Department of Pharmaceutical Technology, Faculty of Pharmacy, Gazi University, Etiler, Ankara, Turkey

A comparative study on the rectal aminopeptidase enzymatic activities of different species

F. Acartürk, Z. İ. Parlatan

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Dr. Füsün Acartürk, Department of Pharmaceutical Technology, Faculty of Pharmacy, Gazi University, TR-06330 Etiler Ankara, Turkey
facar@tr.net

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The aim of the present study was to compare the enzymatic activity of four different aminopeptidases (aminopeptidase N, leucine aminopeptidase, aminopeptidase A, aminopeptidase B) in rectal homogenates from different species: rabbit, rat, guinea-pig, sheep and human. Different substrates were used as the relative specific substrates for the determination of aminopeptidase enzymatic activity. For this purpose, 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 were employed. The rectal aminopeptidase enzymatic activity was determined spectrofluorometrically. The inhibition of activity of aminopeptidase in the presence of bestatin and puromycin inhibitors was also investigated. The results showed the presence of aminopeptidase enzymatic activity in all rectal homogenates. Sheep and guinea-pig had the greatest aminopeptidase activity. The four aminopeptidase activities of rat and rabbit were not significantly different from each other. Human data was not evaluated statistically, due to insufficient sample. But the values of human data was close to those of the rabbit and rat values except for aminopeptidase A. Based on the data of the hydrolysis and inhibition of the 4-methoxy-2-naphthylamide substrates, it was rather difficult to determine the aminopeptidase type in the rectal homogenates of the species studied. It has been found that the aminopeptidase activities of rat and rabbit were not statistically different from each other and the human data were close to them.

1. Introduction

Historically, the rectum has been accepted as a site of drug delivery for local therapy, e.g. haemorrhoids, and for systemic delivery of drugs presenting practical problems for parenteral or oral dosing [1]. For some form of treatment, the rectal route could provide a more acceptable alternative to multiple injections. Recently, the rectum has been considered as a potential absorption site for the delivery of peptide/protein drugs, such as insulin and calcitonin [2–5]. On the other hand, rectal immunization is possible [6]. The advantages for rectal delivery of peptides/proteins are low levels of protease activity, particulary of pancreatic origin, large surface area, potentiated by using spreading/foaming agents and avoidance of first pass metabolism [1].

Although the enzymatic activity of protease is low, exopeptidases, such as aminopeptidases, dipeptidyl dipeptidase, diaminopeptidase, dipeptidyl carboxypeptidase play an important role for the degration of peptide/protein drugs in the rectum [7, 8]. Various comparative studies on the mucosal degradation of peptide and protein drugs on the different mucosae have been carried out [9–14]. It has been reported that the naturally occuring peptide leucine enkephalin showed rapid degradation in the rectal and vaginal homogenates of rabbits, compared to that of nasal homogenate. In addition, assay of enzyme activities in the nasal, rectal and vaginal extracts showed that aminopeptidase does exist in each of the extracts in a quantity of <0.05 units/ml [15].

Stratford and Lee [9] determined the type and activity of aminopeptidases in rabbit conjuctival, nasal buccal, rectal and vaginal homogenates, relative to duodenal and ileal homogenates. It was found that aminopeptidase N, a plasma membrane bound peptidase, was present in all the mucosae. Aminopeptidase A, another plasma membrane bound peptidase appeared 10% or less abundant in the conjunctival, buccal, rectal and vaginal mucosae. Aminopeptidase B was also present in all the mucosae studied.

Zhou and Po [16] investigated the aminopeptidase and esterase activities of dermal, nasal, buccal, rectal and intestinal tissues from the rat, rabbit, guinea-pig and dog. It was found that the ranking was rat < dog < rabbit < guinea-pig for aminopeptidase activity.

Chun and Chien [17] studied the degradation of methionine enkephalin in the mucosal and serosal extracts of the rabbit. They found no significant difference in the degradation rates between mucosal and serosal extracts, regardless of the type of mucosa used. Degradation was most rapid in the extracts of rectal mucosa, followed by vaginal and nasal mucosae.

Han et al. [18] reported that Luteinizing Hormone Releasing Hormone (LHRH) was rapidly degraded in rectal and nasal mucosal homogenates and the half life was 17.9 min and 44.4 min, respectively.

In the previous study, we investigated the specific enzymatic activity of four different aminopeptidases in vaginal homogenates from rabbit, rat, guinea-pig, sheep and humans and found aminopeptidase enzymatic activity in all vaginal homogenates in the order: sheep > guinea-pig > rabbit ≥ human ≥ rat [19].

The aim of this study was to compare the specific enzymatic activity of four different aminopeptidases (aminopeptidase N, leucine aminopeptidase, aminopeptidase A, aminopeptidase B) in rectal homogenates from different species, rabbit, rat, guinea-pig and sheep. The results obtained were compared with human data.

2. Investigations, results and discussion

Measurement of the aminopeptidase activity of the rectal mucosal homogenates involved hydrolysis of the 4-methoxy-2-naphthylamide and monitoring the fluoresence intensity of the solution. 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. The subcellular distribution of aminopeptidase activity based on methionine and leucine enkephalins as substrates reveals that aminopeptidases are distributed throughout the cell to degrade peptides and proteins both during and after absorption into the cell [20]. Aminopeptidase N and A are membrane bound pepdidases, whereas aminopeptidase B is a cytosolic enzyme [9]. The supernatant of the mucosal tissue homogenates contained cystosol and relevant plasma and intracelluler membrane fractions. The supernatants studied would likely contain several enzymes.

Table 1 shows the specific enzymatic activities of the four types of aminopeptidases in the rectal homogenates from various species. Comparison of the results of the hydrolysis of the alanine substrate, which is specific for aminopeptidase N, showed that the values obtained for sheep and guinea-pig were the greatest. The aminopeptidase activity in rat and rabbit rectal homogenates was significantly lower than that of sheep and guinea-pig ($p < 0.001$). Species ranking was in the order: sheep > guinea-pig > rabbit ≥ rat. We had only two human tissue samples, therefore we could not compare the results statistically. However, the value for humans were close to those of rats and rabbits.

In the case of leucine aminopeptidase, lysis of the leucine substrate, which is specific for leucine aminopeptidase, similar statistical results and species ranking was observed. The result for humans was again close to those of the rat and rabbit.

If we compare the results of the glutamic acid substrate, which is specific for aminopeptidase A, the significantly greatest activity was found for sheep among the other species ($p < 0.001$). The specific enzyme activity of aminopeptidase A in the rectal homogenates of the rat, rabbit and guinea-pig was not significantly different from each other ($p > 0.05$). The value obtained for humans was between the value of sheep and other species.

With aminopeptidase B, the results showed that sheep and guinea-pig rectal homogenates had the greatest aminopeptidase B activity compared to those of rat and rabbit ($p < 0.001$), but the values of sheep and guinea-pig were not significantly different ($p > 0.05$). On the other hand, similar statistical results were obtained for rabbits and rats and their values also were not significantly different ($p > 0.05$). The aminopeptidase B activity of humans was found to be close to the value of the rat.

Zhou and Po [16] determined the leucine aminopeptidase activity in rectal homogenates from rat, rabbit, guinea-pig and dog. They compared the absorbance values of the solutions. They reported that the species ranking for protein-normalised aminopeptidase activity was rat < dog < rabbit < guinea-pig. They measured the greatest leucine aminopeptidase activity in the guinea-pig. In our experiments, we have also found the greatest three aminopeptidase activities, mainly aminopeptidase N, leucine aminopeptidase and aminopeptidase B in sheep and guinea-pig.

Stratford and Lee [9] investigated the specific aminopeptidase activity in rectal homogenates from rabbits and they reported that the aminopeptidase ranking was aminopeptidase N > leucine aminopeptidase > aminopeptidase B > aminopeptidase A. We also observed the same aminopeptidase pattern in all species studied (Table 1).

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. The percentage inhibition of the aminopeptidase substrates in the presence of bestatin and puromycin is shown in Table 2. It was found that the hydolysis of all four 4-methoxy-2-naphthylamide substrates in all species rectal hemogenates, was sensitive to both inhibitors and it was difficult to differentiate between them. Therefore, the enyzmatic activity observed was attiributable to aminopeptidases and probably of more than one type.

Due to the insufficient sample of human studies, we could not statistically evaluate the results. On the other hand, it was impossible to obtain rectal tissue samples from healthy volunteers; therefore, the specific enzyme activities of human rectal tissues were measured for only having an idea. The specific enzyme activity of human rectal tissue may be affected by disease state. Based on the general enzymatic aminopeptidase studies in rectal homo-

Table 1: Aminopeptidase activity (μmol substrate hydrolysed min^{-1} (mg protein)$^{-1}$ against 4-methoxy-2-naphthylamide substrates in rectal homogenates from various species(mean ± s.e.m.) (n = 5)

Species	Aminopeptidase N (L-alanine)	Leucine aminopeptidase (L-leucine)	Aminopeptidase A (L-glutamic acid)	Aminopeptidase B (L-arginine)
Rabbit	1.67 ± 0.28	0.969 ± 0.134	0.174 ± 0.009	0.357 ± 0.038
Rat	1.05 ± 0.12	0.968 ± 0.095	0.341 ± 0.036	0.955 ± 0.053
Guinea-pig	8.56 ± 0.63	3.64 ± 0.21	0.352 ± 0.029	3.69 ± 0.426
Sheep	15.7 ± 0.6	6.68 ± 0.77	1.26 ± 0.21	4.85 ± 0.60
Human[a]	2.67 ± 0.47	1.35 ± 0.07	0.641 ± 0.29	0.828 ± 0.14

[a] mean of two data

Table 2: Percent inhibition of rectal aminopeptidase activities by bestatin and puromycin (mean ± s.e.m.) (n = 5)

Species	Bestatin				Puromycin			
	Alanine	Leucine	Glutamic acid	Arginine	Alanine	Leucine	Glutamic acid	Arginine
Rabbit	61.8 ± 15.1	57.6 ± 5.7	71.5 ± 5.6	73.6 ± 5.0	69.1 ± 3.7	47.9 ± 3.9	78.9 ± 2.6	41.6 ± 1.6
Rat	60.3 ± 2.3	42.4 ± 3.8	32.6 ± 4.0	66.4 ± 2.6	62.5 ± 3.4	41.0 ± 3.3	41.1 ± 4.1	46.0 ± 1.9
Guinea-pig	90.1 ± 0.7	73.5 ± 1.2	65.5 ± 2.4	69.1 ± 4.3	37.1 ± 3.0	36.1 ± 2.3	59.8 ± 2.6	60.5 ± 2.8
Sheep	83.9 ± 3.3	70.0 ± 7.4	70.4 ± 2.4	71.9 ± 3.8	84.1 ± 2.2	60.2 ± 1.7	75.6 ± 1.3	50.6 ± 3.2
Human[a]	79.6 ± 3.1	58.6 ± 4.1	63.6 ± 6.1	57.8 ± 1.5	80.4 ± 0.9	51.4 ± 0.3	79.7 ± 7.3	35.5 ± 5.1

[a]mean of two data

genates from rat, rabbit, guinea-pig, sheep and humans, we observed that sheep and guinea pig had the greatest aminopeptidase activity. The four aminopeptidase activities of the rat and rabbit were not significantly different from each other. Meanwhile, the human data was found to be agreement with values of rat and rabbit, except for aminopeptidase A.

3. Experimental

3.1. Materials

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

3.2. Rectal tissue samples

Rectal 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 rectum were obtained immediately after slaughter in the slaughterhouse. At least five animals were used for the experiments. The Ankara University Veterinary Faculty Ethics Committee, in accordance with internationally accepted principles, approved the experimental protocol.
Human rectal tissue samples were obtained from leftover tissues from human with rectum cancer during the rectal surgical operations in the hospital. Only two samples from humans were obtained.

3.3. Preparation of rectal tissue homogenates

After removal of rectal 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 the preparation. The protein concentration of the supernatant was determined using a dye-binding assay [21] with serum albumin from each species as the standard.

3.4. Aminopeptidase activity studies

The specific aminopeptidase activity of tissue samples was determined using the method of Stratford and Lee [9]. Stock solutions of 4-methoxy-2-naphtylamides of L-leucine, L-alanine, L-arginine and L-glutamic acid each at 3 mM were prepared in dimethylformamide. They were stored at −15 °C and used within five days of preparation as reported by Stratford and Lee [9]. 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 (0.01 mM) or puromycin (0.1 mM) was dissolved in dimethylformamide. One hundred microliter of bestatin or puromycin were added to reaction mixture consisting of 100 µl tissue supernatant and 2.7 ml of 0.05 M Tris-maleate buffer 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 ß-naphthylamine and from plots of fluorescence intensity vs. time. Activity was expressed in µmol of substrate hydrolyzed min^{-1} (mg protein)$^{-1}$.

3.5. Statistical analysis

One-way analysis of variance (ANOVA) was used to test for differences in aminopeptidase activity in the tissue homogenates using GraphPad Software (Instat ver. 2.04a). Tukey- Kramer Multiple Comparison Test was performed.

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