3PO_4 to pH 3.5 (flow rate 1.5 ml min^{-1}, system A); 2, CH_3CN (4%)/Et_2N (0.1%)/H_3PO_4 to pH 3.5 (flow rate 1.5 ml min^{-1}, system A); 3, CH_3CN (4%)/Et_2N (0.1%)/H_3PO_4 to pH 3.5 (flow rate 1.5 ml min^{-1}, system B); 4, CH_3CN (3%)/Et_2N (0.1%)/H_3PO_4 to pH 3.5 (flow rate 1.0 ml min^{-1}, system A); 5, CH_3CN (30%)/0.1% TFA (70%); flow rate 1.0 ml min^{-1}, system A). Wavelengths: phenylalanine-containing (F) peptides, 255 nm; tryptophan-containing (W) peptides, 270 nm.

a Waters Chromatography 815 workstation. Mobile phases and retention times are listed in Table 1.

3.2. Enzymic preparations

3.2.1. Preparation of nasal homogenate

The nasal homogenate was prepared by a modification to the method of Stratford and Lee (1986). The heads of recently slaughtered sheep, freshly obtained from a local abattoir, were bisected along the nasal septum to expose the nasal cavity. The nasal mucosa was carefully excised from the nasal septum and turbinates and freed from the underlying cartilage and bone. The tissue was rinsed with phosphate-buffered saline, pooled in glass vials and stored at −70°C. Prior to use, the tissue was thawed at room temperature for about 30 min. The tissue was homogenised in an Omnimixer for 2 min at 4°C and then in a Teflon-glass homogeniser. The homogenate was than centrifuged at $3020 \times g$ at 4°C for 10 min to remove cellular and nuclear debris. The supernatant, which contained cytosolic, plasma and intracellular membrane fractions, was diluted in phosphate buffer and equilibrated at 37°C for 30 min then used immediately. To determine the effect of pH upon the hydrolysis, McIlvaine's buffer (pH 3.4, 5.4, 7.4) was employed to prepare the homogenate (Perrin and Dempsey, 1974).

3.2.2. Preparation of microsomes

Microsomal and cytosolic fractions were prepared by a modification to the method of Larsson et al. (1989). The nasal homogenate was prepared as described but with McIlvaine's buffer (pH 7.4; $\mu = 0.5$). To separate the cytosolic and microsomal fractions, the homogenate was centrifuged in a Beckmann model TL-100 Ultracentrifuge at $100\,000 \times g$ for 1 h at 4°C. The supernatant, which represented the cytosolic fraction, was then removed and the microsomal pellet was resuspended in McIlvaine's buffer (pH 7.4) with a Teflon homogeniser. The cytosolic and microsomal fractions were diluted with McIlvaine's buffer and equilibrated at 37°C for 30 min.

3.2.3. Experiments performed with commercial enzymes

The carboxypeptidase A (CPA; EC 3.4.17.1), purified from bovine pancreas (Sigma), was suspended in an aqueous solution containing toluene and treated with phenylmethylsulphonylfluoride (PMSF) to inhibit serine proteases. Each mg of protein was equivalent to 70.5 units, where each unit was capable of hydrolysing 1.0 μmol of hippuryl-L-phenylalanine per min at pH 7.5 and at a temperature of 25°C. The enzyme preparation contained 16.2 mg ml^{-1} of protein with an activity of 1.14 mmol min^{-1} ml^{-1}. The leucine aminopeptidase (cytosolic) enzyme (LAP-C; EC 3.4.11.1), obtained from porcine kidney (Sigma), was a chromatographically purified suspension in 2.9 M $(NH_4)_2SO_4$, 0.1 M Tris buffer and 5 mM $MgCl_2$ solution at pH 8.0. Each mg of protein was equivalent to 210 units, where each unit was capable of hydrolysing 1.0 μmol of L-leucinamide to L-leucine and ammonia per min at pH 8.5 and at a temperature of 25°C. The enzyme preparation contained 1.9 mg ml^{-1} of protein with an activity of 0.399 mmol min^{-1} ml^{-1}. The leucine aminopeptidase (microsomal) enzyme (LAP-M; EC 3.4.11.2), obtained from porcine kidney microsomes (Sigma) was a suspension in 3.5 M $(NH_4)_2SO_4$ and 10 mM $MgCl_2$ solution at pH 7.7. Each mg of protein was equivalent to 19 units, where each unit was capable of hydrolysing 1.0 μmol of L-leucine-*p*-nitroanilide to L-leucine and *p*-nitroaniline per min at pH 7.2 and at a temperature of 37°C. The enzyme preparation contained 2.1 mg ml^{-1} of protein with an activity of 39.9 mol min^{-1} ml^{-1}. The enzymes were each diluted in 0.1 M phosphate buffer (pH 7.4) to give a final protein concentration of 10 μg ml^{-1} in the incubation mixture. The enzymes were equilibrated at 37°C in a water bath for 30 min before experiments were initiated by addition of the substrate.

3.3. Metabolism studies

All the metabolism studies were carried out in duplicate, in a shaking water bath at 37°C. The concentrations of substrates and metabolites were determined by HPLC as described above.

3.3.1. The effect of storage on the enzymic activity of sheep nasal mucosa

Stock solutions (3 mM, 30 mM) of the tripeptides Trp-Gly-Gly (WGG) and Phe-Gly-Gly (FGG) were prepared in 0.1 M phosphate buffer (pH 7.4). Fresh nasal mucosa was pooled and divided into 1 g portions some of which were stored at −70°C while the remainder were homogenised as above. Sufficient fresh homogenate for a series of experiments was retained while the remainder was stored at −70°C in 0.5 ml volumes. After 1 and 3 week periods, the stored tissues were at room temperature for 30 min and homogenised. The stored, frozen homogenate was similarly defrosted at room temperature. These two enzymic preparations were then used in metabolism studies.

The following protocol was adopted for all the experiments. The enzymic preparation (0.1 ml) was diluted in 3.9 ml phosphate buffer (pH 7.4) and the protein concentration determined. Metabolism was initiated by the addition of 2 ml of the substrate (final concentration 1 or 10 mM). At set time points, 0.4 ml aliquots were removed from the incubation mixture and the reaction quenched on ice by the addition of 0.1 ml of trifluoroacetic acid (TFA). The sample was then centrifuged for 10 min at $9000 \times g$ to separate precipitated protein. Aliquots (100 μl), of each time point sample (comprising 0.4 ml peptide mixture in 0.1 M phosphate buffer (pH 7.4), and 0.1 ml TFA), were injected onto the HPLC column without any sample pretreatment. HPLC standards were prepared in 0.1 M phosphate buffer (pH 7.4). TFA (100 μl) was then added to 0.4 ml aliquots of each standard and 100 μl of the resultant mixture injected onto the analytical column.

3.3.2. Homogenate preparation

The substrates tested comprised a tetrapeptide phenylalanylglycylglycylphenylalanine (FGGF), tripeptides phenylalanylglycylglycine (FGG), glycylglycylphenylalanine (GGF), and tryptophylglycylglycine (WGG) and dipeptides phenylalanylglycine (FG), glycylphenylalanine (GF) and tryptophylglycine (WG). In preliminary experiments to validate the method, 3 mM stock solutions of the substrates were prepared in 0.1 M phosphate buffer (pH 7.4). Subsequently, due to the insolubility of FGGF, 3 mM stock solutions of all the substrates were prepared in 20:80 dimethylsulphoxide/phosphate buffer to enable direct comparisons to be made. The homogenate (0.1 ml) was diluted in 3.9 ml phosphate buffer and the protein concentration determined. Metabolism was initiated by the addition of 2 ml of the substrate (final concentration 1 mM). At set time points (0–420 min), 0.4 ml aliquots were removed from the incubation mixture and the reaction quenched as above. Volumes (100 μl) from each time point sample (comprising 0.4 ml peptide mixture in 6.67% DMSO in 0.1 M phosphate buffer (pH 7.4), and 0.1 ml TFA), were injected onto the HPLC column without any sample pretreatment. HPLC standards were prepared in 6.67% DMSO in 0.1 M phosphate buffer (pH 7.4). TFA (100 μl) was then added to 0.4 ml aliquots of each standard and 100 μl of the resultant mixture injected onto the analytical column.

3.3.3. Effect of pH on the rate of hydrolysis

Solutions of the substrate GGF (3 mM) were prepared in McIlvaine's buffers ($\mu = 0.5$) with pH values of 3.4, 5.4 and 7.4 (Perrin and Dempsey, 1974). The protocol used for the homogenate preparation described above was used except that McIlvaine's buffer, without DMSO, was used.

3.3.4. Experiments performed with enzymic fractions

Each of the three enzymic preparations (0.1 ml) were diluted in 3.9 ml McIlvaine's buffer (pH 7.4) and the protein concentration of the resultant solutions determined. Metabolism was initiated by the addition of 2 ml of the substrate (GGF; 3 mM in McIlvaine's buffer; pH, 7.4; final concentration, 1 mM). At set time points (0–300 min), 0.4 ml volumes were removed from the incubation mixture and the reaction quenched and analysed as described above.

3.3.5. Commercial enzymes

Solutions (3 mM) of the tripeptides FGG, GGF and WGG, and of the dipeptide FG were pre-

pared in 0.1 M phosphate buffer (pH, 7.4). Metabolism was initiated by the addition of 2 ml of the appropriate substrate (final concentration 1 mM) to the diluted enzymic preparation. At set time points (0–420 min), 0.4 ml aliquots were removed from the incubation mixture and the reaction quenched and analysed as described above. Additionally, solutions of GGF (3 mM) were prepared in one of three vehicles: A, 90.6 ml of 0.2 M Na_2HPO_4, diluted to 100 ml with water and adjusted to pH 7.4 with orthophosphoric acid; B, McIlvaine's buffer (pH, 7.4); C, McIlvaine's buffer (pH, 7.4) with KCl to an ionic strength of 0.5 M.

4. Results and discussion

Control experiments demonstrated that all of the peptides studied were stable in 0.1 M phosphate buffer (pH 7.4) with 6.67% DMSO, at 37°C, for the duration of the experiments and that the preparation did not interfere with the HPLC assay. In contrast, all of the substrates tested were hydrolysed in the nasal mucosal homogenate (protein concentration 0.42 mg ml^{-1}) but with a considerable variation in susceptibility to degradation. The enzymic activity against WGG, FGG and GGF, dissolved in phosphate buffer containing 6.67% DMSO, did not differ markedly to the activity in phosphate buffer alone. Freezing the nasal mucosa, in an unprocessed form, had little effect on the protease activity. The rate of degradation of WGG caused by fresh homogenate was 4.87×10^{-6} mol l^{-1} min^{-1} while that from stored whole tissue was 4.66×10^{-6} mol l^{-1} min^{-1}. A similar result was obtained with 1 mM FGG where the rates of degradation of FGG were 2.02×10^{-5} mol l^{-1} min^{-1} (fresh) and 1.90×10^{-5} mol l^{-1} min^{-1} (whole tissue frozen). In contrast, homogenisation of the nasal mucosa prior to storage caused a reduction in enzymic activity. For example, 10 mM FGG degraded with a rate of 8.79×10^{-5} mol l^{-1} min^{-1} when stored unprocessed mucosa was used but this fell to 5.72×10^{-5} mol l^{-1} min^{-1} on treatment with stored homogenate. In the case of WGG the rate of degradation with the stored homogenate was

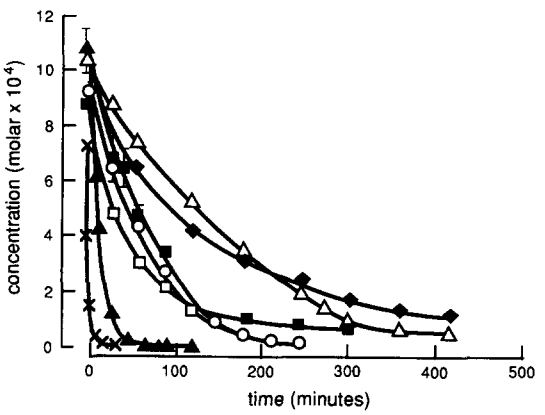


Fig. 1. Degradation of phenylalanylglycylglycylphenylalanine (FGGF), phenylalanylglycylglycine (FGG), phenylalanylglycine (FG), glycylglycylphenylalanine (GGF), glycylphenylalanine (GF), tryptophylglycylglycine (WGG) and tryptophylglycine (WG) by nasal mucosal homogenate in 0.1 M phosphate buffer containing 6.67% DMSO (pH 7.4) at 37°C.

80% of the value obtained with the fresh mucosa. These results are similar to those observed by Stratford and Lee (1986) with rabbit nasal mucosa and, where necessary, tissue was stored in an unprocessed form and was defrosted immediately prior to use.

The degradation profiles of all of the substrates tested are shown in Fig. 1; the dipeptide GF was hydrolysed most rapidly and was undetectable after 10 min whereas the most stable peptides, WG and WGG, were still quantifiable after 420 min. The three tripeptides showed considerable variation in susceptibility with the order of stability being FGG < GGF < WGG with half-lives of ~8, ~40 and ~100 min, respectively. The order of stability for the three dipeptides was GF < FG < WG with half-lives of ~1, ~40 and ~140 min, respectively. The tetrapeptide FGGF was of a similar stability to GGF with a half-life of ~30 min. Both peptides with aliphatic N-terminal amino acids, GF and GGF, were degraded rapidly; GF was least stable ($t_{\frac{1}{2}}$ ~1 min) and GGF was the third least stable ($t_{\frac{1}{2}}$ ~40 min). A similar trend was observed by Sterchi and Woodley (1980), who studied the metabolism of a series of peptides, (including FG, FGG and FGGF) with homogenates of human small intestinal mucosa and found that peptides

Table 2
Michaelis-Menten kinetic parameters determined by the method of half-time analysis for substrates in homogenate preparation

Peptide	V_{max} ($\times 10^5$) (mol l^{-1} min^{-1})	K_m ($\times 10^4$) (M)	$k = V_{max}/K_m$ (min^{-1})
FGGF	1.90	8.23	0.0231
FGG	5.43	4.45	0.122
WGG	0.34	10.08	0.00337
GGF	2.69	12.36	0.0218
GF	33.25	4.25	0.782

with aliphatic, rather than aromatic, amino acids at their N-terminal end were better substrates. In contrast is the observation here that FGG was degraded significantly faster than GGF in the sheep nasal homogenate.

Although the complexity of enzymic preparations precludes a detailed mechanistic interpretation of results, degradation profiles followed the Michaelis-Menten model and the apparent kinetic parameters associated with these reactions could be determined using half-time analysis. These are recorded in Table 2 together with the derived first-order rate constants ($k = V_{max}/K_m$). Theoretical hydrolysis profiles generated with these parameters were in close agreement with the experimental profiles, with the exception of WG. As K_m indicates the affinity of an enzyme for a particular substrate (more properly, K_m^{-1}) and V_{max} indicates the maximum rate of degradation obtainable, a low K_m and a high V_{max} indicate a good enzyme substrate undergoing rapid degradation. The first-order rate constant ($k = V_{max}/K_m$), which combines both terms into a single parameter, gives an overall assessment of the degradation profile and mirrors the rank order stability (Table 2). The substrate GF, which was hydrolysed most rapidly, exhibited a low K_m value (4.25×10^{-4} M) and a large V_{max} value (33.25×10^{-5} mol min^{-1}) indicating that it was a good substrate (k, 0.782 min^{-1}). Conversely, the most stable compound, WGG, showed a low V_{max} (0.34×10^{-5} mol min^{-1}) and a high K_m (10.08×10^{-4} M), confirming that the compound was a poor substrate (k, 0.00337 min^{-1}). The data obtained from the hydrolysis of FG did not produce

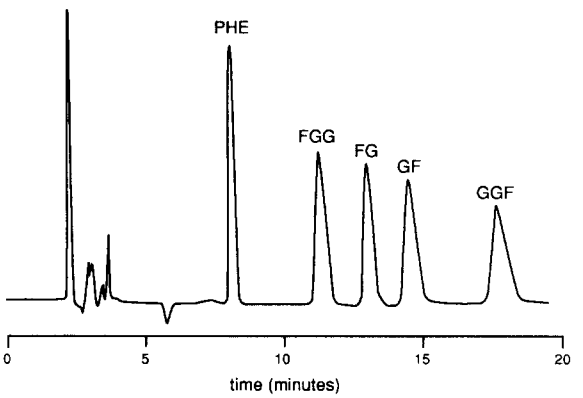


Fig. 2. HPLC chromatogram showing the separation of products of hydrolysis of the tetrapeptide FGGF using mobile phase 4; unchanged FGGF was analysed using mobile phase 5.

linear plots from half-time analysis and consequently Michaelis-Menten kinetic parameters for this substrate were not determined.

HPLC analysis enabled degradation pathways to be elucidated in addition to providing kinetic information for the degradation profile (Fig. 2). The degradation of the tetrapeptide, FGGF, is illustrated in Fig. 3. FGGF was cleaved exclusively at its N-terminal end, as indicated by the absence of the possible metabolites FGG, FG and GF, to generate the amino acid phenylalanine and the tripeptide GGF. The GGF formed

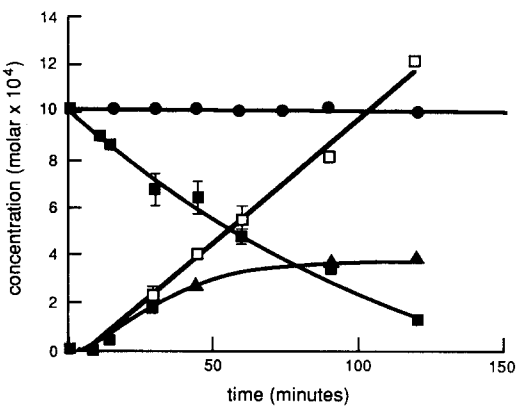
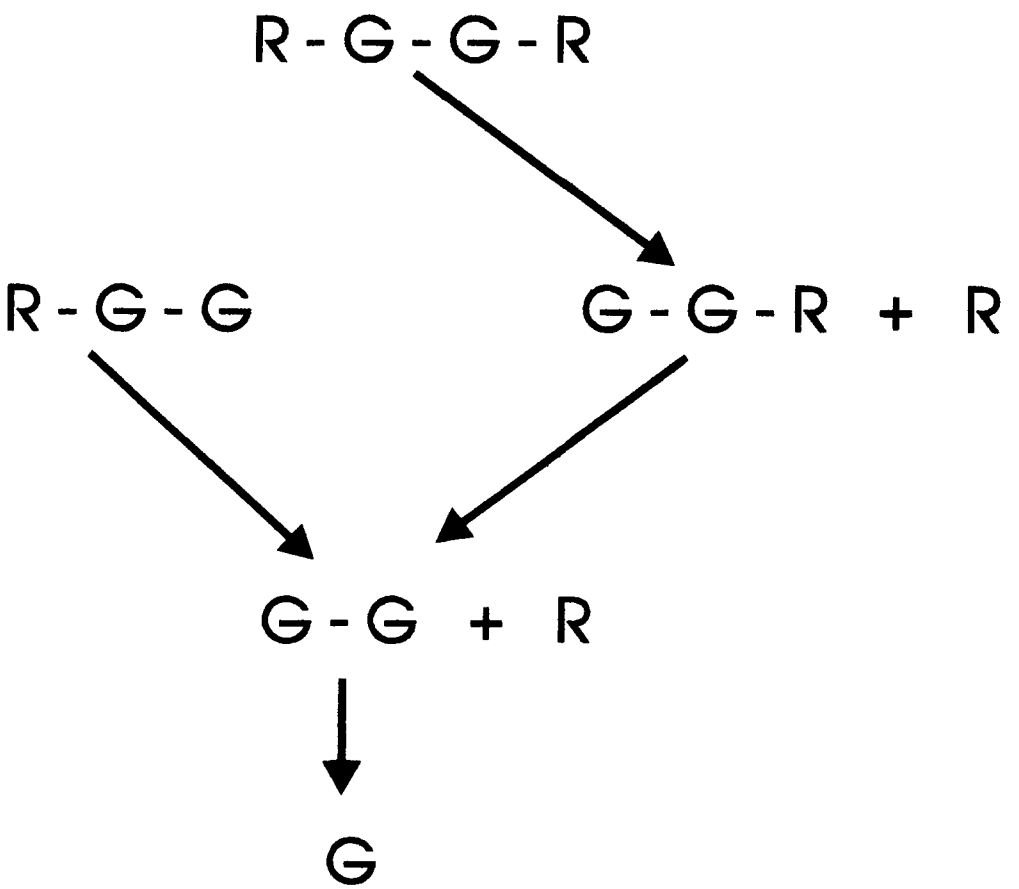


Fig. 3. Hydrolysis profile of the tetrapeptide phenylalanylglycylglycylphenylalanine (FGGF) by nasal mucosal homogenate in 0.1 M phosphate buffer containing 6.67% dimethylsulphoxide at 37°C.

was then cleaved, exclusively at its C-terminal end, to yield phenylalanine, as denoted by the absence of GF. This suggested that the dipeptide product glycylglycine (GG) was the other product of the hydrolysis of GGF, but this was not confirmed experimentally. The tripeptide, GGF, in contrast to FGGF, was degraded exclusively at its C-terminal end to yield phenylalanine as the sole product containing that amino acid. The production of GF was not observed. The theoretical progress curves for FGGF and GGF, using the calculated values of K_m and V_{max} corresponded well with the experimental data for degradation of the substrate and formation of products and suggests that the proposed routes and parameters for the degradation of these compounds are valid.

The hydrolysis of the tripeptide FGG, paralleled that of the tetrapeptide FGGF and occurred exclusively at the N-terminal end to generate phenylalanine. The formation of FG, which would be present as a result of carboxypeptidase action on this compound, was not observed. Similarly, the tripeptide WGG was also hydrolysed exclusively at its N-terminal end to generate tryptophan and the dipeptide GG, with no indication of the metabolite WG. Each of the dipeptides FG, GF and WG was converted to its constituent amino acids; the appearance of glycine was not quantified but this amino acid was detected using an automated amino acid analyser system.

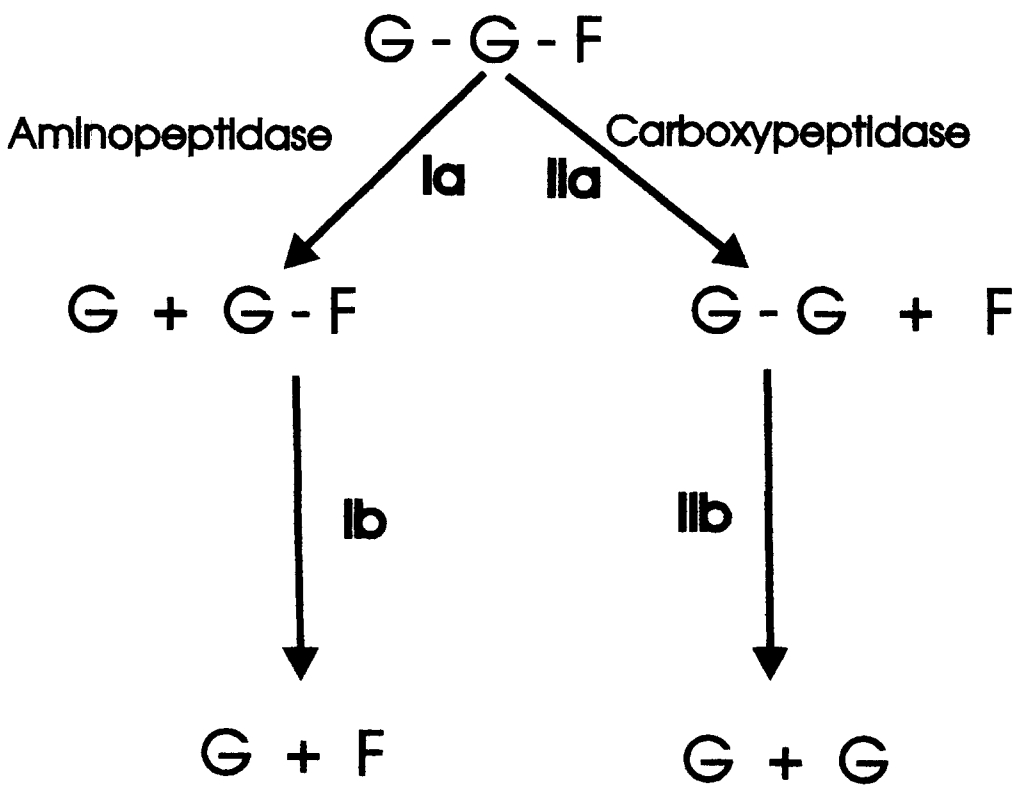
The tetrapeptide, FGGF, and the three tripeptides, FGG, GGF and WGG, were each cleaved exclusively at the peptide bond between the aromatic amino acid and the glycine irrespective of whether the amino acid was at the N-terminal or the C-terminal end of the peptide (Scheme 1).



Scheme 1. Principal degradation pathways observed with the homogenate preparation.

The susceptibility of FGGF, WGG, and FGG to attack at their N-terminal end suggested the presence of at least one aminopeptidase. This class of proteolytic enzymes has been identified in rat and rabbit nasal mucosa (Dodda Kashi and Lee, 1986; Stratford and Lee, 1986). The degradation of GGF at its C-terminal end indicated the presence of a carboxypeptidase the activity of which has been observed in homogenate preparations of buccal and intestinal tissue from rats and hamsters (Garren and Repta, 1988). The fact that no carboxypeptidase activity was observed against FGG and WGG suggested that the enzyme may have some specificity for an aromatic, C-terminal amino acid, perhaps due to carboxypeptidase A or the lysosomal enzyme cathepsin A. Both exhibit a preference for either a branched or an aromatic amino acid in the C-terminal position (Iodice, 1967; Kim and Lipscomb, 1990) but cathepsin A exerts an optimum activity at pH 3–6 and is inactivated at pH 7.4 (Bond and Beynon, 1987). The effect of pH was investigated with the substrate GGF. At pH values 5.4 and 3.4 no degradation of GGF was observed. This suggested that GGF was stable in the presence of the lysosomal enzymes under the experimental conditions.

To probe the activity further, the hydrolysis of GGF was studied using cellular fractions to compare the activity of homogenate with cytosolic and microsomal components. The substrate (GGF) was hydrolysed in all three enzymic preparations and the degradation was found to be linear with time during the course of the experiment. The substrate was hydrolysed most rapidly in the microsomal fraction and slowest in the cytosolic fraction. The rates, determined by linear regression were 0.85 μmol l^{-1} min^{-1} (homogenate), 0.63 μmol l^{-1} min^{-1} (cytosolic) and 1.91 μmol l^{-1} min^{-1} (microsomal). The specific activi-



Scheme 2. Possible hydrolytic pathways for glycylglycylphenylalanine (GGF).

ties were calculated to be 0.27 μmol l^{-1} min^{-1} mg^{-1} (homogenate), 0.25 μmol l^{-1} min^{-1} mg^{-1} (cytosolic) and 0.54 μmol l^{-1} min^{-1} mg^{-1} (microsomal) of protein. The activity in the microsomal fraction was therefore double that in the cytosolic fraction. This result, with greater activity being observed in membrane-bound fractions, is comparable to those obtained by Choi and Lee (1986) using methionine and leucine enkephalin with rabbit nasal mucosa and Yamamoto et al. (1988) using insulin as a substrate and rabbit nasal mucosa. The substrate GGF may be degraded at both its N- and C-terminal ends. Attack at the N-terminal would generate the primary products GF and G, whilst attack at the C-terminal end would yield GG and F (Scheme 2). The hydrolysis profiles are shown in Fig. 4. In all three preparations, the metabolites GF and F were observed. The products GF and F may result from successive aminopeptidase actions or from competitive degradation at both N- and C-termini. The latter pathway may be expected to provide a constant proportion of products and the GF/F ratios are calculated in Table 3. The high GF/F ratios for the microsomal fraction suggest that attack at the N-terminal end, by aminopeptidase action, is the principal site of degradation via routes Ia and Ib. The considerably lower GF/F ratios in the cytosolic and homogenate fractions suggest that degradation may be occurring competitively via routes I and II with route Ib being a minor component of the degradation. This profile contrasts with the previous experiments where GGF was degraded exclusively by carboxypeptidase action to generate F and GG but when GGF hydrolysis by purified CPA was assessed in various buffer systems the rates of degradation were ~ 0.80 μmol l^{-1} min^{-1} for each with attack occurring exclusively at the C-terminal end to produce phenylalanine; no GF was observed.

The hydrolysis of the substrates FGG, GGF, WGG and FG was evaluated using commercially available CPA, cytosolic leucine aminopeptidase (LAP-C) and microsomal leucine aminopeptidase (LAP-M) and the half-lives are recorded in Table 4. Only one of the substrates tested, GGF, was hydrolysed on incubation with CPA. In contrast,

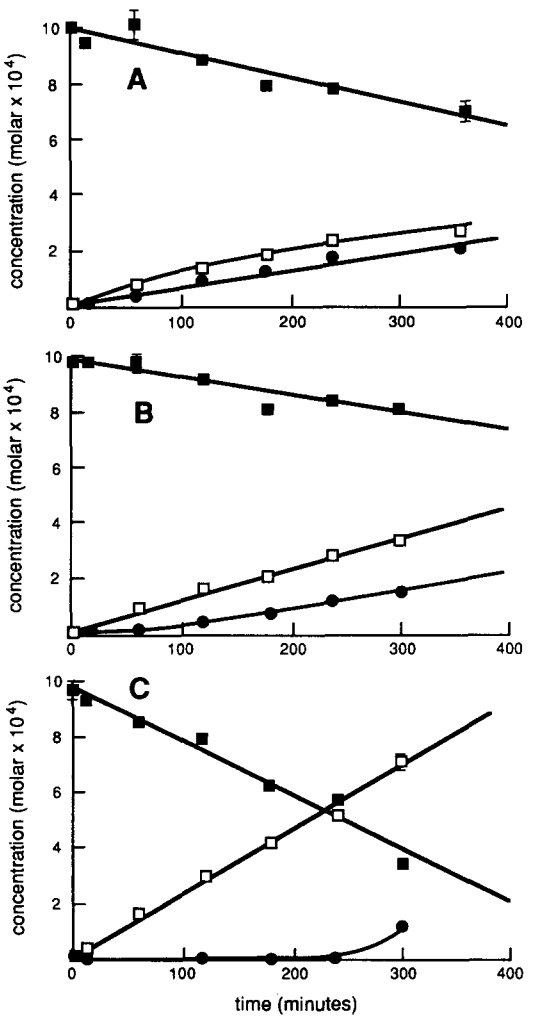


Fig. 4. Hydrolysis profiles of glycylglycylphenylalanine (GGF) in (A) homogenate, (B) cytosolic and (C) microsomal fraction. Experiments were performed in McIlvaine's buffer (pH 7.4), ionic strength 0.5 M at 37°C.

Table 3
Ratios of GF/F produced in the metabolism of GGF in each of the cellular fractions

Time (min)	Homogenate	Cytosolic	Microsomal
0	ND	ND	ND
15	ND	ND	ND
60	2.9	27.1	ND
120	1.6	4.6	40.9
180	1.4	2.9	28.7
240	1.4	2.6	38.0
360	1.3	2.31	5.4

ND, F not detectable.

Table 4
Half-lives for the degradation of peptides by commercial enzymes

Substrate	Half-life (min)		
	CPA	LAP-C	LAP-M
FGG	stable	70	4
WGG	stable	79	3
GGF	287	stable	fast
GF	fast	fast	122
FG	stable	567	88

all of the substrates, with the exception of GGF, were hydrolysed by LAP-C but with variations in their susceptibility to degradation. The order of stability was FG > FGG > WGG. In the studies with microsomal leucine aminopeptidase, GGF surprisingly was the most rapidly degraded, but the same order of stabilities was observed for the other substrates as with the cytosolic enzyme. The rates of degradation were significantly higher with the microsomal aminopeptidase than the cytosolic aminopeptidase for all of the peptides studies.

The stability of WGG, FGG and FG with CPA is not surprising in view of the fact that CPA has substrate preferences which include an L-branched or aromatic amino acid at the C-terminus (Kim and Lipscomb, 1990). The order of stability of the substrates with LAP-C parallels the observation that activity is substrate-dependent with those containing an N-terminal leucyl group being preferred, and those with hydrophobic residue at this position being hydrolysed rapidly (Delange and Smith, 1971). The crystallographic structure of bovine lens and hog kidney leucine aminopeptidases has recently been reported (Taylor et al., 1984; Burley et al., 1990) and may lead to the rationalisation of mechanistic studies. The stabilities of WGG < FGG < FG exhibited by LAP-C and LAP-M differ from those observed in the sheep nasal homogenate where FGG < FG < WGG. These differences may reflect that the substrates are being degraded by more than one enzyme in the homogenate or that sheep nasal aminopeptidases may have different enzyme preferences to those isolated from porcine kidney. The hydrolysis profiles for FGG in the

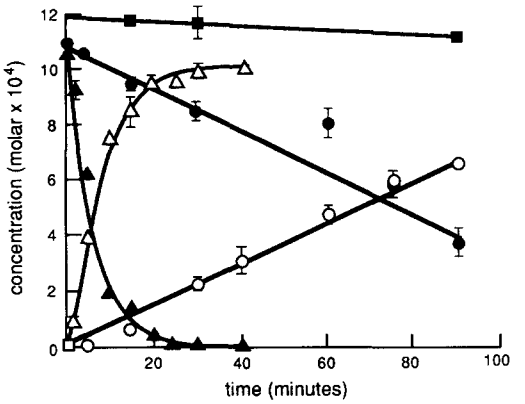


Fig. 5. Hydrolysis profile of phenylalanylglycylglycine (FGG) in the presence of carboxypeptidase A (CPA), cytosolic leucine aminopeptidase (LAP-C) and microsomal leucine aminopeptidase (LAP-M) in 0.1 M phosphate buffer at 37°C.

presence of the three enzymes are illustrated in Fig. 5 which show it to be stable in the presence of CPA but degraded by both LAP-C and LAP-M. Attack by both enzymes occurred exclusively at the N-terminal end of the molecule to generate phenylalanine. Analogous degradation of WGG, to yield tryptophan, was also observed; no WG was detected. The tripeptide GGF was the only substrate hydrolysed on incubation with CPA. Additionally, this was the only substrate stable in the presence of LAP-C. In contrast, it was rapidly hydrolysed by LAP-M to G and GF, the latter

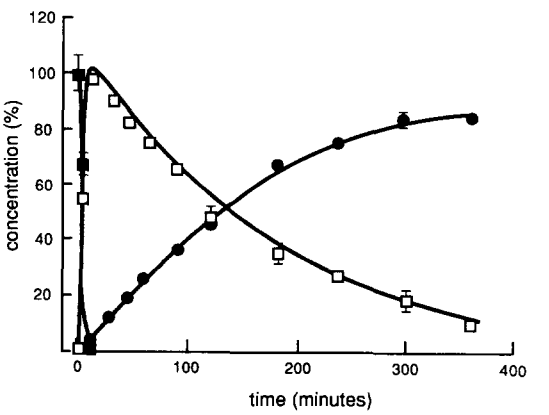


Fig. 6. Hydrolysis profile of glycylglycylphenylalanine (GGF) in the presence of microsomal leucine aminopeptidase (LAP-M) in 0.1 M phosphate buffer at 37°C.

being slowly degraded to phenylalanine and glycine. This behaviour resulted in a biexponential curve, characteristic of an A → B → C kinetic scheme, being observed for GF (Fig. 6). The dipeptide FG was stable in the presence of CPA but was degraded by both aminopeptidases.

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