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Comparison of vaginal aminopeptidase enzymatic activities in various animals and in humans

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Abstract

The specific enzymatic activity of four different aminopeptidases (aminopeptidase N, leucine aminopeptidase, aminopeptidase A and aminopeptidase B) in vaginal homogenates from rabbit, rat, guinea-pig, sheep and humans was compared. The purpose of the study was to find an appropriate animal model that can be used in degradation studies of protein and peptide drugs. Different substrates were used as the relative specific substrates for the determination of aminopeptidase enzymatic activity: 4-methoxy-2-naphthylamide of L-alanine for aminopeptidase N, 4-methoxy-2-naphthylamide of L-leucine for leucine aminopeptidase, 4-methoxy-2-naphthylamide of L-glutamic acid for aminopeptidase A and 4-methoxy-2-naphthylamide of L-arginine for aminopeptidase B. The vaginal aminopeptidase enzymatic activity of different species was determined spectrofluorometrically. The inhibition of aminopeptidase activity in the presence of bestatin and puromycin inhibitors was also investigated. The results showed the presence of aminopeptidase enzymatic activity in all vaginal homogenates in the order: sheep $>$ guinea-pig $>$ rabbit $\geqslant$ human $\geqslant$ rat. Based on the results of the hydrolysis and inhibition of the 4-methoxy-2-naphthylamide substrates, it was difficult to have an exact decision on the aminopeptidase type in the vaginal homogenates from the species studied. It was found that the aminopeptidase activity in rat, rabbit and humans was not statistically different. Therefore, we suggest that rats and rabbits could be used as model animals for vaginal enzymatic activity studies and for determination of the degradation of protein and peptide drugs in the vagina.

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Introduction

With the advent of biotechnology, increasing attention has been paid to peptide and protein drug production and delivery. However, there are many problems in the systemic administration of peptide and protein drugs. Almost all peptide and protein drugs are administered via the parenteral route. However, because this route is invasive, various alternative routes, including oral (Geary & Schlameus 1993), buccal (Ishida et al 1981; Hoogstraate et al 1996), nasal (Adjei et al 1992; Merkus et al 1996), vaginal (Richardson et al 1992a; Nakada et al 1993), rectal (Watanabe et al 1991), pulmonary (Patton et al 1994) and ocular (Sasaki et al 1997), have been investigated.

Penetration and enzymatic barriers are two major barriers for absorption of peptide and protein drugs from mucosal sites. Enzymatic barriers comprise exo- and endopeptidases that cleave peptides and proteins at their N- and C-termini and at an internal peptide bond, respectively. Examples of exopeptidases are aminopeptidase, dipeptidyl dipeptidase, diaminopeptidase, dipeptidyl carboxypep-

tidase, serine carboxypeptidase, metallocarboxypeptidase and cysteine carboxypeptidase.

Various studies on the mucosal degradation of peptide and protein drugs have been reported. Kashi & Lee (1986) studied the mucosal metabolism of various enkephalins in rabbits and suggested that at least three peptidases, including aminopeptidases, dipeptidyl peptidase and dipeptidyl carboxypeptidase, play an important role in hydrolysis of enkephalins. Among these enzymes, aminopeptidases were the major enzymes for methionine and leucine enkephalin, whereas dipeptidyl carboxypeptidase was important for the D-ala-met-enkephalin. The highest activity of dipeptidyl carboxypeptidase was found to be in the order: buccal > rectal > vaginal > nasal.

Sayani et al (1993) reported that aminopeptidases exist in rabbit nasal, rectal and vaginal extracts in concentrations of < 0.05 U mL^{-1}, with the greatest concentration in the vaginal extract (0.045 U mL^{-1}). A small concentration of carboxypeptidase was also detected (0.05 U mL^{-1}). They showed that, based on the degradation rate constants of leucine enkephalin, the level of enzymes involved was greatest in rectal and vaginal mucosae, and least in the nasal mucosae.

It has been reported that the degradation profile of luteinizing hormone releasing hormone (LHRH) in rabbit rectal, nasal and vaginal mucosal homogenates, based on protein concentration of the supernatants, follows first-order kinetics and accelerates with an increase in protein concentration. The degradation order was: rectal > nasal > vaginal. The half-life of LHRH in vaginal homogenates was 9–12 times longer than that in rectal homogenates and 3–4 times greater than in nasal homogenates (Han et al 1995).

Stratford & Lee (1986) determined the type and activity of aminopeptidases in rabbit conjunctival, nasal, buccal, rectal and vaginal homogenates, relative to duodenal and ileal homogenates. It was found that aminopeptidase N was present in all these mucosae to the extent of 50–100% with respect to ileal activity, whereas aminopeptidase A was present to the extent of 4–20% with respect to ileal activity. Acartürk & Robinson (1996) compared the aminopeptidase activity in vaginal homogenates from normal and ovariectomized rabbits. They reported that the activity of leucine aminopeptidase, aminopeptidases B and A was the same in vaginal tissue homogenates and whole tissue specimens in both normal and ovariectomized rabbits, whereas the activity of aminopeptidase N was significantly decreased in ovariectomized compared with normal rabbits.

The vagina is a potential site for systemic absorption of therapeutic peptides and proteins owing to its large surface area and rich vascularity. However, peptide and protein drugs are degraded by vaginal enzymes. Aminopeptidases, dipeptidyl peptidases and dipeptidyl carboxypeptidases are the major enzymes at mucosal sites. In particular, aminopeptidases have an important role in the degradation of peptide and protein drugs in the vagina. Various animal models, including rodent (Hsu et al 1983; Durrani et al 1985; Nakada et al 1993), sheep (Richardson et al 1992b), rabbit and monkey (Chen et al 1993), have been used in vaginal absorption studies. Rabbits have been used for comparative vaginal enzymatic activity studies in the literature (Stratford & Lee 1986; Acartürk & Robinson 1996). Although a number of vaginal enzymatic activity studies have been performed in rabbits, to our knowledge no comparative study with other species, including humans, has been reported.

In this study, the specific enzymatic activity of four different aminopeptidases (aminopeptidase N, leucine aminopeptidase, aminopeptidase A, aminopeptidase B) in vaginal homogenates from different species, including rabbit, rat, guinea-pig and sheep, was determined. The results obtained were compared with human data to find an appropriate animal model that could be used in vaginal absorption and degradation studies of protein and peptide drugs.

Materials and Methods

Materials

Aminopeptidase substrates (4-methoxy-2-naphthylamides of L-leucine, L-alanine, L-glutamic acid and L-arginine) and inhibitors (bestatine and puromycin) were purchased from Sigma Chemical Co., (Ankara, Turkey). All other materials and solvents were of analytical grade.

Vaginal tissue samples

Vaginal tissue samples were obtained from female Albino rabbits (1900–4800 g), Wistar rats (145–165 g) and guinea-pigs (270–350 g) after a lethal injection of sodium pentobarbital solution either into a marginal ear vein or intraperitoneally. Samples of sheep vagina were obtained immediately after slaughter in the slaughterhouse. The Ankara University Veterinary Faculty Ethics Committee in accordance with internationally accepted principles approved the experimental protocol.

Human vaginal tissue samples were obtained from

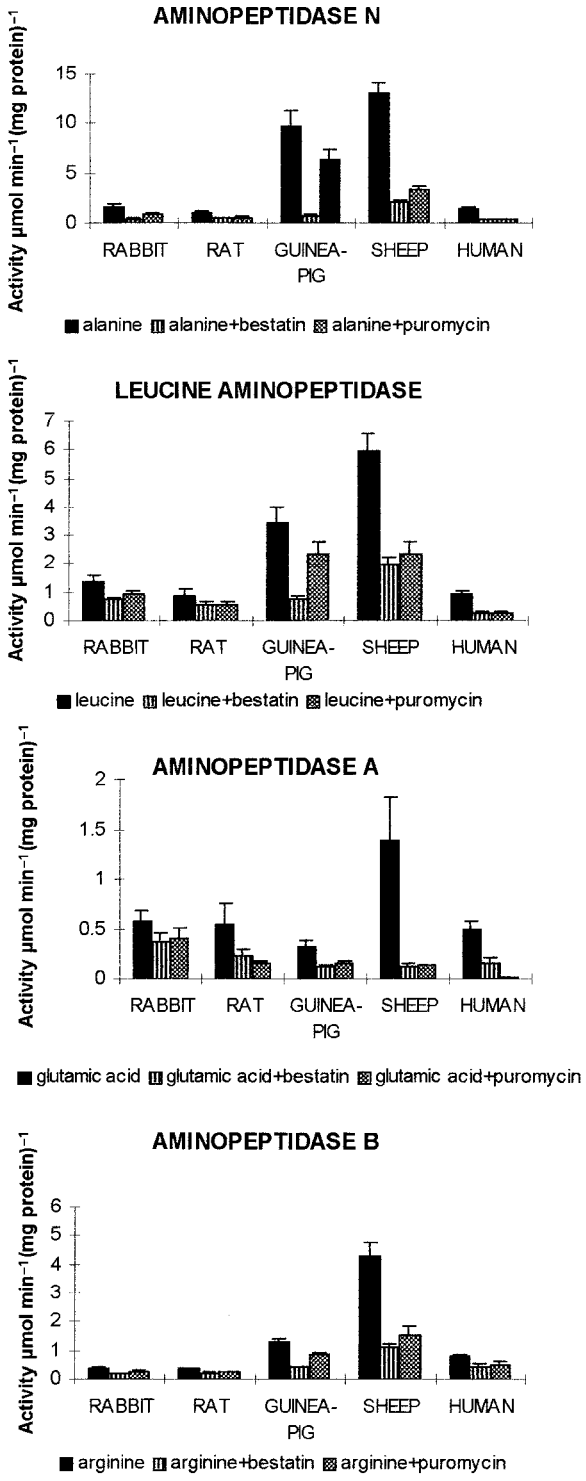

Figure 1 Activity (μmol substrate hydrolysed min^{-1} (mg protein)$^{-1}$) of aminopeptidase N, leucine aminopeptidase, aminopeptidase A and aminopeptidase B, with and without enzyme inhibitors (bestatin and puromycin) against 4-methoxy-2-naphthylamide substrates in vaginal homogenates from various species. Each bar represents the mean $\pm$ s.e.m. of five samples.

left-over tissues from women of reproductive age during vaginal surgical operations in the hospital.

Preparation of vaginal tissue homogenates

After removal of vaginal mucosae, samples were rinsed with ice-cold saline solution and then frozen in liquid nitrogen and stored at -60°C. For the experiments, the tissues were thawed at room temperature and cut into 1-mm cubes and then homogenized in cold 0.05 M Tris-maleate buffer (pH 7.4) using a Teflon homogenizer for 2 min. The supernatant was separated by centrifugation at 1000 *g* for 1 min. The supernatants were kept on ice and used within 4 h of preparation. The protein concentration of the supernatant was determined using a dye-binding assay (Bradford 1976) with serum albumin from each species as the standard.

Aminopeptidase activity studies

The specific aminopeptidase activity of tissue samples was determined using the method of Stratford & Lee (1986). Stock solutions of 4-methoxy-2-naphthylamides of L-leucine, L-alanine, L-arginine and L-glutamic acid were prepared in 3 mM dimethylformamide. They were stored at -15°C and used within five days of preparation. The reaction was initiated by the addition of 100 μL of the substrate solution to the reaction mixture, consisting of 100 μL tissue supernatant and 2.8 mL 0.05 M Tris-maleate buffer (pH 7.4). The reaction mixture was pre-incubated at 37°C for 15 min. Fluorescence intensity was monitored at an excitation wavelength of 342 nm and an emission wavelength of 426 nm for 5 min (Shimadzu RF 5000 fluorescence spectrophotometer). Duplicate or triplicate incubations were performed for each sample.

Aminopeptidase assays were also performed in the presence of aminopeptidase inhibitors. Bestatin or puromycin (100 μL) at 0.14 or 1.4 mM, were added to 2.8 mL of the tissue mixture and incubated for 15 min at 37°C. After incubation, 100 μL of the substrate solution was added and the fluorescence intensity was monitored as described above. Initial velocities were calculated from the standard curves for fluorescence intensity vs mol β-naphthylamine and from plots of fluorescence intensity vs time. Activity was expressed as μmol substrate hydrolysed min^{-1} (mg protein)$^{-1}$.

Statistical analysis

One-way analysis of variance was used to test for differences in aminopeptidase activity in the tissue homogenates using GraphPad Software (Instat ver.

2.04). Student-Newman-Keuls multiple comparison test was also performed.

Results and Discussion

Aminopeptidases are one of the major enzymes for peptide and protein degradation. The aminopeptidase substrates, 4-methoxy-2-naphthylamides of L-alanine, L-leucine, L-glutamic acid and L-arginine are relatively specific for aminopeptidase N, leucine aminopeptidase, aminopeptidase A and aminopeptidase B, respectively. Aminopeptidases N and A are membrane-bound peptidases, whereas aminopeptidase B and leucine aminopeptidase are cystosolic enzymes. Aminopeptidase activity is principally cystosolic in the vaginal mucosae (Lee & Yamamoto 1990). The supernatant of the mucosal tissue homogenates, obtained by low speed centrifugation (1000 ***g***), contained cystosol and relevant plasma and intracellular membrane fractions, and therefore this supernatant would likely contain several enzymes.

Figure 1 and Table 1 show the specific enzyme activities of the four types of aminopeptidases, with and without enzyme inhibitors, in vaginal homogenates from the various species. If we compare the results of the hydrolysis of the alanine substrate, which is specific for aminopeptidase N, the values obtained for sheep and guinea-pig were the greatest. The aminopeptidase activity in rat, rabbit and human vaginal homogenates was significantly lower than that of sheep and guinea-pig ($P < 0.001$). However, there was no statistical difference among the aminopeptidase N activity in these three species. Species ranking was in the order: sheep > quinea-pig > rabbit $\geqslant$ human $\geqslant$ rat.

The second aminopeptidase, leucine aminopeptidase, gave a similar statistical result and species ranking. No statistical difference was found among leucine aminopeptidase activity in rat, rabbit and human vaginal homogenates.

In the case of aminopeptidase A, the lowest activity was seen in vaginal homogenates from guinea-pig. A statistically significant difference was only found between the values of sheep and guinea-pig ($P < 0.05$). The hydrolysis of glutamic acid substrate in vaginal homogenates from the other species was not significantly different ($P > 0.005$).

Arginine was the substrate of the aminopeptidase B and the results showed that sheep vaginal homogenates had the greatest aminopeptidase B activity. The aminopeptidase B activity in vaginal homogenates from rat, rabbit and humans was not statistically different ($P > 0.05$). The activity values in homogenates from human and guinea-pig were also not statistically different.

The peptidase activity of vaginal tissues from different species, with the exception of rabbits, has not been reported, and a direct knowledge of human vaginal tissue peptidase activity has been lacking. The peptidase activity of vaginal tissues has been described as significant relative to that of intestinal tissues by Stratford & Lee (1986). They reported the presence of all of the aminopeptidases, except aminopeptidase B, in vaginal homogenates from rabbits. On the other hand, Acartürk & Robinson (1996) showed the presence of all the aminopeptidases in vaginal homogenates and whole tissue samples in normal and ovariectiomized rabbits.

In our study, the values of specific aminopeptidase activity in rabbit vaginal homogenates were found to be greater than those reported by Stratford & Lee (1986), but lower than those reported by Acartürk & Robinson (1996). This difference may be owing to inter-species variation. Given the lack of information about the aminopeptidase activity in vaginal homogenates from rat, guinea-pig, sheep and humans, we could only compare the results among each other. The results showed that the four types of aminopeptidase activity in vaginal

Table 1 Aminopeptidase activity (μmol substrate hydrolysed min^{-1} (mg protein)$^{-1}$) against 4-methoxy-2-naphthylamide substrates in vaginal homogenates from various species.

Species	Aminopeptidase N (L-alanine)	Leucine aminopeptidase (L-leucine)	Aminopeptidase A (L-glutamic acid)	Aminopeptidase B (L-arginine)
Rabbit	1.67 ± 0.30	1.37 ± 0.21	0.575 ± 0.103	0.380 ± 0.067
Rat	1.03 ± 0.26	0.849 ± 0.248	0.545 ± 0.214	0.371 ± 0.021
Guinea-pig	9.65 ± 1.63	3.44 ± 0.57	0.321 ± 0.058	1.30 ± 0.095
Sheep	13.1 ± 1.0	5.94 ± 0.61	1.38 ± 0.44	4.29 ± 0.47
Human	1.38 ± 0.20	0.906 ± 0.108	0.496 ± 0.083	0.823 ± 0.057

Data are mean ± s.e.m.

Table 2 Percentage inhibition of vaginal aminopeptidase activity by bestatin and puromycin.

Species	Bestatin				Puromycin			
	L-Alanine	L-Leucine	L-Glutamic acid	L-Arginine	L-Alanine	L-Leucine	L-Glutamic acid	L-Arginine
Rabbit	72.2±4.9	46.5±6.5	35.6±9.2[a]	51.0±8.1	39.5±8.0	31.7±5.3	33.4±10.4[a]	32.2±5.6[a]
Rat	52.0±6.6[a]	32.4±6.0[a]	46.0±11.9[a]	47.9±8.0	41.5±6.1[a]	32.4±5.3[a]	84.6±2.4[a]	77.7±3.6
Guinea-pig	92.5±0.4	78.0±1.5	59.1±3.3	66.6±2.3	34.1±2.8[a]	32.9±5.0[a]	49.3±3.4	37.1±5.3
Sheep	84.0±2.5	66.9±2.8	88.0±3.2	72.5±3.9	74.3±1.8	62.8±6.4	87.8±2.1	61.1±10.5
Human	73.0±3.6	67.9±4.2	68.4±10.9	43.1±9.0	78.1±3.1	69.0±4.2	95.2±1.2	41.1±2

Data are mean±s.e.m. [a]Not significantly different compared with control ($P > 0.05$).

homogenates from rabbit, rat and humans were not statistically different ($P > 0.05$).

The results of the specific enzymatic activity, with and without enzyme inhibitor, in vaginal homogenates from various animals and humans according to species are shown in Table 1. Vaginal homogenates showed more or less a similar pattern of aminopeptidase activity in all species, that is aminopeptidase N was the most active enzyme, followed by leucine aminopeptidase and aminopeptidases A and B or B and A. The results showed that aminopeptidase N activity was significantly greater than that of leucine aminopeptidase, aminopeptidase A and aminopeptidase B ($P < 0.01$ to $P < 0.05$) in vaginal homogenates from all species, except rat.

The values of aminopeptidase A and B were not statistically different among all species, except sheep. The only statistical difference was between leucine aminopeptidase and aminopeptidase A or B in rabbit and sheep ($P < 0.001$). Therefore, it was difficult to give an exact species ranking for the activity of these enzymes.

Aminopeptidase assays were also performed for each substrate in the presence of an aminopeptidase inhibitor, bestatin or puromycin. Bestatin inhibits aminopeptidase N, leucine aminopeptidase and aminopeptidase B activity, puromycin does not inhibit leucine aminopeptidase, and neither inhibitor has an effect on aminopeptidase A. Table 2 shows the percentage inhibition of the aminopeptidase substrates in the presence of aminopeptidase inhibitors. The results showed that the hydrolysis of all four 4-methoxy-2-naphthylamide substrates in all homogenates was sensitive to both inhibitors and it was difficult to differentiate between them. For example, the significant inhibition of the alanine substrate in the vaginal homogenates from rabbit, sheep and humans by bestatin and puromycin suggested the presence of aminopeptidase N in these species. Both inhibitors also significantly inhibited the hydrolysis of the glutamic acid substrate, which is the specific substrate of aminopeptidase A in vaginal homogenates from all species, except rabbit and rat. The results suggested that the enzymatic activity observed was attributable to aminopeptidases and probably of more than one type.

Based on the general enzymatic aminopeptidase studies in vaginal homogenates from rat, rabbit, guinea-pig, sheep and humans, we conclude that the aminopeptidase activity in rat, rabbit and humans was not significantly different. Therefore, the rat and the rabbit may be used as model animals for vaginal enzymatic activity studies and for the determination of degradation of protein and peptide drugs in the vagina.

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